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Pathophysiological roles, pharmacological inhibition and cellular regulation of the cardiac sarcolemmal sodium/hydrogen exchanger

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Pathophysiological Roles, Pharmacological Inhibition and Cellular Regulation of the Cardiac Sarcolemmal Sodium/Hydrogen Exchanger

submitted by Professor Metin Avkiran
for the degree of DSc
of the University of Bath
2002

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5. Full List of Main Publications 272

1. Acknowledgements:

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Firstly, I must thank my mother and late father, for their love and their moral and financial support during my School and University education in England – I was supposed to return to Cyprus after getting a "good education", but I know that they have taken pride and satisfaction in my achievements, despite my failing to fulfil that particular expectation.

I could not have achieved half of what I have, without the love, devotion and understanding of my wife Diana. I met Diana during the "sandwich year" of my undergraduate studies and she commuted untiringly between London and Bath on most weekends for the subsequent 4 years, during which I completed my BSc and then my PhD at The University of Bath. However, my greatest gratitude to her is for our son Timur, whose arrival in 1993 gave me a new outlook and fresh energy in all aspects of my life, including my work.

Professionally, I will always remain grateful to Dr Brian Woodward, for inspiring my interest in cardiovascular pharmacology during my undergraduate studies, for subsequently taking me on as a PhD student, for allowing me to develop as an independent investigator, and for supplying me with some outstanding co-workers after I established my own laboratory in London.

Perhaps the greatest single influence on the development of my career as an academic scientist has been Professor David Hearse, under whose guidance I have progressed from postdoctoral fellow to group head, advancing along the way through the ranks of Lecturer, Senior Lecturer and Reader. David has been an extraordinary mentor, always positive and encouraging (especially at times of self-doubt) and unselfishly nurturing the development of my own ideas and directions. I cannot thank him enough for all he has done and continues to do in support of my career.

Finally, I wish to thank the many excellent research fellows and students with whom I have been fortunate to work over the past 15 years, for their hard work, loyalty and friendship.

2. Brief Curriculum Vitae:

EDUCATION AND QUALIFICATIONS:

1983-86 School of Pharmacy and Pharmacology, University of Bath.
PhD in Pharmacology.

1979-83 School of Pharmacy and Pharmacology, University of Bath.
BSc Hons in Pharmacology.

1977-79 Bedford School, Bedford, England.
GCE 'A' levels (Oxford & Cambridge) in Biology, Chemistry, Physics.

1972-77 The English College, Nicosia, Cyprus.
Nine GCE 'O' levels (University of London).

CURRENT POST:

Centre for Cardiovascular Biology and Medicine, King's College London, St Thomas' Hospital, London.
Professor of Molecular Cardiology (since 2001).

PREVIOUS POSTS:

Centre for Cardiovascular Biology and Medicine, King's College London, St Thomas' Hospital, London.
Reader in Molecular Cardiology (1999-2001).

Cardiovascular Research, King's College London (formerly UMDS), St Thomas' Hospital, London.
Honorary Senior Lecturer in the Divisions of Medicine (Cardiology) (1997-1999), Biomolecular Sciences (1995-1999) and Pharmacology & Toxicology (1995-1999).
Honorary Lecturer in the Divisions of Biochemistry & Molecular Biology and Pharmacology &Toxicology (1991-95).
Research Fellow (1986-90).

SELECTED INVITED LECTURES AT INTERNATIONAL CONFERENCES:

International Conference on *Advances in Na-H Exchange Research: From Molecular Regulation to Therapeutic Development*, London - Ontario, July 2002.
ISHR European Section, Szeged, July 2002.

2nd International Göttingen Heart Failure Conference, Schloß Waldeck, May 2002.
74th Scientific Sessions, American Heart Association, Anaheim, November 2001.
XVII World Congress of the ISHR, Winnipeg, July 2001.
German Cardiac Society Annual Meeting, Mannheim, April 2001.
XXII Congress of the European Society of Cardiology, Amsterdam, August 2000.
British Cardiac Society Annual Conference, Glasgow, May 2000.
XXI Congress of the European Society of Cardiology, Barcelona, August 1999.
Congress of the Federation of European Physiological Societies, Prague, July 1999.
Trends in Cardiovascular Pharmacotherapy, Orlando, May 1999.
American College of Cardiology Scientific Session, New Orleans, March 1999.
71st Scientific Sessions, American Heart Association, Dallas, November 1998.
XX Congress of the European Society of Cardiology, Vienna, August 1998.
ISHR American Section, Ann Arbor, August 1998.
III International Congress of Pathophysiology, Lahti, June/July 1998.
XVI World Congress of the ISHR, Rhodes, May 1998.
British Cardiac Society Annual Conference, Glasgow, May 1998.
XIII World Congress of Cardiology, Rio de Janeiro, April 1998.
Annual Scientific Meeting of the Japanese Circulation Society, Tokyo, March 1998.
ISHR European Section, Versailles, July 1997.
XVIII Congress of the European Society of Cardiology, Birmingham, August 1996.
ISHR European Section, Bologna, June 1996.
ISHR American Section, Chicago, June 1996.
XVII Congress of the European Society of Cardiology, Amsterdam, August 1995.
XV World Congress of the ISHR, Prague, July 1995.
Congestive Heart Failure: From Molecular Biology to the Clinic, Venice, June 1995.
ISHR American Section, London - Ontario, July 1994.
Cellular Interactions in Cardiac Pathophysiology, Smolenice, June 1994.
ISHR European Section, Copenhagen, June 1994.
International Conference on Heart Failure, Winnipeg, May 1994.
XV Congress of the European Society of Cardiology, Nice, September 1993.
Myocardial Ischemia and Arrhythmia, Gargellen, June 1993.
Cardiovascular Therapeutics: New Drug Activity, London, October 1992.

PROFESSIONAL ACTIVITIES:

Committees:

Member of World Council, International Society for Heart Research (2001 to date).

Chairman, British Society for Cardiovascular Research (2000 to date).

Member of Council, British Cardiac Society (2000 to date).

Member of NHLBI (NIH, USA) Grant Review Panel (2002).

Member of Selection Committee for the Outstanding Investigator Prize of the ISHR (2002).

Member of International Scientific Program Committee, ISHR 2002 European Section Meeting (2000-02).

Member of Selection Committee for Richard Bing Young Investigator Award, XVII World Congress of the ISHR (2001).

Member of European Section Council, ISHR (1995-2001).

Member of NIH (USA) Advisory Panel on Myocardial Resuscitation (2000).

Member of Selection Committee, Junior Cardiac Club Young Investigator Competition (1997).

Secretary, British Society for Cardiovascular Research (1996-98).

Member of Scientific Program Committee, XVI World Congress of the ISHR (1996-98).

Member of Scientific Advisory Committee, ISHR 1997 European Section Meeting (1996-97).

Member of Study Group on Arrhythmia Research, European Society of Cardiology (1994-95).

Member of Committee, British Society for Cardiovascular Research (1992-95).

Chairman of The Pharmacological Society, University of Bath (1984-85).

Editorial:

Member of Editorial Board, *British Journal of Pharmacology* (2002 to date).

Associate Editor, *Pharmacology & Therapeutics* (2001 to date)

Member of Editorial Board, *Circulation* (2000 to date).

Member of Editorial Board, *Journal of Molecular and Cellular Cardiology* (1999 to date).

Guest Editor, *European Heart Journal Supplements* (1999).

Guest Editor, *CardioExchange* (1999).
Associate Editor, *Cardiovascular Research* (1991-95).
Editor, Bulletin of the British Society for Cardiovascular Research (1987-91).

AWARDS:

Pfizer Academic Award in Biology [for "*work on the signal transduction pathways involved in the regulation of the cardiac Na^+/H^+ exchanger and the cellular mechanisms underlying cardiac ischaemia/reperfusion injury*"– from *Nature*, 27 July 2000, p37] (2000).
Alberta Heritage Foundation Visiting Professorship, University of Alberta, Canada (1994).
Young Investigator Travel Award, American Heart Association Conference on Molecular Biology of the Normal, Hypertrophied and Failing Heart, Asilomar, USA (1993).
Bayer Young Investigator Award (with Dr Masahiro Yasutake), St Thomas' Hospital (1993).
Young Investigator Travel Award, International Society for Heart Research World Congress, Kobe (1992).
Smith Kline & French Prize for Best Industrial Placement Report, University of Bath (1983).

MEMBERSHIP OF PROFESSIONAL BODIES:

British Society for Cardiovascular Research.
British Cardiac Society.
British Pharmacological Society.
International Society for Heart Research (ISHR), European Section.
Council on Basic Cardiovascular Sciences, American Heart Association (Fellow).
Working Group on Cellular Biology of the Heart, European Society of Cardiology.
American Physiological Society.

3. Summary and Achievements of the Submitted Research Work:

This thesis comprises a selection of papers, based on my work on the pathophysiological roles, pharmacological inhibition and cellular regulation of the cardiac sarcolemmal sodium/hydrogen exchanger (also referred to as the Na^+/H^+ exchanger, or NHE), which have been published in the biomedical literature during the period 1991-2002. I am the senior and corresponding author on each of these papers, and I have been principally responsible for the design, execution and/or supervision, analysis and interpretation of the studies described in them, with the invaluable support of the various students and fellows who worked in my laboratory over the relevant period.

My interest in the sarcolemmal NHE was initially triggered by the possibility that its activity may be a critical determinant of cardiac susceptibility to severe ventricular arrhythmias, particularly during reperfusion following a brief period of ischaemia. In the late 1980s, the prevailing view was that the rapid washout during reperfusion of H^+ ions that accumulated extracellularly during the preceding ischaemia would create a trans-sarcolemmal H^+ gradient (higher concentration (i.e. lower pH) inside than outside the myocardial cells), thereby leading to increased sarcolemmal NHE activity and consequently intracellular Na^+ and Ca^{2+} overload, with arrhythmogenic consequences. To study the relative contribution of putative arrhythmogenic processes (such as H^+ washout) during ischaemia and reperfusion, I developed a technique that allowed the independent perfusion of left and right coronary arteries in isolated hearts, through the use of a novel dual-lumen aortic cannula.[1] This technique enabled, for the first time, the selective manipulation of the rate of washout of metabolites (such as H^+ ions) from the extracellular space within the involved zone, in isolated hearts subjected to regional ischaemia. Using this model, we were the first to show that limiting the rate at which extracellular pH is restored during reperfusion, by transient acidic reperfusion of the ischaemic zone, suppresses the occurrence of ventricular fibrillation (VF) and promotes spontaneous reversion to normal sinus rhythm in isolated rat and rabbit hearts subjected to regional ischaemia.[2] Subsequently, we were able to show, in the same model, that the anti-fibrillatory effect of transient acidic reperfusion was mediated by the high H^+ concentration, rather than the low HCO_3^- concentration, of the initial reperfusion

medium.[3] Furthermore, we demonstrated that at least 2 min of acidic reperfusion was required for sustained protection against VF, which was accompanied by enhanced recovery of sarcolemmal Na^+/K^+ ATPase activity.[4] These findings led us to suggest that the ability of transient acidic reperfusion to suppress VF was meditated largely through the inhibition of sarcolemmal NHE activity, with a possible contribution from enhanced recovery of sarcolemmal Na^+/K^+ ATPase activity. In 1994, we were the first academic laboratory to use a novel, selective inhibitor of the sarcolemmal NHE, 3-methylsulphonyl-4-piperidinobenzoyl guanidine (HOE-694), to determine the role of the exchanger in reperfusion-induced arrhythmogenesis. This work revealed that the selective intracoronary infusion of HOE-694 into the ischaemic and reperfused zone of isolated rat hearts, through the use of the dual perfusion model, was as effective as transient acidic reperfusion in suppressing reperfusion-induced VF.[5] Having shown that interventions that inhibited sarcolemmal NHE activity (i.e. acidic reperfusion and HOE-694) suppressed reperfusion-induced VF, we then tested whether the reciprocal relationship also applied, that is whether stimuli that increased sarcolemmal NHE activity produced a pro-arrhythmic effect. In this work, we were able to show that α_1-adrenergic agonists, which had been associated with increased sarcolemmal NHE activity, did indeed increase the incidence of reperfusion-induced VF when infused selectively into the involved zone of isolated rat hearts, through mechanisms that were likely to involve stimulation of the α_{1A}-adrenoceptor subtype and thereby sarcolemmal NHE activity.[6] Taken together, this body of work established the role of H^+ washout and increased NHE activity in reperfusion-induced arrhythmogenesis, and instigated our publication of a review in 1996, which summarised our findings in this area as well as pertinent work by others.[7]

Our work on the role of the sarcolemmal NHE in arrhythmogenesis, summarised above, as well as data emerging from other laboratories, made us query a broader role for exchanger activity in mediating myocardial injury during ischaemia and reperfusion. On the basis of the experience we had gained with the then novel NHE inhibitor HOE-694 in our arrhythmia work,[5] we then tested whether this compound would affect the contractile dysfunction that arises from global ischaemia and reperfusion in isolated rat hearts and, specifically, whether any benefit would arise from NHE inhibition only during early reperfusion. To our surprise, and against the prevalent dogma of the time, the administration of HOE-694 at

reperfusion afforded no benefit when used in the presence of a physiological buffer (HCO_3^-); in contrast, when given from before the onset of ischaemia, the NHE inhibitor produced a significant improvement in the post-ischaemic recovery of left ventricular contractile function.[8] Thus, it appeared that, while NHE activity during reperfusion after a short period (≤10 min) of regional ischaemia was an important determinant of the severity of reperfusion-induced arrhythmias, such activity during reperfusion after longer periods (≥20 min) of global ischaemia did not play a key role in determining the recovery of contractile function. Instead, our data suggested that, in the latter setting, NHE activity during the prolonged ischaemic period was the more important factor, necessitating the pre-ischaemic administration of the NHE inhibitor to preserve myocardium and improve the recovery of contractile function during reperfusion. We next explored the inter-relationship between NHE activity and ischaemic preconditioning, a powerful cardioprotective intervention which has been shown to slow the progression of myocardial injury during ischaemia, again in rat hearts subjected to prolonged global ischaemia and subsequent reperfusion. This work led us to conclude that NHE inhibition and ischaemic preconditioning afforded comparable and additive cardioprotective benefit (as reflected by improved contractile function and reduced enzyme leakage) and suggested that increased NHE activity may be a detrimental "side effect", rather than a protective mechanism (as had been suggested by others), of ischaemic preconditioning.[9] This work inspired several subsequent studies in other laboratories, including some in large animal *in vivo* models, with essentially similar findings; one such publication was accompanied by an editorial which I was invited to write and which provided a critical assessment of the available evidence in the pertinent area.[10] In related work, we also evaluated the efficacy of NHE inhibition as an adjunct or additive to hypothermic cardioplegic arrest, which is used commonly for intra-operative myocardial protection during cardiac surgery. In this study, we used a structural congener of HOE-694, 4-isopropyl-3-methylsulphonylbenzoyl guanidine (HOE-642, now known by the proprietary name cariporide), which exhibited greater potency and selectivity as an inhibitor of the sarcolemmal NHE. The data provided by this study allowed us to conclude that the use of cariporide as an adjunct or additive to cardioplegia afforded significant protective benefit on ischaemic and reperfused myocardium (again, as reflected by improved functional recovery and reduced enzyme leakage), under not only normothermic but also moderately and severly

hypothermic conditions.[11] Intriguingly, the efficacy of the adjunctive use of cariporide (i.e. the inhibitor given at the time of reperfusion after cardioplegic arrest) appeared to be enhanced in hearts subjected to cardioplegic arrest under conditions of moderate or severe hypothermia. Our more recent work suggests that this may be related to the effects of episodes of hypothermia and rewarming on sarcolemmal NHE activity and may have clinical relevance (see later). My views on the rationale behind the potential therapeutic application of pharmacological NHE inhibitors and their likely mechanisms of action during myocardial ischaemia and reperfusion have been described in recent invited reviews.[12,13]

The apparent involvement of the sarcolemmal NHE in myocardial injury during ischaemia and reperfusion, as well as other emerging evidence that the sarcolemmal NHE may also modulate myocardial contractile and growth responses to neurohormonal and mechanical stimuli, revealed the need to explore and understand the cellular and molecular mechanisms that regulate exchanger activity. To facilitate such work, I established a new technique in my laboratory, which enabled the measurement of intracellular pH in single cardiac ventricular myocytes by microepifluorescence, using a pH-sensitive intracellular dye. In initial studies that employed this technique, we determined the effects on sarcolemmal NHE activity of thrombin, a multifunctional protease which, in addition to its established roles in thrombus formation and blood coagulation, induces a variety of cellular responses through a family of thrombin receptors. This work provided the first evidence that adult rat ventricular myocytes express a functional thrombin receptor (now known as protease-activated receptor 1, or PAR1), whose stimulation produces increased activity of the sarcolemmal NHE, through a mechanism mediated via protein kinase C (PKC).[14] This finding led us to explore the regulation of sarcolemmal NHE activity through a variety of other G protein-coupled receptors which are stimulated by endogenous neurohormonal mediators of pathophysiological significance. Our work on the regulation of the sarcolemmal NHE by angiotensin II revealed that this peptide hormone exerted a stimulatory effect on sarcolemmal NHE activity via the AT_1 receptor subtype, through intracellular signalling mechanisms that required the activation of PKC and the extracellular signal-regulated kinase (ERK) pathway.[15] Intriguingly, the positive effect of AT_1 receptor stimulation on sarcolemmal NHE activity was counteracted by AT_2 receptor stimulation, through a mechanism that did

not involve direct inhibition of the exchanger or attenuation of ERK activation.[15] Following on from our earlier work on the pro-arrhythmic effects of selective α_{1A}-adrenoceptor stimulation within ischaemic and reperfused myocardium,[6] we also investigated the potential involvement of the various cloned α_1-adrenoceptor (α_1-AR) subtypes (α_{1A}-, α_{1B}- and α_{1D}-AR) in the regulation of sarcolemmal NHE activity in adult rat ventricular myocytes. Our analysis of EC_{50} and IC_{50} values obtained for a variety of α_1-AR agonists and antagonists in ventricular myocytes, in relation to the relative affinities of these agents for recombinant rat α_1-AR subtypes, revealed that α_1-adrenergic stimulation of sarcolemmal NHE activity was likely to be mediated selectively by the α_{1A}-AR subtype.[16] In view of the fact that the α_{1A}-AR is the predominant α_1-AR subtype expressed in human myocardium, we have since continued to spend considerable effort to delineate the molecular mechanisms that mediate sarcolemmal NHE stimulation via this receptor. Our more recent data have shown that, like the angiotensin II AT_1 receptor-mediated response, α_{1A}-AR-mediated stimulation of the sarcolemmal NHE in adult rat ventricular myocytes requires activation of the ERK pathway and that the ERK-mediated effect is likely to occur via activation of the 90 kDa ribosomal S6 kinase ($p90^{rsk}$), a putative NHE kinase.[17] This work has also shown that α_{1A}-AR-mediated stimulation of sarcolemmal NHE activity also requires PKC activation, but that PKC-mediated regulation occurs through an ERK-independent pathway.[17] Having demonstrated that stimulation of a variety of G protein-coupled receptors that signal via G_q proteins (e.g. PAR1, AT_1 and α_{1A}-AR) invariably increases sarcolemmal NHE activity, commonly through the activation of ERK and PKC signalling pathways, we have also recently investigated the effects of stimulation of receptors that signal via other G proteins. This work has revealed that stimulation of a G_i protein-coupled receptor, the adenosine A_1 receptor, does not affect sarcolemmal NHE activity alone, but opposes α_{1A}-AR-mediated stimulation of the exchanger, thus suggesting the existence of a novel inhibitory receptor crosstalk mechanism.[18] Our current work is aimed at identifying the molecular mechanisms that underlie such crosstalk, focusing on the role of proteins of the "regulators of G protein signalling (RGS)" family and altered activation of ERK and PKC.

Our work described above, and complementary studies by others, have

indicated that the ERK pathway is a key mediator of sarcolemmal NHE stimulation through a variety G_q protein-coupled receptors and that such stimulation is likely to occur through the downstream activation of $p90^{rsk}$, which in turn phosphorylates the NHE regulatory domain. However, the molecular mechanism(s) through which sarcolemmal NHE activity is regulated by PKC, which our work has suggested also plays a key role but does not directly phosphorylate the NHE regulatory domain, are unclear. We have explored the possibility that a recently discovered kinase, protein kinase D (PKD), may be involved in PKC-mediated regulation of sarcolemmal NHE activity. Our work has shown that PKD (which is also known as PKCμ) is indeed expressed in myocardium, where its activity is increased by α_1-AR stimulation and PKC activation.[19] In the absence of specific pharmacological modulators of PKD activity, in order to explore the functional role of PKD in regulating NHE activity we have adapted our microepifluorescence technique for the selective measurement of NHE activity in cells transfected with plasmids containing different PKD cDNA constructs. In this work, we transfected COS-7 and A-10 cells (which are easier to transfect than cardiac ventricular myocytes) with wild-type PKD or a kinase-inactive mutant (which acts in dominant-negative fashion to inhibit endogenous PKD), together with green fluorescent protein (GFP) to allow identification of transfected cells for measurement of NHE activity. Contrary to our initial expectation, this work suggested that PKD may be an inhibitory, rather than a stimulatory, regulator of NHE activity.[20] This raises the intriguing possibility that PKC activation may lead to the activation of both NHE-*inhibitory* (e.g. PKD) and NHE-*stimulatory* (unknown) downstream signalling moieties, potentially through distinct PKC isoforms, and that the overall outcome may depend on the specific cellular context. Currently, we are extending this work to investigate the roles of different PKC isoforms in the regulation of sarcolemmal NHE activity, through the use of membrane-permeant, PKC isoform-specific agonist and antagonist peptides, as well as myocytes from the hearts of knock-out mice with targeted disruption of genes encoding selected PKC isoforms. Additionally, we are testing the applicability of our findings in COS-7 and A-10 cells[20] to the myocardium, through the use of adenovirus-mediated gene transfer to modulate PKD expression and function in rat ventricular myocytes.

As part of our effort aimed at the determination of the cellular and molecular mechanisms that regulate the sarcolemmal NHE, we have also investigated the roles

of other factors such as age, temperature and oxidative stress. For example, our work has shown that the sarcolemmal NHE is subject to developmental regulation, with a progressive decline in the postnatal period in both expression (at the mRNA level) and activity.[21] With regard to the effects of temperature, we have shown that moderate hypothermia (as may be encountered by the myocardium during cardiac surgery) produces a significant, but partial, inhibition of sarcolemmal NHE activity, but does not affect the NHE-inhibitory potency of cariporide.[22] The latter observation may be of therapeutic significance, since cariporide is being tested in current clinical trials for potential application for cardioprotection in high-risk patients undergoing coronary artery bypass surgery, during which hypothermic arrest is commonly employed. We have recently extended this work to investigate the effects of different degrees of hypothermia followed by rewarming on sarcolemmal NHE activity and NHE-regulatory signalling pathways. This work suggests that, while hypothermia itself undoubtedly inhibits sarcolemmal NHE activity, episodes of hypothermia followed by rewarming may lead to a significant *increase* in such activity, which is likely to be of relevance to myocardial protection during cardiac surgery and transplantation (manuscript currently under review). Most recently, we have shown that oxidative stress (induced by exogenous hydrogen peroxide) increases sarcolemmal NHE activity through a variety of signalling mechanisms, some of which (e.g. the ERK/p90^{rsk} and PKC pathways) resemble those utilised by G_q protein-coupled receptors to produce a similar response.[23] The various mechanisms through which sarcolemmal NHE activity may be regulated and the potential pathophysiological significance of such regulation have been reviewed in several articles which I have been invited to write in recent years.[24-26]

A considerable amount of information has emerged in recent years, from work in our laboratory and many others, on the pathophysiological roles of the sarcolemmal NHE, the therapeutic potential of its inhibitors and the cellular and molecular mechanisms that regulate its expression and activity. Nevertheless, notwithstanding recent clinical trials with NHE inhibitors, this information has been obtained almost exclusively from work utilising hearts and cardiac myocytes from a variety of animal species, and its relevance to humans is unconfirmed. Recently, we have begun to address this issue, by determining, for the first time, sarcolemmal NHE activity in human ventricular myocytes.[27] This work has revealed that, in

human ventricular myocardium, sarcolemmal NHE activity arises from the NHE1 isoform and is indeed inhibited for cariporide, accurately reflecting previous findings from animal studies; intriguingly, our work with human myocytes (obtained from explanted recipient and unused donor hearts) has also suggested that hearts with end-stage heart failure may exhibit increased sarcolemmal NHE activity, through altered post-translational regulation of the exchanger.[27] Taken together with recent animal studies, this work is consistent with an additional pathophysiological role for NHE activity in heart failure, and may indicate a novel therapeutic application for NHE inhibitors.

As outlined above, my interest in the sarcolemmal NHE arose initially from its putative role in mediating myocardial injury and dysfunction during ischaemia and reperfusion. Progress in this field has been rapid, such that several clinical trials have recently been completed with NHE inhibitors in patients at risk of such injury and dysfunction, while others are currently underway. As we have reviewed very recently,[28] the findings of the clinical trials that have been completed to date have been mixed, but wholly consistent with the relevant scientific knowledge base. Ongoing clinical trials will establish whether NHE inhibitors will find therapeutic utility as cardioprotective agents in the clinical setting. In the meantime, on the basis of the strong evidence that NHE activity contributes not only to myocardial injury during ischaemia and reperfusion but also to the regulation of myocardial contractility, growth, hypertrophy and failure, our efforts continue towards the delineation of the cellular and molecular mechanisms that regulate NHE expression and activity in the heart, as well as the determination of the functional consequences of spontaneous or induced changes in these parameters.

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4. Submitted Papers (in order of citation in 3.):

special communications

Independent dual perfusion of left and right coronary arteries in isolated rat hearts

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AVKIRAN, METIN, AND MICHAEL J. CURTIS. *Independent dual perfusion of left and right coronary arteries in isolated rat hearts.* Am. J. Physiol. 261 (Heart Circ. Physiol. 30): H2082–H2090, 1991.—A novel dual lumen aortic cannula was designed and constructed to permit independent perfusion of left and right coronary beds in isolated rat hearts without necessitating the cannulation of individual arteries. Stability of the dual-perfusion preparation was shown to be similar to that of the conventional Langendorff preparation, in terms of coronary flow, heart rate, and high-energy phosphate content. The independence of left and right perfusion beds was confirmed by unilateral infusion of disulfine blue dye and spectrophotometric detection of the dye in ventricular homogenates. Transient cessation of flow to the left coronary bed resulted in severe ventricular arrhythmias upon reperfusion, as in conventional models of regional ischemia and reperfusion. The dual-perfusion model is technically undemanding, reproducible, inexpensive, and can be used in several species. It enables studies with *1)* regional low flow ischemia, *2)* regional zero-flow ischemia without coronary ligation (with attendant damage to vasculature), *3)* selective application of drugs or interventions to the ischemic-reperfused zone, and *4)* selective application of components of ischemia and reperfusion to a site anatomically relevant to ischemic heart disease.

models; regional ischemia; reperfusion; arrhythmias

MYOCARDIAL ISCHEMIA and subsequent reperfusion lead to an impairment of cellular metabolic and ionic homeostasis, which is associated with contractile dysfunction and the development of ventricular arrhythmias. Isolated Langendorff-perfused hearts from small mammals are commonly used to study the pathophysiology of ischemia and reperfusion by regional or global manipulation. Current models, however, have several limitations. Global ischemia does not adequately mimic the condition of regional ischemia in the human, although it is relevant as a model of cardiac arrest during surgery. Although regional ischemia can be readily induced in small mammalian hearts by coronary artery ligation, there are problems associated with this technique. First, coronary ligation is an all or none process, and the degree of flow reduction cannot be regulated (thus low flow regional ischemia cannot be modeled). Second, coronary ligation itself may cause vascular damage and may disrupt venous and lymphatic drainage.

Limitations of current models extend to their use in assessment of the pathophysiological role of individual components of ischemia and reperfusion (defined as metabolic or ionic factors whose homeostasis is impaired). Although global manipulation of components may be satisfactory for the assessment of their effects on myocardial metabolism and contractile function, it is not so within the context of arrhythmogenesis where regional heterogeneities may play a critical role (5, 6). A large number of components have been implicated as arrhythmogenic factors. During ischemia, such components include catecholamines (22–24), thromboxane A_2 (3), adenosine 3′,5′-cyclic monophosphate (cAMP) (20), lipid metabolites (4), platelet-activating factor (25), calcium (11), potassium (14, 15, 17, 19), and protons (15, 17). During reperfusion, components include oxygen free radicals (9, 28), cAMP (21), and intracellular calcium (18). Although several studies have been carried out with the local infusion of components of ischemia into selected coronary arteries of large mammalian hearts such as those of the dog and the pig, both in vivo and in vitro (11, 17, 19), comprehensive studies have not been possible because of cost and technical limitations. In species such as the dog, the issue is further complicated by the difficulty in regulating the extracellular concentration of the substance under investigation (e.g., 19) owing to significant collateral communication between adjacent coronary beds (16). Studies with isolated hearts of smaller mammals such as the rat and the rabbit, which have negligible collateral flow, have been limited almost exclusively to the use of global interventions (e.g., 1, 13, 29), since manipulation of regional flow has hitherto not been possible.

The unavailability of a technique that permits manipulation of regional flow in isolated small mammalian hearts has also limited the utility of such hearts in studies of the mechanism and site of action of putative therapeutic agents. Thus, in studies employing regional ischemia and reperfusion, selective regional application of agents to the involved zone has not been possible, necessitating the use of global application.

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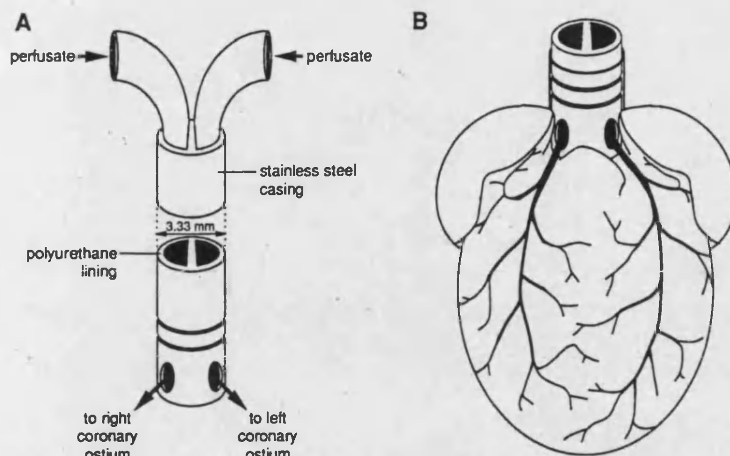

FIG. 1. Schematic diagrams depicting design of dual-perfusion cannula (*A*), and correct position of cannula in ascending aorta of an isolated heart (*B*). Each hemicylinder receives an independent supply of perfusate from distal end. Orifices near proximal end are located to appose left and right coronary ostia when cannula is in position in ascending aorta. For details of availability of cannula, contact author.

In view of the above, a recent attempt was made to develop a model using the isolated rabbit heart in which adjacent coronary beds could be perfused independently (5). However, the model has several potential disadvantages: *1*) it is technically complex, since it requires cannulation of the left coronary artery, *2*) constant pressure perfusion is precluded owing to the necessarily small bore (and hence high resistance) of the cannula, *3*) its utility for arrhythmia studies is limited by an intrinsically low susceptibility to ischemia-induced and reperfusion-induced arrhythmias (cf. 6 and 26), and *4*) rabbit costs are high in comparison with smaller species.

The objective of the present study was to overcome the disadvantages of the rabbit dual-perfusion model. We opted to use the rat heart, which is relatively inexpensive versus the rabbit yet has a similar paucity of collateral anastomoses (16). To achieve our objective we developed a novel dual lumen aortic cannula, which permits independent perfusion of left and right coronary arteries via their ostia without the necessity of intracoronary cannulation. This paper describes and characterizes the new model.

MATERIALS AND METHODS

Design of Aortic Cannula

A fundamental problem in developing a rat dual-perfusion model is how to achieve an independent supply of perfusate to left and right coronary arteries. The insertion of cannulas into individual coronary arteries can be ruled out, since the cannulas would, by necessity, have a very narrow bore that would not permit constant pressure perfusion at physiological pressures (60–90 mmHg) or constant flow perfusion at flow rates (8–12 $ml \cdot min^{-1} \cdot g^{-1}$) sufficient to provide adequate oxygenation with crystalloid solutions. We therefore adopted an alternative approach and designed an aortic cannula that would enable the perfusion of left and right coronary arteries via their ostia, without the necessity of intracoronary cannulation.

The coronary anatomy of the rat heart has been described in detail by Halpern (10). The two ventricles and the septum are supplied by right and left coronary arteries arising from the ascending aorta. The septal artery is usually presented as the first branch of either the left or the right coronary artery. In the majority of hearts, single main left and right trunks arise from single ostia (2). Occasionally the septal artery, the circumflex artery, or the conus artery present as separate arteries originating from the aorta with their own ostium. The aortic cannula was designed to accommodate these possible variations in coronary anatomy.

The design of the cannula is depicted in Fig. 1. The standard cannula consists of stainless steel tubing of 3.33 mm external diameter. This cannula size fits snugly into the ascending aorta of a heart from a 220- to 280-g rat, stretching the aorta taut and forming a tight seal between the cannula and the aorta. The cylindrical interior of the cannula is lined with custom-made medical grade polyurethane and divided into two equal hemicylinders by a central septum. Each hemicylinder receives an independent supply of solution from the proximal end. The distal end is sealed, and two bore holes are made through the stainless steel and polyurethane near the distal end to allow exit of solution from each hemicylinder. These holes are located to appose the right and left coronary ostia when the cannula is inserted into position in the aorta. The openings are sufficiently large (~1.0–1.2 mm in diam) enough to span either an individual ostium (in the case of single left and right ostia) or pairs of ostia (in the event that coronary arteries arise from a pair of adjacent ostia, as is sometimes the case). The physical characteristics of the dual-perfusion cannula are such that flow through each hemicylinder of the cannula exceeds 50 ml/min with a pressure head of 100 cmH_2O (75 mmHg) and, within the range of flow rates normally encountered in individual coronary beds (3–8 ml/min), the pressure drop across each hemicylinder is <5 mmHg (Fig. 2).

Perfusion Apparatus

Each hemicylinder was supplied with an oxygenated perfusion solution from a temperature-regulated reservoir (37°C) at a constant pressure of 100 cmH_2O (Fig.

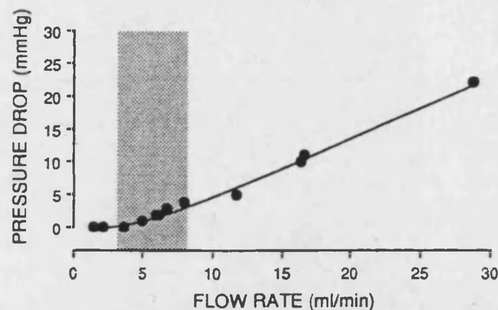

FIG. 2. Characterization of pressure gradient across individual hemicylinder of dual-perfusion cannula in absence of a heart. Pressure was simultaneously recorded proximal to point of entry and distal to point of exit of perfusion fluid, at different flow rates. Perfusion fluid was supplied from a reservoir 100 cm above cannula. Shaded area represents range of flow rates normally encountered in individual coronary beds; within this range, pressure drop across cannula remained <5 mmHg.

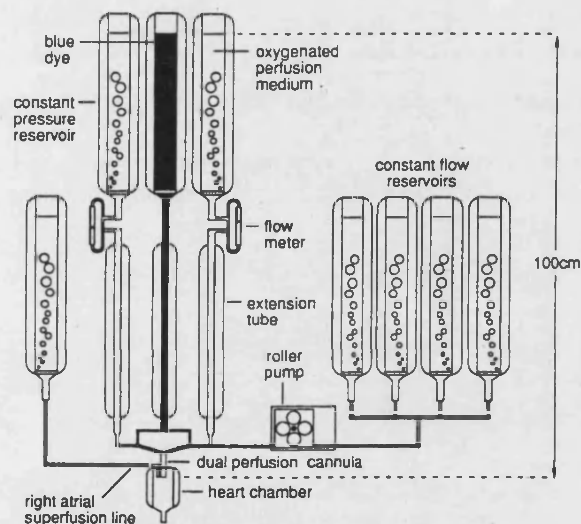

FIG. 3. Perfusion apparatus used in conjunction with dual-perfusion cannula. Perfusion of either coronary bed can be switched from constant pressure to constant flow at required flow rate through use of a roller pump. Randomized concentration-response studies can be performed through use of multiple reservoirs, as illustrated. Disulfine blue dye can be administered unilaterally to enable delineation of left and right coronary beds.

3). The flow through each hemicylinder was continuously monitored using two in-line flowmeters (Meterate, Jencons Scientific, Leighton Buzzard, UK, detection range 0.25–12.00 ml/min). Before use the flowmeters were calibrated at 37°C, using a precision roller pump (Gilson Minipuls 3). A central reservoir held buffer containing disulfine blue dye (0.016% wt/vol), which could be infused through either side of the cannula to delineate the two perfusion beds. The heart was housed in a temperature-regulated chamber and maintained at 37°C. To maintain sinus rate, the right atrium and sinoatrial node were continuously superfused with oxygenated perfusion medium (37°C) at a constant flow rate of 5 ml/min (7).

Perfusion Medium

All hearts were perfused with Krebs-Henseleit solution, modified to the following composition (in mmol/l): 118.5 NaCl, 25.0 $NaHCO_3$, 3.2 KCl, 1.2 $MgSO_4$, 1.2 KH_2PO_4, 1.4 $CaCl_2$, and 11.0 glucose. The solution (pH 7.4 at 37°C) was continously gassed with carbogen (95% O_2-5% CO_2).

Perfusion Protocol

Male Wistar rats (Bantin and Kingman) were anesthetized with diethylether after which 50 U of heparin sodium was injected into the left femoral vein. The chest was opened and the heart excised and immersed in perfusion medium at 4°C. Within 30 s of excision, a dual-perfusion cannula was inserted into the ascending aorta (in exactly the same way that a conventional Langendorff cannula is inserted) and secured in position with a braided silk suture. Coronary perfusion was then begun at a perfusion pressure of 100 cmH_2O, and coronary ostia were aligned with the cannula orifices, using in-line monitoring of left and right coronary flows as a guide. The aorta was rotated on the cannula until flow in each perfusion bed reached a maximum, a procedure that could be accomplished within 20 s after insertion of the cannula. The pulmonary artery was cut near its origin, and a stainless steel needle was inserted into the left ventricle via the apex to enable adequate drainage of coronary and thebesian venous effluent. The heart was then subjected to one of the following protocols.

Stability of preparation. A protocol was designed to test the hemodynamic and metabolic stability of the dual-perfusion preparation relative to the Langendorff preparation. To enable such an assessment, some hearts were cannulated with a standard single-lumen aortic cannula of equivalent diameter (3.33 mm OD) and perfused in the Langendorff mode with perfusion solution supplied from the two reservoirs used for dual perfusion.

Hearts (n = 8 per group) were subjected to 60 min of perfusion in the dual perfusion or the Langendorff mode with continuous monitoring of heart rate and coronary flow. At the end of the perfusion period the hearts were rapidly frozen using a pair of stainless steel clamps precooled in liquid nitrogen. The hearts were then stored in liquid nitrogen for subsequent analysis of tissue ATP and creatine phosphate (CrP) content.

Independence of right and left coronary artery perfusion. This protocol was designed to test the adequacy of the seal formed between the aorta and the dual-perfusion cannula by assessment of the extent of cross-flow between the two orifices of the cannula at this level.

Hearts (n = 8) were perfused in the dual-perfusion mode with oxygenated bicarbonate buffer for 15 min during which period heart rate and coronary flow were monitored. The perfusion fluid supplied to one coronary bed was then switched for 1 min to a similar solution containing 0.016% wt/vol disulfine blue dye. Four hearts received dye to the right coronary bed and the other four to the left. The heart was then removed from the perfusion apparatus, the atria and mediastinal tissue were removed, and the dyed ventricular tissue was carefully

dissected away from the remainder. The dyed and nondyed sections of tissue, corresponding to the ventricular masses supplied by the left and right coronary beds, were then lightly blotted and weighed. Samples of ~140 mg were then dissected from the left and right ventricular free walls for spectrophotometric detection of the presence of blue dye (see below).

Left-right coronary collateral communication during coronary occlusion in the rat heart provides to the ischemic zone <10% of normal flow (16). We, therefore, expected to detect dye in the nominally dye-free bed at a concentration significantly <10% of that in the dye-perfused bed (since hydrostatic pressure in the nominally dye-free bed must exceed that in an ischemic zone after coronary occlusion). Any value exceeding this would indicate an inadequate seal between the dual-perfusion cannula and the aorta. To test the sensitivity of the dye-detection method and to provide a calibration for 10% cross-flow, an additional heart received a 1:10 dilution of dye (0.0016% wt/vol) simultaneously into each perfusion bed for 1 min. Left and right ventricular free wall samples of similar size (~140 mg) obtained from this heart were then processed and underwent spectrophotometric dye detection in an identical fashion to the other samples.

Effects of transient regional ischemia and reperfusion. This protocol was designed to test the arrhythmogenic effect of transient cessation of flow in the left coronary bed and to compare the pattern of reperfusion-induced arrhythmogenesis with that previously reported in conventional models of regional ischemia and reperfusion.

Hearts (n = 12) were perfused in the dual-perfusion mode with oxygenated bicarbonate buffer for 15 min after which flow to the left coronary bed was stopped while the right coronary bed continued to be perfused at constant pressure. After 10 min of ischemia, the left coronary bed was reperfused at constant pressure for 5 min, during which arrhythmias were assessed. At the end of each experiment, the perfusion fluid supplied to the left coronary bed was switched for 1 min to a solution containing 0.016% wt/vol disulfine blue dye after which the heart was removed from the perfusion apparatus. The atria and mediastinal tissue were removed and the dyed tissue, representing the ventricular myocardium subjected to ischemia and reperfusion, was carefully dissected away from the remainder. The dyed and nondyed sections of tissue were then lightly blotted and weighed. Ischemic zone size was expressed as a percentage of total ventricular weight.

Measured Variables

Coronary flow, heart rate, and electrogram. Coronary flow was measured using the in-line flowmeters, and heart rate was determined from a unipolar electrogram. The electrogram was obtained using a silver electrode inserted into the left ventricular free wall and a reference electrode connected to the aorta via the stainless steel casing of the aortic cannula. The electrogram signal was amplified (Gould Universal Amplifier), continuously monitored on a digital storage oscilloscope (Gould type 1421), and recorded on an ink-jet recorder (Gould 2200S).

Tissue ATP and CrP content. Frozen ventricular tissue samples were lyophilized (Edwards Modulyo Freeze Dryer) before assay in duplicate. ATP and CrP were extracted into perchloric acid and quantified using a spectrophotometric hexokinase/glucose-6-phosphate dehydrogenase assay, as described by Hearse (12). Analysis of samples was performed in a blinded manner.

Tissue disulfine blue dye content. First, the maximum absorbance wavelength of disulfine blue dye (650 nm) was determined from scanning wavelength spectra of several concentrations of the dye in deionized water, using a Pye Unicam SP8–100 UV spectrophotometer. Ventricular samples were then homogenized in 3 ml of deionized water and centrifuged at 3,000 rpm for 10 min. Scanning spectra of the supernatants were obtained by recording their absorbance between the wavelengths of 800 and 500 nm. Blanks were prepared from ventricular homogenates from hearts not perfused with dye. The presence of a discernible absorption peak at 650 nm was taken to indicate the presence of disulfine blue dye. The peaks observed in nominally dye-free samples were quantified in relation to the size of the peak obtained from the appropriate sample receiving a 1:10 dilution of the dye. In this manner we obtained a semiquantitative assessment of the extent of cross-flow.

Arrhythmias. The electrogram was analyzed for the incidence and duration of ventricular tachycardia (VT) and ventricular fibrillation (VF) in accordance with the Lambeth Conventions (27). VT was defined as four or more consecutive premature beats of ventricular origin, and VF was defined as a signal in which individual QRS deflections could no longer be distinguished from one another and for which rate could not be determined.

RESULTS

Stability of Preparation

The hearts used for Langendorff perfusion or dual perfusion in this protocol were obtained from two groups of rats (n = 8 per group) with mean body weights of 266 ± 10 and 262 ± 9 g, respectively.

Figure 4 shows the temporal changes in coronary flow and heart rate in the two groups of hearts perfused for 60 min in the Langendorff or the dual-perfusion mode. Total coronary flow profiles were similar in the two groups, declining slightly during the first 15 min of perfusion and remaining stable thereafter. There was no significant difference between the groups at any time point. Heart rate profiles in each group were also similar with no significant difference between groups at any time point.

Tissue high-energy phosphate (ATP and CrP) contents after 60 min of aerobic perfusion were similar in the two groups. Tissue ATP was 19.2 ± 0.2 and 19.5 ± 0.3 μmol/g dry wt, and tissue CrP was 22.0 ± 1.1 and 19.3 ± 0.9 μmol/g dry wt in the Langendorff and dual-perfusion groups, respectively.

Independence of Right and Left Coronary Artery Perfusion

The hearts perfused in the dual-perfusion mode for this protocol were obtained from rats (n = 8) with a

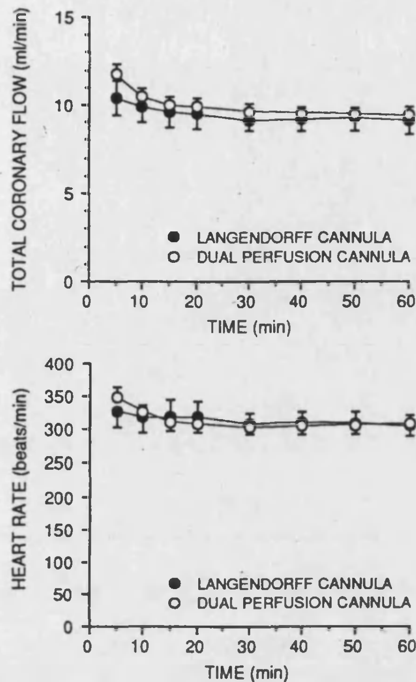

FIG. 4. Profiles of total coronary flow (*top*) and heart rate (*bottom*) during 60 min of perfusion in Langendorff or dual-perfusion modes (n = 8 per group). There were no significant differences in either variable between the groups at any time point.

mean body weight of 231 ± 2 g. Mean total ventricular wet weight was 0.83 ± 0.02 g, and mean heart rate at the end of 15 min aerobic perfusion was 315 ± 17 beats/min. Despite the variability in the size of the left and right coronary artery beds (due to variable septal artery origin), the mean coronary flows normalized for the wet weight of each bed were similar at 11.4 ± 1.6 $ml \cdot min^{-1} \cdot g^{-1}$ in the left coronary bed and 12.2 ± 1.0 $ml \cdot min^{-1} \cdot g^{-1}$ in the right coronary bed.

Figure 5 shows the typical delineation of the two coronary beds after unilateral perfusion with disulfine blue dye. The outline of the border between the two coronary beds was identical regardless of the site of dye infusion, confirming the presence of two discrete perfusion beds. Figure 6 shows the absorption spectra obtained from left and right ventricular free wall homogenates. Large absorption peaks were observed at a wavelength of 650 nm in samples perfused unilaterally with disulfine blue dye. Only very small absorption peaks were detectable at 650 nm in contralateral samples (not perfused with dye), and these were significantly smaller than the peaks obtained in the corresponding samples that received a 1:10 dilution of dye.

Unilateral perfusion with disulfine blue dye permitted determination of whether the interventricular septal artery arose from the left or right coronary artery. Left dominance was found in 50% of hearts. In these hearts the mean weights of ventricle supplied by the left and right coronary artery beds, as a percentage of total ventricular weight, were 71 ± 5 and 29 ± 5%, respectively. In the remaining 50% of hearts that exhibited right dominance, the corresponding values were 46 ± 3 and 54 ± 3%, respectively.

Reproducibility of Preparation

To date, a total of 153 hearts with a mean total ventricular wet weight of 0.85 ± 0.01 g have been perfused in various experimental protocols, using the dual-perfusion cannula. Of these hearts, 33% (51/153) displayed left dominance and 58% (88/153) displayed right dominance. In the remaining 9% (14/153) the interventricular septum was supplied by both left and right coronary arteries. The left and right coronary beds supplied 72 ± 1 and 28 ± 1% of the ventricular weight, respectively, in hearts with left dominance (n = 51), and 52 ± 1 and 48 ± 1% of the ventricular weight, respectively, in hearts with right dominance (n = 88). These values are very similar to those obtained from the much smaller (n = 4) population samples described above. The left and right coronary beds supplied 57 ± 3 and 43 ± 3% of the ventricular weight, respectively, in those hearts that did not display a clear septal dominance (n = 14). The mean coronary flows normalized for tissue wet weight were

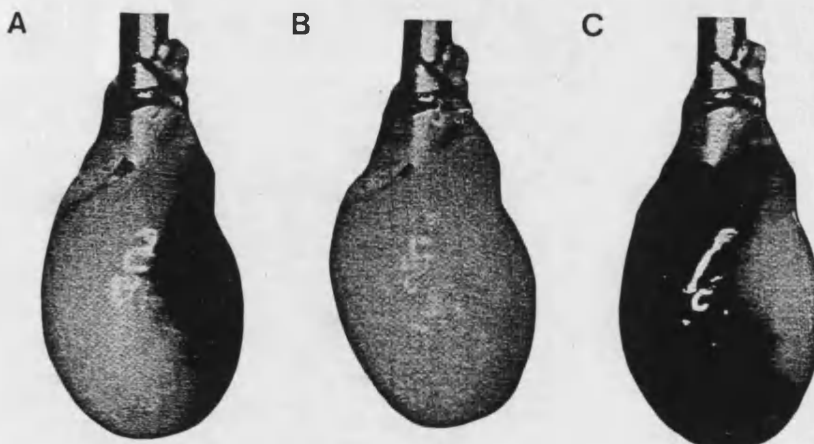

FIG. 5. Typical delineation of left and right coronary beds by unilateral infusion of disulfine blue dye. Same heart is shown during dye infusion into left coronary bed (*A*), after washout of dye (*B*), and during dye infusion into right coronary bed (*C*). Right coronary ostial orifice of dual-perfusion cannula is also visible (by right atrial appendage).

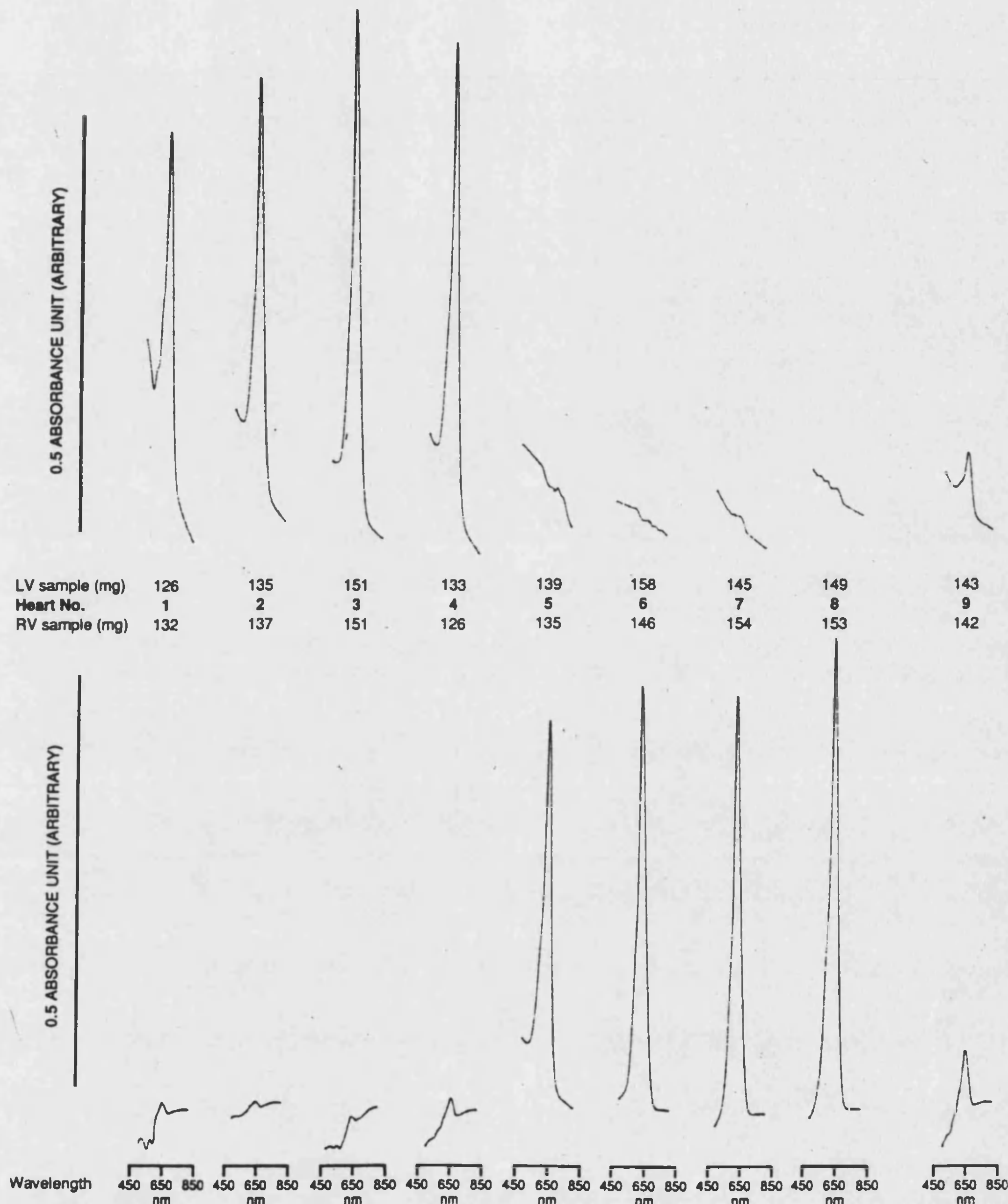

FIG. 6. Scanning absorption spectra (500–800 nm) obtained from homogenates of left ventricular (LV, *top*) and right ventricular (RV, *bottom*) free wall samples from 9 hearts. *Hearts 1–4* received disulfine blue dye only to left coronary bed and *hearts 5–8* only to right coronary bed. *Heart 9* received a 1:10 dilution of dye in both coronary beds. Left and right ventricular samples were, by design, of similar weight.

11.9 ± 0.2 ml·min^{-1}·g^{-1} in the left coronary bed and 12.3 $\pm$ 0.3 ml·min^{-1}·g^{-1} in the right coronary bed ($n = 153$). These values are also very similar to those obtained from the sample of eight hearts described above.

Effects of Transient Regional Ischemia and Reperfusion

The hearts used in this protocol ($n = 12$) were obtained from rats with a mean body weight of 236 $\pm$ 3 g. Mean total ventricular wet weight was 0.70 $\pm$ 0.02 g and mean size of the left coronary bed, representing the ischemic zone, was 54 $\pm$ 3% of total ventricular weight. Mean values for heart rate and coronary flow in the left and right coronary beds, measured at the end of the initial 15-min period of aerobic perfusion, were 326 $\pm$ 6 beats/min, 11.6 $\pm$ 0.6 ml·min^{-1}·g^{-1}, and 10.2 $\pm$ 0.7 ml·min^{-1}·g^{-1}, respectively. Heart rate and flow in the right coro-

nary bed remained stable during the period of regional ischemia and were 334 ± 11 beats/min and 10.1 ± 0.8 $ml \cdot min^{-1} \cdot g^{-1}$, respectively, 1 min before the onset of reperfusion. Heart rate could not be measured during reperfusion because of severe ventricular arrhythmias. Coronary flow increased to 24.4 ± 2.5 $ml \cdot min^{-1} \cdot g^{-1}$ in the reperfused zone 1 min after the onset of reperfusion, indicating reactive hyperemia. Reduced extravascular compression, due to loss of coordinated contractile activity (see below), probably contributed to the increased flow, since flow also increased in the adjacent bed during reperfusion, albeit to a lesser extent (16.4 ± 1.5 $ml \cdot min^{-1} \cdot g^{-1}$ at 1 min).

All hearts were in sinus rhythm immediately before reperfusion. Upon reperfusion, all hearts developed VT within a few beats. In 1 heart VT reverted to normal sinus rhythm, while in the remaining 11 hearts it degenerated into VF (Fig. 7). In 10 hearts, VF was sustained until the end of the 5-min reperfusion period (Fig. 7).

DISCUSSION

We have developed and characterized a dual-perfusion model for small mammalian hearts. The model utilizes a novel aortic cannula that permits independent perfusion of left and right coronary arteries, yet is no more technically demanding than the conventional Langendorff model. The model has potential utility in the study of regional myocardial ischemia beyond the scope of the Langendorff model.

The Need for New Models

Despite intensive research, progress in the development of new therapies for ischemic heart disease has yielded only limited clinical success, and the disease remains a major cause of mortality. The development of new animal models, which in addition to adequately mimicking the human disease also function as reliable bioassays, may accelerate progress in this area (8).

A bioassay must possess certain attributes in order for it to have utility; low cost, ease of use, and reproducibility. If we define the primary objectives of any model for the study of regional ischemia and reperfusion as being *1*) the determination of the relative contribution of components of ischemia and reperfusion to cardiac dysfunction, and *2*) the assessment of the extent and mechanism of action of putative therapeutic interventions, then the potential utility of the dual-perfusion model relative to conventional models is self-evident. The potential limits of the model are primarily related to the unusual characteristics of rat cardiac electrophysiology. However, despite these characteristics, the consequences of ischemia and reperfusion do not appear to be anomalous in the rat heart in that contractile dysfunction, arrhythmias, and infarction all occur (8). Nevertheless, the dimensions of the new cannula can be adapted to enable its use with other small mammalian hearts.

Advantages and Utility of Dual-Perfusion Model

The described model offers several advantages over conventional models by enabling the independent perfusion of left and right coronary arteries in isolated hearts of small mammals. Such procedures have hitherto been restricted to hearts from larger, more expensive animals (5, 17). The use of the rat heart makes the model relatively inexpensive, the only extra source of cost compared with the conventional Langendorff preparation being the dual-perfusion cannula. The novel design of the aortic cannula overcomes the need to cannulate individual coronary arteries, making the model comparable to the Langendorff preparation in its ease of use. The model is also highly reproducible, as illustrated by its characterization. Low standard errors for all variables (coronary flow, heart rate, tissue ATP and CrP contents, size of perfusion beds) and the absence of any significant difference in hemodynamic or metabolic stability from conventional Langendorff hearts indicate that the model is sufficiently stable and reproducible for use in bioassay.

Reperfusion after transient regional ischemia, induced by cessation of flow to the left coronary bed, resulted in the almost instantaneous induction of VT, which rapidly degenerated into VF in 11 of 12 hearts. This pattern of reperfusion-induced arrhythmogenesis is almost identical to that previously reported in isolated rat hearts subjected to transient regional ischemia of identical duration by proximal ligation of the left main coronary artery (29). This confirms the validity of the dual-perfusion preparation and illustrates its usefulness in the study of reperfusion-induced arrhythmogenesis. The incidence of sustained VF was higher in the present study compared with that reported previously (29); this can be explained by the greater mean occluded zone size in our study, since occluded zone size has been shown to be a determinant of the incidence of reperfusion-induced sustained ventricular fibrillation in the isolated rat heart (7).

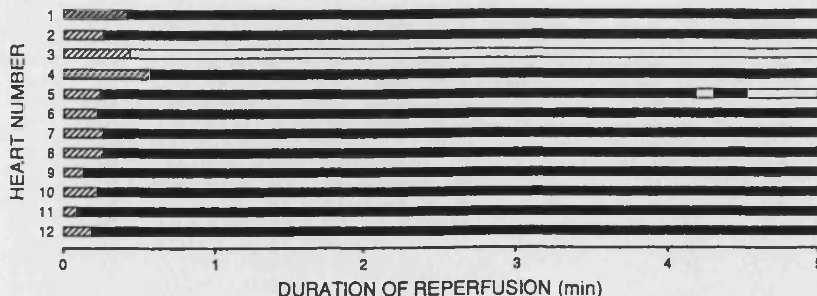

FIG. 7. Time course of reperfusion-induced ventricular tachycardia (VT) and fibrillation (VF) in 12 hearts subjected to cessation of flow to left coronary bed for 10 min followed by 5 min of reperfusion. Horizontal bars, individual hearts with reperfusion initiated at *time 0*; open bars, normal sinus rhythm; hatched bars, VT; filled bars, VF. All hearts were in sinus rhythm immediately before onset of reperfusion.

The utility of the new model is enhanced by its wide scope of application. Possible applications include *1*) studies of the consequences of regional low-flow (as opposed to zero-flow) ischemia at any chosen percentage of basal flow, by switching perfusion of the selected coronary bed from the constant pressure mode to the constant flow mode (Fig. 3); *2*) studies of the consequences of regional zero-flow ischemia without the necessity of coronary artery ligation (with attendant damage to the vasculature); *3*) assessment of the effectiveness and site of action (ischemic vs. nonischemic region) of drugs by their selective administration, with scope for randomized concentration-response studies through the use of multiple reservoirs (Fig. 3); *4*) studies with interventions selectively applied to the reperfused zone at the onset of reperfusion; and *5*) assessment of the effects of individual components of ischemia and reperfusion by their regional application, with selection of the site of administration (left or right coronary bed) and the size of the perfusion bed (hearts with left or right dominance).

Concluding Comments

The dual-perfusion preparation described in this study provides an inexpensive, technically undemanding and reproducible model for use in studies of aspects of regional myocardial ischemia and reperfusion, and their modification. The new model has many potential applications, and its use should assist identification of the relative importance of different factors in the development of ischemia and reperfusion-induced cardiac dysfunction, including arrhythmias, and the evaluation of putative therapeutic interventions.

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Reperfusion-Induced Arrhythmias

A Role for Washout of Extracellular Protons?

Metin Avkiran and Chikao Ibuki

Rapid washout of extracellular H^+ accumulated during preceding ischemia (i.e., the abrupt restoration of extracellular pH) has been implicated as an arrhythmogenic factor during reperfusion. Therefore, we hypothesized that by limiting the rate at which extracellular pH was restored during early reperfusion it should be possible to protect against reperfusion-induced arrhythmias. To test this, we used isolated rat hearts (n=12 per group) and a novel dual coronary perfusion cannula that permitted the induction of regional ischemia (10 minutes) and the selective reperfusion (8 minutes) of the ischemic zone with modified solutions. We examined the antiarrhythmic efficacy of 1) acidic (pH 6.6) reperfusion with stepwise restoration of extracellular pH to 7.4 (stepped pH) and 2) transient (2-minute) acidic (pH 7.1, 6.8, 6.6, or 6.4) reperfusion with subsequent abrupt restoration of extracellular pH to 7.4. Hearts in two contemporary control groups were reperfused with solution at pH 7.4 throughout. In all groups, 100% of hearts exhibited ventricular tachycardia (VT) on reperfusion. VT degenerated into ventricular fibrillation (VF) in 100% of hearts in the control group but in only 42% of hearts in the stepped-pH group ($p<0.05$). In the groups subjected to transient acidic reperfusion, there was a pH-dependent prolongation of VT cycle length (measured at 15 seconds of reperfusion), which was 47.1±3.9, 51.1±5.5, 56.0±1.9, 60.4±2.8 ($p<0.05$), and 68.8±5.0 ($p<0.05$) msec in the pH 7.4 (control), 7.1, 6.8, 6.6, and 6.4 groups, respectively. In these groups, VT degenerated into VF in 92%, 92%, 83%, 42% ($p<0.05$), and 33% ($p<0.05$) of hearts, respectively. In conclusion, limiting the rate at which extracellular pH is restored during early reperfusion does not affect the rapid induction of VT but inhibits the degeneration of VT into VF and promotes spontaneous reversion to normal sinus rhythm. This is consistent with a major arrhythmogenic role, during uncontrolled reperfusion, for the rapid washout of extracellular H^+. (*Circulation Research* 1992;71:1429–1440)

KEY WORDS • regional ischemia • reperfusion-induced arrhythmias • ventricular fibrillation • protons • dual coronary perfusion • rats

Reperfusion of myocardium subjected to a transient period of ischemia rapidly induces severe ventricular arrhythmias (for review, see Manning and Hearse[1]). Although the precise mechanisms have not been elucidated, several factors have already been implicated in reperfusion arrhythmogenesis,[1] including the generation of free oxygen radicals,[2-4] the rapid washout of K^+ from the extracellular space,[5] and the accumulation of Ca^{2+} in the intracellular space.[6] However, as recently pointed out,[7] the fundamental question of whether the arrhythmogenic process is initiated by the washout of substances (e.g., K^+) accumulated during ischemia or by the resupply of substances (e.g., oxygen) absent during ischemia remains to be resolved. Yamada and colleagues[8] have reported that, whereas reestablishment of flow and readmission of oxygen were independent determinants of reperfusion-induced arrhythmias, the latter was not a prerequisite for arrhythmogenesis (since anoxic reperfusion did not significantly alter the incidence of reperfusion-induced ventricular fibrillation), indicating that multiple mechanisms may be involved. In this context, the recent study by Curtis[5] on the effects of regional infusion and washout of high [K^+] solutions in the isolated rabbit heart suggests that the rapid washout of extracellular K^+ could be a significant arrhythmogenic factor.

Protons (H^+) also accumulate in the extracellular space during ischemia,[9] and their rapid washout may be another factor. Lazdunski and colleagues[10] suggested in 1985 that the rapid washout of extracellular H^+ on reperfusion may create an intracellular to extracellular H^+ gradient, resulting in an influx of Na^+ via the Na^+-H^+ exchanger. Such an influx of Na^+, in the face of Na^+,K^+-ATPase inhibition caused by the preceding ischemia, could result in an increase in intracellular [Na^+], which in turn would favor an increase in intracellular [Ca^{2+}] via the Na^+-Ca^{2+} exchanger.[10] Increase in intracellular [Ca^{2+}] on reperfusion, which can be ameliorated by inhibition of Na^+-H^+ exchange with amiloride[11] or by acidic reperfusion,[12] has been demonstrated in the isolated rat heart. Such an increase in intracellular [Ca^{2+}] has been proposed as a potential culprit for reperfusion arrhythmogenesis.[6] Indeed, the

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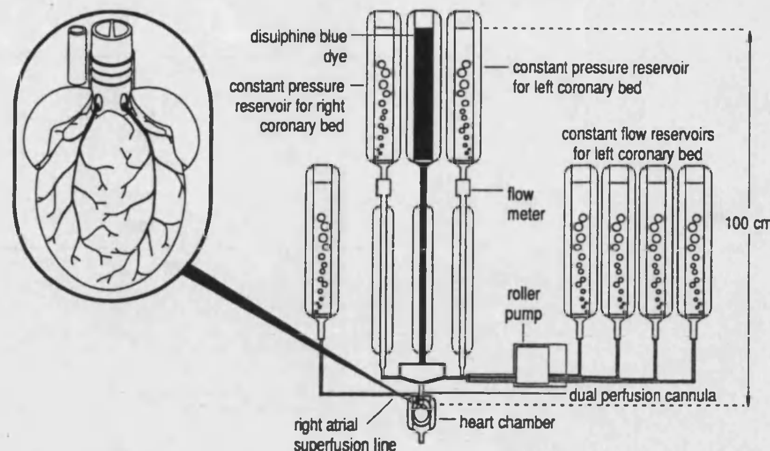

FIGURE 1. *Diagrams illustrating the perfusion apparatus and the correct position of the dual-perfusion cannula in the aorta of an isolated heart (inset). A divided lining to the lumen of the cannula permits each hemicylinder to deliver an independent supply of perfusate. The orifices near the proximal end are designed to be opposite the left and right coronary ostia when the cannula is in position. Regional ischemia was induced by stopping flow to the left coronary bed. During reperfusion, the left coronary bed was perfused at the preischemic flow rate via a roller pump, with perfusion solution from one of a bank of four reservoirs. At the end of each experiment, disulfine blue dye was infused into one coronary artery to delineate the left and right coronary beds, thus enabling the size of the ischemic zone to be quantified.*

recent study by Dennis and colleagues,[13] who used solutions containing various buffer concentrations and amiloride analogues to modify the activity of the Na^+-H^+ exchanger, provided support for an arrhythmogenic role for Na^+-H^+ exchange–mediated mechanisms during reperfusion.

We hypothesized that, if the rapid washout of extracellular H^+ (i.e., the abrupt restoration of extracellular pH) were a major arrhythmogenic factor during reperfusion, then limiting the rate of washout of extracellular H^+ (i.e., the rate of restoration of extracellular pH) during early reperfusion should afford protection. To test this, we used isolated rat hearts and a recently developed dual perfusion cannula[14] that permits the induction of regional ischemia and the selective reperfusion of the ischemic zone with modified solutions. We examined the antiarrhythmic efficacy of 1) acidic (pH 6.6) reperfusion with stepwise restoration of extracellular pH to 7.4 by sequential perfusion with solutions of increasing pH and 2) transient acidic (pH 7.1, 6.8, 6.6, or 6.4) reperfusion followed by an abrupt restoration of extracellular pH to 7.4.

Materials and Methods

Dual Coronary Perfusion of Isolated Rat Hearts

Independent dual perfusion of left and right coronary arteries in isolated rat hearts was performed as described in detail by Avkiran and Curtis.[14] In brief, male Wistar rats (Bantin and Kingman, Ltd., N. Humberside, UK) were anesthetized by inhalation of diethyl ether, and then 50 units sodium heparin was injected into the left femoral vein. The chest was opened, and the heart was excised and immersed in perfusion medium at 4°C. Within 30 seconds of excision, a dual perfusion cannula was inserted into the ascending aorta (Figure 1) and secured in position with a braided silk suture. The pulmonary artery was cut near its origin, and a stainless-steel needle was inserted into the left ventricle through the apex to allow adequate drainage of coronary and thebesian venous effluent. Perfusion of both coronary beds was then initiated at a perfusion pressure equivalent to 100 cm H_2O. Alignment of the coronary ostia with the orifices of the cannula was achieved using in-line monitoring of left and right coronary flow as a guide, the aorta being rotated on the cannula until flow in each perfusion bed reached a maximum.

Perfusion Apparatus

Each coronary bed was initially supplied with oxygenated perfusion solution from a temperature-regulated reservoir (37°C) at constant pressure (Figure 1). The flow to each bed was continuously monitored using two in-line flowmeters (Meterate, Jencons Scientific Ltd., Leighton Buzzard, UK) with a detection range of 0.25–12.00 ml/min. When required, the left coronary bed could be perfused at constant flow, via a roller pump (Gilson Minipuls 3), with perfusion solution from any one of a bank of reservoirs. A central reservoir held perfusion solution containing disulfine blue dye (0.016% [wt/vol]), which could be infused unilaterally to delineate the two perfusion beds. The heart was housed in a temperature-regulated chamber and maintained at 37°C. Because the atria in the rat are supplied by extracardiac arteries,[15] the right atrium was continuously superfused with oxygenated perfusion medium (37°C) at a constant flow rate of 10 ml/min to maintain sinus rate.[14]

Perfusion Solutions

All solutions were continuously gassed with 95% O_2–5% CO_2 and filtered (pore size, 5 μm) before use. The standard perfusion solution had a pH of 7.4 and

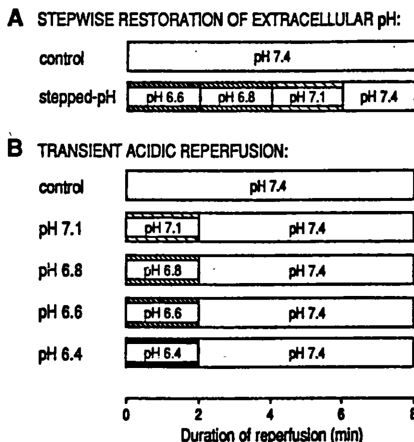

FIGURE 2. *Protocols for stepwise restoration of extracellular pH* (panel A) *and transient acidic perfusion followed by abrupt restoration of extracellular pH* (panel B) *during selective reperfusion (8 minutes) of the left coronary bed after 10 minutes of zero-flow ischemia (n=12 per group). The left coronary bed was perfused at the preischemic flow rate, via a roller pump, throughout reperfusion.*

was of the following composition (mmol/l): NaCl 118.5, $NaHCO_3$ 25.0, KCl 3.2, $MgSO_4$ 1.2, KH_2PO_4 1.2, $CaCl_2$ 1.4, and glucose 11.0. Solutions at pH 7.1, 6.8, 6.6, and 6.4 were obtained by reducing the concentration of $NaHCO_3$ to 12.5, 6.3, 4.0, and 2.5 mmol/l, respectively. The concentration of sodium and the osmolarity were kept constant in the solutions by substitution of $NaHCO_3$ with NaCl as necessary.

Experimental Protocols

After 15 minutes of perfusion of both coronary beds with the standard perfusion solution (pH 7.4) at constant pressure, regional ischemia was induced by clamping the perfusion line supplying the left coronary bed. The right coronary bed continued to receive the standard perfusion solution at constant pressure throughout the experiment. Regional ischemia was maintained for 10 minutes (the duration that results in a maximum incidence of reperfusion-induced ventricular fibrillation in the isolated rat heart[1]); then the heart was randomly assigned to one of the study groups (n=12 per group), and the left coronary bed was reperfused at constant flow (100% of its preischemic flow rate) via the roller pump according to the following protocols.

Stepwise restoration of extracellular pH. This protocol (Figure 2A) was designed to test the effects of stepwise restoration of extracellular pH in the reperfused zone on the severity of reperfusion-induced arrhythmias. During 8 minutes of reperfusion, the left coronary bed received either the standard perfusion solution at pH 7.4 throughout (control group) or solution at pH 6.6 for the first 2 minutes of reperfusion, pH 6.8 from 2 to 4 minutes, pH 7.1 from 4 to 6 minutes, and finally pH 7.4 from 6 to 8 minutes.

Transient acidic reperfusion. This protocol (Figure 2B) was designed to test the antiarrhythmic efficacy of transient acidic reperfusion and to determine the pH–response characteristics of any such effect. In the control group, the left coronary bed received the standard perfusion solution at pH 7.4 throughout 8 minutes of reperfusion, as above. In four transient acidic reperfusion groups, the left coronary bed received the solution at pH 7.1, 6.8, 6.6, or 6.4 for the first 2 minutes of reperfusion, followed by the standard solution at pH 7.4 from 2 to 8 minutes.

Measured Variables

Arrhythmias. Arrhythmias were diagnosed from a unipolar electrocardiogram (ECG), which was obtained using a silver electrode inserted into the free wall of the left ventricle and a reference electrode connected to the aorta. The ECG was continuously monitored on a digital storage oscilloscope (model 1421, Gould Electronics Ltd., Ilford, UK) and recorded on an ink-jet recorder (model 2200S, Gould). Chart speed was set at 50 mm/sec a few seconds before reperfusion so as to obtain a permanent high-speed recording of the changes in the ECG during early reperfusion. The ECG was retrospectively analyzed, in a blinded manner, for the incidence, time to onset, and duration of ventricular tachycardia (VT) and ventricular fibrillation (VF). All analyses were carried out in accordance with the Lambeth Conventions.[16] VT was defined as four or more consecutive premature beats of ventricular origin, and VF was defined as a signal in which individual QRS deflections could no longer be distinguished from one another and for which the rate could not be determined.

VT cycle length. In hearts subjected to transient acidic reperfusion and contemporary controls, average VT cycle length was determined during reperfusion with the solution at pH 7.4, 7.1, 6.8, 6.6, or 6.4. VT cycle length was determined after 15 seconds of reperfusion, because at this time a significant number of hearts (n=6–12) were in VT in each study group. VT cycle length was calculated from the number of QRS deflections over a 2-second interval using the ECG tracing.

Coronary flow and heart rate. Throughout the experimental protocol, coronary flow was monitored by the in-line flowmeters, and heart rate was determined from the ECG.

Size of ischemic zone. At the end of each experiment, the left coronary bed was perfused for 3 minutes with a solution containing 0.016% disulfine blue dye at 100 cm H_2O perfusion pressure. The heart was then removed from the perfusion apparatus, the atria and mediastinal tissue were removed, and dye-stained tissue, representing ventricular myocardium subjected to ischemia and reperfusion, was carefully dissected away from the remainder. The stained and unstained tissues were lightly blotted and weighed. The size of the ischemic zone, expressed as a percentage of total ventricular weight, was calculated from the following equation: (weight of stained tissue/total ventricular weight)×100.

Exclusion Criteria

Exclusion criteria for the present study, selected to minimize variations in heart rate and size of the ischemic zone (due to atypical coronary anatomy) among the hearts, were based on our previous experience[14,17] with the model. These criteria demanded that hearts were excluded if 1) heart rate was less than 280 or more than 420 beats per minute during the preischemic

period, or 2) the size of the ischemic zone was found to be greater than 70% of total ventricular weight after the end of the experiment. Hearts were also excluded if there was cross flow between the right and left coronary ostia. To verify whether significant cross flow occurred, the perfusion line to one bed was clamped for 10 seconds at the beginning of each experiment. If flow to the contralateral bed increased by more than 10% of the preceding flow in the occluded bed, the heart was excluded from the study (because in the rat heart collateral flow alone could not have been responsible for such an increase[18]). In addition, hearts that exhibited ventricular arrhythmias during the final 3 seconds of ischemia before reperfusion were not included in the analysis of reperfusion-induced arrhythmias, because in those hearts it would have been impossible to differentiate arrhythmias induced by reperfusion from those induced by ischemia. Of 116 hearts entered into the study, four were excluded on the basis of heart rate, 10 were excluded on the basis of the size of the ischemic zone, nine were excluded because of cross flow, and nine were excluded because of arrhythmias during the final 3 seconds of ischemia.

Data Analysis

The general approach to statistical analysis adopted the guidelines described by Wallenstein and colleagues.[19] Gaussian-distributed variables were expressed as mean±SEM and were subjected to analysis of variance. If a difference among mean values was established with one-way analysis of variance, comparison with controls was performed using Dunnett's test. Binomially distributed variables, such as the incidence of VT or VF, were compared using the χ^2 test for a $2\times n$ table, followed by a sequence of 2×2 χ^2 tests with Yates's correction. A value of $p<0.05$ was considered significant.

Results

Ischemia-Induced Arrhythmias

The objective of the present study was to assess the effects of interventions applied after ischemia on the incidence and severity of reperfusion-induced arrhythmias. However, arrhythmias were also quantified in the preceding period of ischemia (during which time the composition of the perfusate was identical in all study groups) to confirm that all groups were, in fact, identical before the onset of reperfusion.

Stepwise restoration of extracellular pH. The incidence of VT during the 10-minute period of ischemia preceding reperfusion was similar in both the control (92%) and the stepped-pH (100%) groups, and all episodes of VT were nonsustained. None of the hearts in either group exhibited ischemia-induced VF. There was no significant difference between the control and stepped-pH groups in the mean time to onset of ischemia-induced VT (520±13 and 531±13 seconds, respectively) or in the mean total duration of nonsinus rhythm (consisting of ventricular premature beats and VT) during the 10-minute period of ischemia (25±6 and 24±5 seconds, respectively).

Transient acidic reperfusion. The incidences of VT during the 10-minute period of ischemia preceding reperfusion were 92%, 83%, 75%, 50%, and 67% in the control, pH 7.1, pH 6.8, pH 6.6, and pH 6.4 groups, respectively (p=NS). Again, all episodes of VT were nonsustained, and none of the hearts in the five study groups exhibited ischemia-induced VF. There were no significant differences between control, pH 7.1, pH 6.8, pH 6.6, and pH 6.4 groups in the mean time to onset of ischemia-induced VT (534±12, 470±23, 536±15, 487±15, and 536±16 seconds, respectively). The mean total duration of nonsinus rhythm (consisting of ventricular premature beats and VT) during the 10-minute period of ischemia was less than 35 seconds in all groups.

Reperfusion-Induced Arrhythmias

Reperfusion of the ischemic region resulted in the rapid (within a few beats) induction of VT (Figure 3A) regardless of the pH of the reperfusion solution. Reperfusion-induced VT was generally polymorphic in nature (Figure 3A), and episodes of reperfusion-induced VT were uninterrupted (see Figures 4 and 5) until either spontaneous reversion to normal sinus rhythm or degeneration into VF, as illustrated in Figures 3B and 3C, respectively.

Stepwise restoration of extracellular pH. Figure 4 shows the time course of reperfusion-induced arrhythmias in control hearts in which the left coronary bed was reperfused with solution at pH 7.4 throughout and in hearts in which the left coronary bed was subjected to a stepwise restoration of extracellular pH (stepped pH) by sequential perfusion with solutions at pH 6.6, 6.8, 7.1, and 7.4 during the reperfusion period. All hearts in both groups developed VT within the first 3 seconds of reperfusion. In all hearts in the control group, VT degenerated into VF within the first 30 seconds of reperfusion. In contrast, only 42% of hearts in the stepped-pH group exhibited VF during reperfusion ($p<0.05$). Among hearts that exhibited VF, the mean time to onset of VF was significantly prolonged from 14±2 seconds in the control group (n=12) to 90±23 seconds in the stepped-pH group (n=5). By the end of reperfusion, 92% of hearts in the stepped-pH group were in normal sinus rhythm compared with only 8% of hearts in the control group ($p<0.05$).

Transient acidic reperfusion. Figure 5 shows the time course of reperfusion-induced arrhythmias in control hearts in which the left coronary bed was reperfused with solution at pH 7.4 throughout and in hearts in which the left coronary bed was subjected to transient (2-minute) acidic reperfusion with the solution at pH 7.1, 6.8, 6.6, or 6.4. There was a 100% incidence of VT in all five groups. In the control group, VT degenerated into VF within the first 30 seconds of reperfusion in 92% of the hearts; in the remaining heart, VT converted to sinus rhythm within 2 minutes of reperfusion. Transient acidic reperfusion resulted in a pH-dependent reduction in the incidence of VF (Figure 6), with 92%, 83%, 42% ($p<0.05$), and 33% ($p<0.05$) of hearts exhibiting VF in the pH 7.1, 6.8, 6.6, and 6.4 groups, respectively. Among hearts that exhibited VF during reperfusion, the mean time to onset was significantly prolonged (Figure 6) from 14±1 seconds in the control group (n=11) to 55±22 seconds in the pH 6.8 group (n=10), 64±24 seconds in the pH 6.6 group (n=5), and 190±42 seconds in the pH 6.4 group (n=4). The mean time to onset of VF was not significantly different from the control value in the pH 7.1 group (18±2 seconds). In

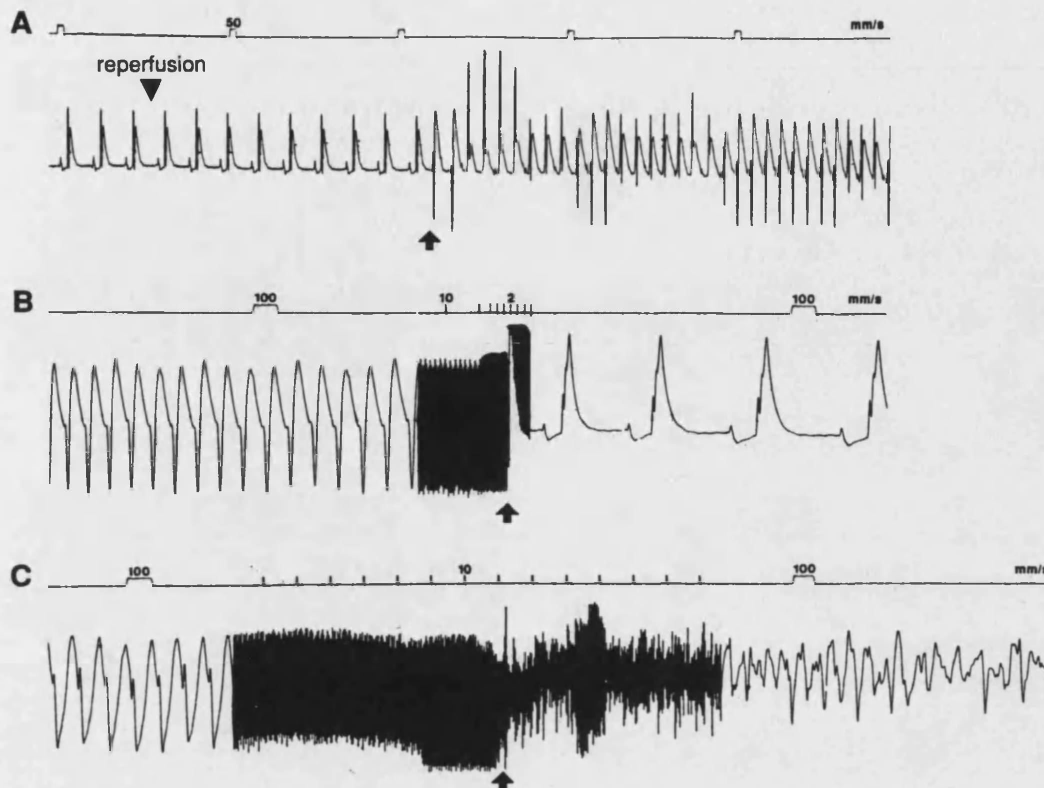

FIGURE 3. *Representative electrocardiographic tracings illustrating the rapid induction and polymorphic nature of reperfusion-induced VT* (panel A), *spontaneous reversion of reperfusion-induced VT to normal sinus rhythm* (panel B), *and degeneration of reperfusion-induced VT into VF* (panel C). *Arrows indicate the moments of initiation/degeneration of arrhythmia or reversion to normal sinus rhythm. Chart speeds are shown on the horizontal lines above each panel.*

control, pH 7.1, pH 6.8, pH 6.6, and pH 6.4 groups, 8%, 25%, 58% ($p<0.05$), 83% ($p<0.05$), and 67% ($p<0.05$) of hearts, respectively, were in normal sinus rhythm by the end of the reperfusion period.

VT Cycle Length

VT cycle length was measured in control hearts and those subjected to transient acidic reperfusion to determine whether it was affected by the pH of the initial reperfusion solution. As illustrated in Figure 7, acidic reperfusion resulted in a pH-dependent prolongation of VT cycle length at 15 seconds of reperfusion. VT cycle length at this time point was significantly greater in the two groups reperfused with solution at pH 6.6 or 6.4 than in the control group reperfused with solution at pH 7.4. Mean VT cycle length at 15 seconds of reperfusion was also calculated for two subpopulations of hearts in which VT subsequently either reverted to normal sinus rhythm ($n=20$) or degenerated into VF ($n=30$). Mean VT cycle length was 67.5±3.2 msec in the former and 52.1±1.8 msec in the latter subpopulations ($p<0.05$).

Coronary Flow, Heart Rate, and Size of Ischemic Zone

Stepwise restoration of extracellular pH. Preischemic flow was similar in control and stepped-pH groups in both right (14.1±0.7 and 12.9±0.7 ml/min per gram, respectively) and left (10.9±0.5 and 10.8±0.7 ml/min per gram, respectively) coronary beds. During regional ischemia induced by cessation of flow to the left coronary bed, flow in the right coronary bed did not change significantly (12.9±0.5 and 13.0±0.7 ml/min per gram after 9 minutes of ischemia in control and stepped-pH groups, respectively). During the first minute of reperfusion of the left coronary bed (at 100% of preischemic flow), flow in the right coronary bed increased in both control (19.5±0.7 ml/min per gram) and stepped-pH (16.5±0.8 ml/min per gram) groups. This increase in right coronary flow was maintained throughout reperfusion in the control group, with a flow of 17.6±0.9 ml/min per gram recorded after 7 minutes of reperfusion. In contrast, right coronary flow in the stepped-pH group reverted to the prereperfusion value within 3 minutes of reperfusion (11.6±0.8 ml/min per gram).

The preischemic heart rate was similar in control and stepped-pH groups (323±7 and 324±6 beats per minute, respectively). Regional ischemia had no effect on heart rate, which was 320±11 beats per minute in the control group and 328±8 beats per minute in the stepped-pH group after 9 minutes of ischemia. Sinus heart rate could not be measured during early reper-

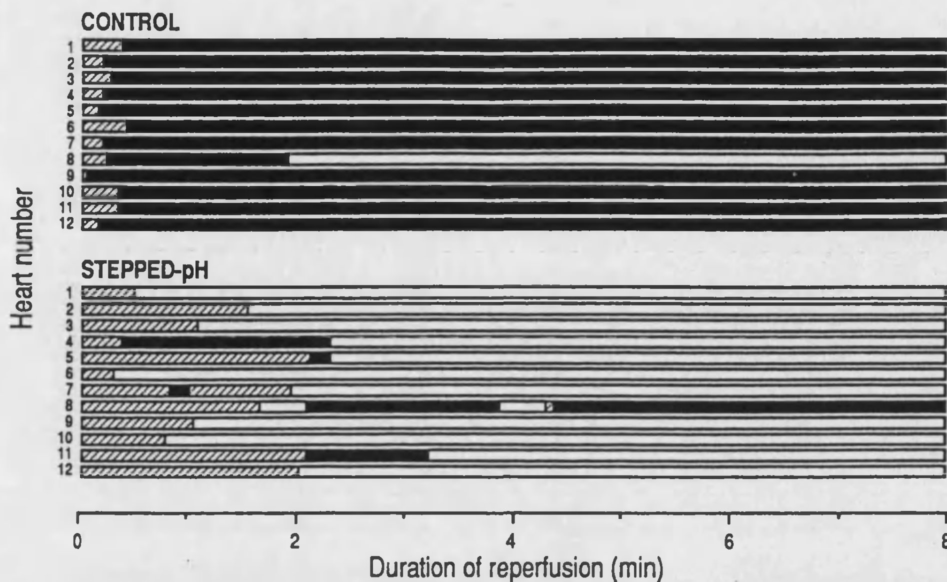

FIGURE 4. *Time course of reperfusion-induced ventricular tachycardia and ventricular fibrillation in individual hearts in the control group and in the group in which the left coronary bed underwent stepwise restoration of extracellular pH (stepped-pH group). All hearts (n=12 per group) were subjected to 10 minutes of regional ischemia. Reperfusion began at time zero at the preischemic flow rate and was continued for 8 minutes. All hearts were in sinus rhythm at the moment of reperfusion. Open bars represent normal sinus rhythm, hatched bars represent ventricular tachycardia, and filled bars represent ventricular fibrillation. The hearts in each group are arranged in the order in which the experiments were carried out (the study was randomized).*

fusion because of the almost immediate onset of VT and VF.

The size of the ischemic zone was similar in control and stepped-pH groups at 56±2% and 57±3% of the total ventricular weight, respectively.

Transient acidic reperfusion. Preischemic flow was similar in control, pH 7.1, pH 6.8, pH 6.6, and pH 6.4 groups in both right (11.3±0.5, 12.9±0.8, 13.3±1.6, 13.4±0.6, and 12.6±0.9 ml/min per gram, respectively) and left (11.2±1.2, 10.2±0.7, 9.7±0.5, 10.7±0.6, and 9.9±0.8 ml/min per gram, respectively) coronary beds. The time course of the changes in right coronary flow during regional ischemia and reperfusion are illustrated in Figure 8. During the first minute of reperfusion of the left coronary bed, flow in the right coronary bed increased in all groups. This increase was maintained throughout reperfusion in the control and pH 7.1 groups. In contrast, right coronary flow returned toward prereperfusion values within 3 minutes of reperfusion in the pH 6.8, pH 6.6, and pH 6.4 groups.

The preischemic heart rate was similar in control, pH 7.1, pH 6.8, pH 6.6, and pH 6.4 groups, at 323±10, 334±10, 326±12, 330±8, and 325±10 beats per minute, respectively. Regional ischemia had no effect on heart rate, and the corresponding values after 9 minutes of ischemia were 335±8, 333±10, 326±9, 320±12, and 327±7 beats per minute, respectively. Sinus heart rate could again not be measured during early reperfusion because of the almost immediate onset of VT and VF.

The size of the ischemic zone was also similar in the control, pH 7.1, pH 6.8, pH 6.6, and pH 6.4 groups at 55±3%, 53±2%, 53±3%, 55±3%, and 51±2% of total ventricular weight, respectively.

Discussion

The results of the present study indicate that acidic reperfusion of the ischemic rat myocardium does not affect the incidence of reperfusion-induced VT but inhibits the degeneration of VT into VF and promotes spontaneous reversion to normal sinus rhythm. Within the pH range of 7.4–6.4, the protective effect appeared to be pH dependent, with the most significant protection obtained by initial reperfusion with solution at pH 6.6 or 6.4. In these two groups, VT cycle length during early reperfusion was significantly prolonged relative to the control group. The protection afforded by acidic reperfusion was not associated with changes in antecedent variables such as heart rate, the size of the ischemic/reperfused zone, and the rate of reflow, because there were no differences between the groups in the former two variables and reperfusion of the left coronary bed was performed at 100% of preischemic flow in all cases.

Interestingly, initial acidic reperfusion with solution at pH 6.6 was equally protective regardless of whether the subsequent restoration of extracellular pH to 7.4 occurred in an abrupt or a stepwise manner. Thus, although the results of the present study support a profibrillatory role for the rapid restoration of extracellular pH, they also suggest that this effect is evident only when such restoration occurs within the first 2 minutes of reperfusion.

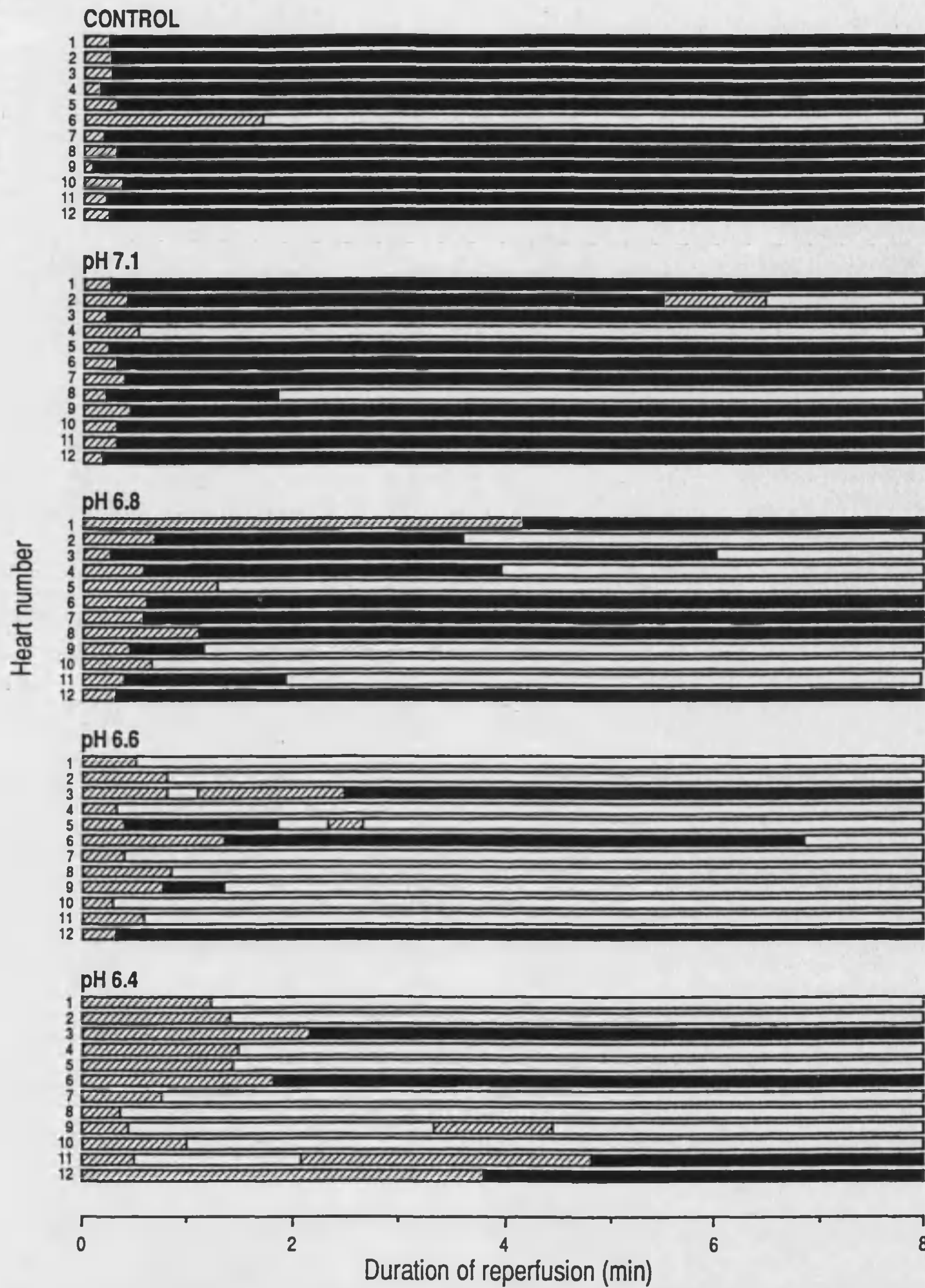

FIGURE 5. *Time course of reperfusion-induced ventricular tachycardia and ventricular fibrillation in individual hearts in the control group and in the groups in which the left coronary bed underwent transient acidic reperfusion with solution at pH 7.1, 6.8, 6.6, or 6.4. All hearts (n=12 per group) were subjected to 10 minutes of regional ischemia. Reperfusion began at time zero at the preischemic flow rate and was continued for 8 minutes. All hearts were in sinus rhythm at the moment of reperfusion. Open bars represent normal sinus rhythm, hatched bars represent ventricular tachycardia, and filled bars represent ventricular fibrillation. The hearts in each group are arranged in the order in which the experiments were carried out (the study was randomized).*

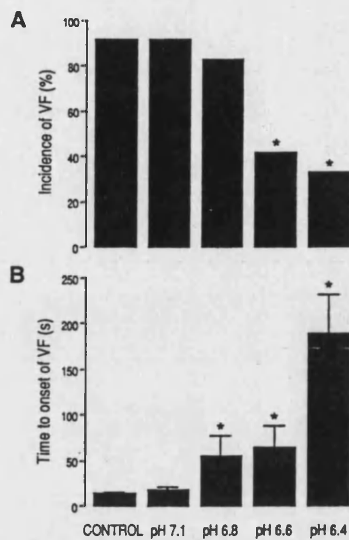

FIGURE 6. *Bar graphs showing the incidence of reperfusion-induced ventricular fibrillation (VF)* (n=12 *per group,* panel A) *and mean time to onset of VF among those hearts that exhibited reperfusion-induced VF* (n=4–11 *per group,* panel B) *in the control group and in the groups in which the left coronary bed underwent transient acidic reperfusion with solution at pH 7.1, 6.8, 6.6, or 6.4.* *$p<0.05$ *compared with the control group.*

Possible Mechanisms of the Protective Effect of Acidic Reperfusion

Role of Na^+-H^+ exchange. As noted earlier, the rapid washout during reperfusion of H^+, accumulated in the extracellular space during the preceding period of ischemia,[9] has been thought to result in Na^+ influx via Na^+-H^+ exchange and consequently an increase in intracellular [Ca^{2+}] via Na^+-Ca^{2+} exchange.[10] Increased intracellular [Ca^{2+}] ("Ca^{2+} overload") on reperfusion has been implicated as a causal factor in several manifestations of reperfusion injury (for review, see Hearse and Bolli[20]). These include myocardial stunning[21,22] and ventricular arrhythmias,[6] the latter possibly occurring via the induction of oscillatory release of Ca^{2+} from the sarcoplasmic reticulum and the subsequent activation of the Ca^{2+}-induced transient inward current.[6,23] However, beat-to-beat measurements of calcium transients by Lee et al[24] and Kihara et al[25] in isolated hearts (using indo 1 or aequorin, respectively) have not shown an increase in intracellular [Ca^{2+}] during reperfusion. Instead, intracellular [Ca^{2+}] was shown to increase dur-

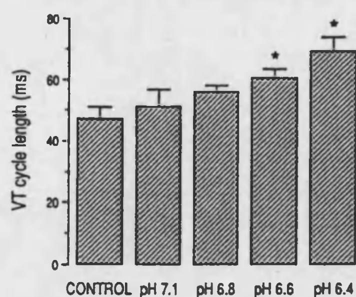

FIGURE 7. *Bar graph showing the mean ventricular tachycardia (VT) cycle length at 15 seconds of reperfusion (in those hearts that were in VT at that time,* n=6–12 *per group) in the control group and in the groups in which the left coronary bed underwent transient acidic reperfusion with solution at pH 7.1, 6.8, 6.6, or 6.4.* *$p<0.05$ *compared with the control group.*

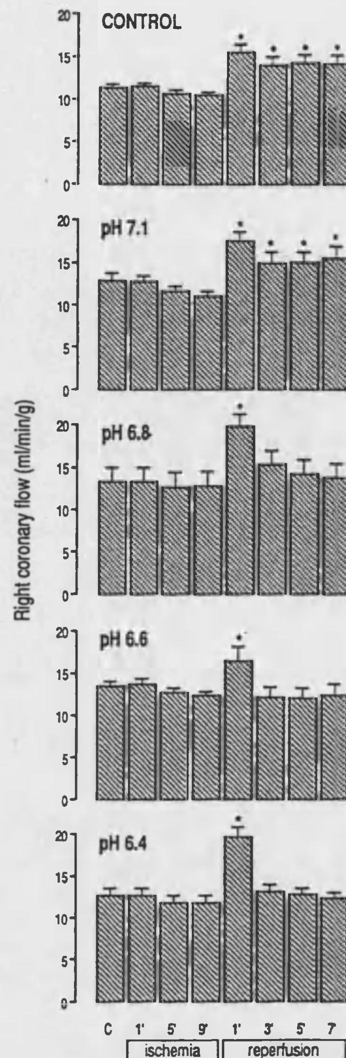

FIGURE 8. *Bar graphs showing the right coronary flow rate before regional ischemia (C), during regional ischemia induced by cessation of flow in the left coronary bed, and during reperfusion of the left coronary bed at the preischemic flow rate in the control group and in the groups in which the left coronary bed underwent transient acidic reperfusion with solution at pH 7.1, 6.8, 6.6, or 6.4.* *$p<0.05$ *compared with the value after 9 minutes of ischemia* (n=12 *per group).*

ing ischemia but normalize on reperfusion. Although this appears to contradict a major role for Ca^{2+} overload in reperfusion-induced cardiac dysfunction, it should be noted that such dysfunction would not be expected to occur after the short durations of ischemia (up to 3 minutes) used in the studies of Lee et al[24] and Kihara et al.[25] Indeed, a more recent study by Kihara and Morgan,[26] in a model identical to that used in their earlier study,[25] has shown that reperfusion after 20 minutes of ischemia results in a further increase in intracellular [Ca^{2+}] and that this is associated with transition into VF.

Other recent studies have supported a role for Na^+-H^+ exchange–mediated mechanisms in postreperfusion Ca^{2+} overload and contractile dysfunction. Thus, inhibition of Na^+-H^+ exchange by amiloride or its analogues has been shown to attenuate reperfusion-induced Ca^{2+} overload[11] and to improve functional recovery.[11,27,28] Within the context of the present discussion, amiloride analogues have also been shown to inhibit reperfusion-induced arrhythmias in both rat[13] and guinea pig[29] hearts. Although acidic reperfusion has previously been shown to attenuate reperfusion-induced Ca^{2+} overload[12] and to improve postischemic recovery of function,[30–33] the present study is the first to report the antifibrillatory efficacy of selective acidic reperfusion of the ischemic zone after a period of regional ischemia.

Vaughan-Jones and Wu[34] have shown, in sheep Purkinje fibers, that H^+ extrusion and Na^+ influx via Na^+-H^+ exchange during recovery from any given intracellular acid load is inhibited by lowering extracellular pH. Assuming that in a preparation perfused with CO_2/HCO_3^-–buffered solution, as in the present study, intracellular pH would be reduced to approximately 6.6 after 10 minutes of ischemia,[35] certain estimates may be made on the basis of the findings of Vaughan-Jones and Wu.[34] Thus, at an extracellular pH of 6.4–6.6 (i.e., the pH of the acidic solutions shown to exhibit the most significant antiarrhythmic efficacy in the present study), H^+ extrusion and Na^+ influx via Na^+-H^+ exchange would be expected to be inhibited to approximately 20–25% of that at an extracellular pH of 7.4. Similar estimates may also be obtained on the basis of the reported sensitivity of the Na^+-H^+ exchanger to extracellular pH in isolated rat myocytes.[36]

It should also be noted that Na^+-Ca^{2+} exchange has been shown[37] to be inhibited by low intracellular pH, with 50% inhibition at pH 6.7. Inhibition of Na^+-H^+ exchange by acidic reperfusion would be expected not only to inhibit Na^+ influx but also to maintain intracellular acidosis for a longer period during reperfusion, which may directly inhibit Na^+-Ca^{2+} exchange. Although Na^+-Ca^{2+} exchange largely mediates Ca^{2+} efflux under normal conditions, its reversal potential is readily attainable even under physiological conditions and is significantly affected by small changes in intracellular [Na^+].[38] Therefore, under conditions of increased intracellular [Na^+] and membrane depolarization, as may be prevalent during the early moments of reperfusion, Na^+-Ca^{2+} exchange may primarily mediate Ca^{2+} influx,[39,40] and its inhibition by maintained intracellular acidosis during early reperfusion may contribute to an attenuation of Ca^{2+} overload.

In light of these observations, it is feasible to propose that the protective effect of acidic reperfusion reported in the present study may result from an attenuation of Ca^{2+} overload via Na^+-Ca^{2+} exchange that is due to reduced Na^+ influx and maintained intracellular acidosis by inhibition of Na^+-H^+ exchange. If, however, the rapid washout of extracellular H^+ and the subsequent influx of Na^+ (with concomitant recovery from intracellular acidosis) is a major arrhythmogenic factor during reperfusion, two questions remain to be answered: 1) Why did acidic reperfusion suppress the incidence of reperfusion-induced VF but not that of reperfusion-induced VT? 2) Why did the abrupt change to pH 7.4 after transient (2-minute) acidic (pH 6.6 or 6.4) reperfusion seldom initiate de novo arrhythmias in those hearts that were in normal sinus rhythm at that time?

In relation to the first question, the recent studies of Kihara and Morgan[26] in the intact ferret heart have shown that spontaneous transitions to VF do not occur unless a state of Ca^{2+} overload is present and that diastolic Ca^{2+} oscillations temporally precede such transitions. Kihara and Morgan concluded that impaired Ca^{2+} homeostasis might be a crucial factor for the initiation of VF. In support of this, the recent studies of Thandroyen and colleagues[41] in isolated spontaneously beating ventricular myocytes have led them to suggest that increased intracellular [Ca^{2+}] may be a causal factor in the degeneration of VT into VF. Therefore, it is possible that acidic reperfusion may preferentially inhibit the transition to VF by inhibition of Ca^{2+} overload and consequent Ca^{2+} oscillations.

The answer to the second question is likely to be found in the extent of inhibition of sarcolemmal Na^+,K^+-ATPase activity by ischemia and the rate of its recovery during reperfusion. Components of ischemia such as acidosis, depletion of ATP, and accumulation of inorganic phosphate are known to inhibit Na^+,K^+-ATPase activity,[42] as can free oxygen radicals.[43] It has been proposed that, during the early moments of uncontrolled reperfusion, any Na^+ entering the myocyte via Na^+-H^+ exchange cannot be extruded by Na^+,K^+-ATPase and exits via Na^+-Ca^{2+} exchange, with a concomitant rise in intracellular Ca^{2+}.[44] Indeed, a recent study[45] in isolated rabbit myocytes has shown that, during recovery from intracellular acidosis in the presence of partial inhibition of Na^+,K^+-ATPase, a large increase in intracellular Ca^{2+} occurs and that this can be inhibited by inhibition of Na^+-H^+ exchange with an amiloride analogue. Other recent studies using pharmacological inhibition of Na^+,K^+-ATPase support a key role for the activity of this enzyme during reperfusion in determining not only the severity of postischemic contractile dysfunction[46] but also the incidence of reperfusion-induced VF.[47] The period of acidic reperfusion with oxygenated perfusate used in the present study may allow sufficient recovery of Na^+,K^+-ATPase activity to enable the Na^+ entering via Na^+-H^+ exchange during the subsequent abrupt return to pH 7.4 to be extruded by Na^+,K^+-ATPase rather than via Na^+-Ca^{2+} exchange. Although Na^+,K^+-ATPase is inhibited by low pH, in the presence of optimal ATP and inorganic phosphate concentrations it retains approximately 82% of its maximal activity even at pH 6.5.[42] Therefore, it is reasonable to propose that the absence of additional arrhythmias on switching the perfusion solution from

pH 6.6 or 6.4 to pH 7.4 after 2 minutes of reperfusion may reflect significant recovery of Na^+,K^+-ATPase activity by that time.

Role of prolongation of VT cycle length. In the present study, the pH of the initial reperfusion solution did not influence the rapid induction of VT but had a significant effect on VT cycle length; this was prolonged with decreasing pH such that it was significantly greater at pH 6.6 and 6.4 than at pH 7.4. As noted earlier, the greatest reductions in the incidence of degeneration from VT into VF were also observed in the two groups initially reperfused with pH 6.6 or 6.4 solution. This may suggest a triggering role for rapid VT in the initiation of VF, which is suppressed by prolongation of VT cycle length. Indeed, in support of an important role for VT cycle length in determining vulnerability to VF, VT cycle length during early reperfusion was found to be significantly shorter in hearts in which VT subsequently degenerated into VF than in those in which VT subsequently reverted to normal sinus rhythm.

It is known[48] that acidosis causes reductions in the maximum rate of rise of the action potential and conduction velocity and may also prolong action potential duration. Such effects on action potential morphology and propagation probably underlie the prolongation of VT cycle length by acidic reperfusion observed in the present study. In addition to imposing a further burden on the metabolic deficit from antecedent ischemia, rapid VT during early reperfusion may also result in a further increase in intracellular $[Ca^{2+}]$, thereby increasing the probability of degeneration into VF.[41] Therefore, it follows that slower VT during reperfusion with acidic solutions may allow enhanced metabolic and electrophysiological recovery and that this mechanism may contribute, at least in part, to the antifibrillatory efficacy of acidic reperfusion.

Role of differences in coronary flow. Although there were no significant differences between the groups in left coronary flow rate at any point during the experimental protocol, there were differences in the profiles of right coronary flow rate during reperfusion of the left coronary bed at 100% of its preischemic flow rate (Figure 8). However, the intergroup differences in right coronary flow rate are unlikely to be causally related to the differences in the severity of reperfusion-induced arrhythmias. Rather, the differences in right coronary flow rate probably reflect the differences in the severity and duration of reperfusion-induced arrhythmias (Figure 5). Severe ventricular arrhythmias result in reduced extravascular compression (due to loss of coordinated contractile activity), which in turn may result in increased flow in the zone not subjected to ischemia and reperfusion.[14] Thus, although the right coronary flow rate was significantly elevated in all groups during the first minute of reperfusion, during which all hearts exhibited episodes of VT or VF (or both), by 3 minutes of reperfusion, flow had returned toward the prereperfusion value in those groups (pH 6.8, 6.6, and 6.4) in which a significant proportion of the hearts had reverted to normal sinus rhythm.

Other possible mechanisms. There are a number of other possible mechanisms by which the acidic reperfusion procedure used in the present study may suppress reperfusion-induced VF. The acidic solutions used were obtained by lowering the HCO_3^- concentration of the standard perfusion solution. It has recently been shown that recovery from an intracellular acid load in cardiac myocytes is mediated not only by Na^+-H^+ exchange but also by Na^+-HCO_3^- cotransport,[49] a process that may also result in an elevated intracellular $[Na^+]$ in the presence of inhibited Na^+,K^+-ATPase activity. Therefore, the protective effect observed in the present study may have been a property of the low $[HCO_3^-]$ rather than the high $[H^+]$ of the acidic reperfusion solution. However, our recent studies[50] that showed acidic reperfusion to be equally protective in the absence of HCO_3^- would argue strongly against such a possibility. These studies also showed that reperfusion with solution at pH 7.4 was equally arrhythmogenic in the presence of either 25 mmol/l HCO_3^- (gassed with 5% CO_2) or 5 mmol/l HEPES as buffer, despite a much reduced buffering capacity in the latter case. Therefore, it is unlikely that the protective effect of acidic reperfusion (with low $[HCO_3^-]$ solutions) observed in the present study was due to the reduced buffering capacity of the reperfusion solutions.

Acidosis inhibits a number of membrane currents, including the slow inward Ca^{2+} current[51] and the inwardly rectifying potassium current (I_{KI}).[52] The inhibition of the calcium current by acidic reperfusion, however, is unlikely to account for the antifibrillatory effect observed in the present study, because pharmacological inhibition of this current, when applied only during reperfusion, has been shown to be ineffective against reperfusion-induced arrhythmias.[53,54] In contrast, inhibition of the potassium current may well play a role in the protective effect of acidic reperfusion, because selective inhibition of this current by a novel pharmacological agent has recently been shown to abolish reperfusion-induced VF in the isolated rat heart.[55] Acidosis also inhibits the release of Ca^{2+} from the sarcoplasmic reticulum.[56] As discussed earlier, oscillatory release of Ca^{2+} from the sarcoplasmic reticulum has been implicated in reperfusion-induced arrhythmogenesis, and the inhibition of this process by acidic reperfusion may be expected to afford protection. In this context, it is worth noting that ryanodine, which also inhibits the release of Ca^{2+} from the sarcoplasmic reticulum, has been shown to prevent the degeneration of VT into VF during reperfusion of the regionally ischemic rat heart.[57]

Relevance to the Mechanism of Reperfusion-Induced Arrhythmias

Whereas the results of the present study indicate the rapid washout of extracellular H^+ to be an important arrhythmogenic factor during early reperfusion, they do not preclude a significant role for other factors in reperfusion-induced arrhythmogenesis. Indeed, whereas acidic reperfusion significantly inhibited reperfusion-induced VF, the incidence of reperfusion-induced VT remained at 100%, thus supporting the argument that multiple factors are involved. The recent study by Curtis[5] has demonstrated that the rapid washout of extracellular K^+, in the absence of ischemia and reperfusion, is sufficient to produce ventricular arrhythmias. In addition, studies with free oxygen radical–generating and –scavenging systems[3,4] suggest a causal role for free radicals in reperfusion-induced arrhythmias. In support of this, a burst of free radical production during early

reperfusion has been demonstrated,[2] and other studies have shown that reactive oxygen intermediates are sufficient to generate ventricular arrhythmias in the absence of ischemia and reperfusion[58,59] ahd to exacerbate reperfusion-induced arrhythmias.[60]

It is indeed possible that the induction of oxidant stress and the rapid washout of extracellular H^+ on reperfusion may act in a synergistic manner to disrupt Ca^{2+} homeostasis,[61] with arrhythmogenic consequences. In this context, oxidant stress has been shown to inhibit Na^+,K^+-ATPase activity,[43] stimulate sarcolemmal Na^+-Ca^{2+} exchange,[62] and increase the open probability of the sarcoplasmic reticular Ca^{2+} release channel.[63] In addition, the recent studies of Shattock and Matsuura[64,65] have shown that reactive oxygen intermediates can induce arrhythmogenic oscillations in membrane potential, indicative of intracellular Ca^{2+} overload, in both isolated ventricular muscles[64] and isolated myocytes.[65]

Limitations of the Study

Although the present study supports a major role for the rapid washout of H^+ in reperfusion arrhythmogenesis, it does not permit the delineation of the precise mechanism(s) by which maintained extracellular acidosis during early reperfusion exerts its protective effect. As discussed above, several possible mechanisms may be involved, and the role of each must be methodically examined before novel therapeutic approaches can be formulated. In addition, although the model used in the present study offers many advantages over conventional models[14,17] and enables the use of groups of adequate size (because of the low cost of the preparation), the use of the rat heart (which has unusual electrophysiological characteristics, such as a short action potential duration and a high heart rate) may limit the applicability of the present findings to other species. While acknowledging this, it should be noted that the response of the rat heart to ischemia and reperfusion and to many protective interventions resembles that of other mammalian species.[66] Indeed, in preliminary studies using isolated rabbit hearts with dual coronary perfusion, we have made observations similar to those reported here in that transient acidic (pH 6.6) reperfusion of the ischemic bed did not significantly reduce the incidence of reperfusion-induced VT (six of eight versus eight of eight in control hearts) but reduced the incidence of reperfusion-induced VF from eight of eight in control hearts to only one of eight (authors' unpublished observations).

Concluding Comments

The present study has shown that the selective reperfusion with acidic solutions of the ischemic rat myocardium, after 10 minutes of regional ischemia, does not affect the rapid induction of VT but significantly suppresses the degeneration of VT into VF and promotes spontaneous reversion to normal sinus rhythm. This protective effect is observed in the absence of intergroup differences in the size of the ischemic zone, the rate of reflow, and oxygen tension of the perfusate. These findings are consistent with a major arrhythmogenic role, during uncontrolled reperfusion, for the rapid washout of extracellular H^+.

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Mechanisms of antifibrillatory effect of acidic reperfusion: role of perfusate bicarbonate concentration

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Ibuki, Chikao, David J. Hearse, and Metin Avkiran. Mechanisms of antifibrillatory effect of acidic reperfusion: role of perfusate bicarbonate concentration. *Am. J. Physiol.* 264 (*Heart Circ. Physiol.* 33): H783-H790, 1993.—Transient (2 min) acidic (pH 6.6) reperfusion with low $[HCO_3^-]$ solution suppresses reperfusion-induced ventricular fibrillation (VF) in the isolated rat heart. Using this preparation, we tested whether the effect was mediated by the high $[H^+]$ or the low $[HCO_3^-]$ of perfusate. Left and right coronary beds were independently perfused with HCO_3^--containing (25.0 mmol/l) solution at pH 7.4. Regional ischemia was then induced by stopping flow to the left coronary bed for 10 min. Hearts were subsequently assigned to four groups ($n = 12$ hearts/group), and the left coronary bed was reperfused with either HCO_3^--containing (25.0 or 4.0 mmol/l) or HCO_3^--free (5.0 mmol/l HEPES) solution, at pH 7.4 throughout (control reperfusion) or at pH 6.6 for the first 2 min and at pH 7.4 from 2 to 5 min (acidic reperfusion). Regardless of the buffer, controls exhibited a high (92 and 100%) incidence of VF; this was reduced to 42% in both of the acidic reperfusion groups ($P < 0.05$). There were no intergroup differences in heart rate, coronary flow, or size of ischemic zone. Thus high $[H^+]$, rather than low $[HCO_3^-]$, appears to mediate the antifibrillatory effect of transient acidic reperfusion.

regional ischemia; dual coronary perfusion; reperfusion arrhythmias; ventricular fibrillation; protons; buffering capacity

REPERFUSION OF MYOCARDIUM subjected to a transient period of regional ischemia can result in the rapid generation of severe ventricular arrhythmias (for review, see Ref. 25). Putative arrhythmogenic factors during reperfusion include the rapid washout of K^+ from the extracellular space (7), the generation of oxygen-derived free radicals (4, 38), and the accumulation of intracellular Ca^{2+} (31). Irrespective of the identity of the metabolic or ionic trigger, the fundamental question that remains to be resolved is whether the arrhythmogenic process is initiated by the washout of components accumulated during ischemia (e.g., K^+) or by the resupply of components depleted during ischemia (e.g., oxygen) (30). In this context, Yamada and colleagues demonstrated that the readmission of oxygen was not a prerequisite for arrhythmogenesis (39), whereas a cycle of elevation and rapid normalization of extracellular K^+ was sufficient to induce arrhythmias even in the absence of ischemia and reperfusion (7).

In addition to K^+, protons (H^+) also accumulate in the extracellular space during ischemia (16), and their rapid washout has been proposed to result in Na^+ influx via Na^+-H^+ exchange with a subsequent increase in intracellular $[Ca^{2+}]$ via Na^+-Ca^{2+} exchange (23). Postischemic washout of extracellular H^+ has been implicated as a contributory factor to several sequelae of reperfusion, including myocardial stunning (17, 27) and arrhythmias (10). In support of the latter, our recent studies (2, 18) employing acidic reperfusion have shown that limitation of the rate at which extracellular pH is restored during early reperfusion can reduce the incidence of reperfusion-induced ventricular fibrillation (VF). The protective effect of acidic reperfusion was pH dependent over the range of 7.1-6.4, with maximum protection at pH 6.4 (18).

The acidic solutions employed in our studies were prepared by reducing the bicarbonate (HCO_3^-) content of a standard perfusion solution (metabolic acidosis), and as such the antifibrillatory effect might be attributable to the reduction of $[HCO_3^-]$ rather than the elevation of $[H^+]$ in the reperfusion solution. In addition to the potential antiarrhythmic effect of reduced extracellular buffering capacity (10), low $[HCO_3^-]$ may also limit Na^+ influx during reperfusion by a mechanism independent of Na^+-H^+ exchange. Liu et al. (24), using cultured chick heart cells, have shown that in CO_2-HCO_3^--buffered media, recovery from intracellular acidosis is partly mediated by a Cl^--HCO_3^- exchange process, which is accompanied by Na^+ influx. Observations consistent with this concept have also been made in sheep Purkinje fibers (9). Na^+-dependent Cl^--HCO_3^- exchange has been shown to be inhibited not only by the removal of extracellular HCO_3^- but also by the application of stilbene derivatives (24). It is interesting to note that the stilbene derivatives 4-acetamido-4'-isothiocyanostilbene-2,2'-disulfonic acid (SITS) and 4,4'-diisothio-cyanostilbene-2,2'-disufonic acid (DIDS) have both been shown to inhibit reperfusion-induced arrhythmias (5, 13), which might support a role for Cl^--HCO_3^- exchange in reperfusion arrhythmogenesis.

With the above observations in mind, the present study was designed to establish the relative contributions of perfusate $[H^+]$, $[HCO_3^-]$, and buffering capacity to the antifibrillatory effect of acidic reperfusion.

METHODS

Animals and Perfusion Technique

The investigation was performed in accordance with the Home Office Guidance on the Operation of the Animals (Scientific Procedures) Act 1986, published by Her Majesty's Stationery Office (London, UK). Male Wistar rats (220-280 g body wt, Bantin and Kingman) were anesthetized with diethyl ether, and heparin sodium (200 IU) was injected intravenously. Thirty seconds later, the heart was excised and immersed in perfusion solution at 4°C to induce rapid cardiac arrest. The aorta was then cannulated with a dual perfusion cannula, and right and left coronary arteries were perfused independently at constant perfusion pressure (100 cmH_2O), as described by Avkiran and Curtis (1) (Fig. 1). Each coronary bed was perfused with oxygenated perfusion solution from a temperature-regulated reservoir (37°C), and flow to each bed was continuously monitored using in-line flowmeters. Solution perfusing the left coronary bed could be changed, as required, by switching the inflow line between five reservoirs while still maintaining a constant perfusion pressure. The heart was housed in a temperature-regulated chamber kept at 37°C throughout the experiment. To maintain a stable sinus rate, the right atrium was continuously

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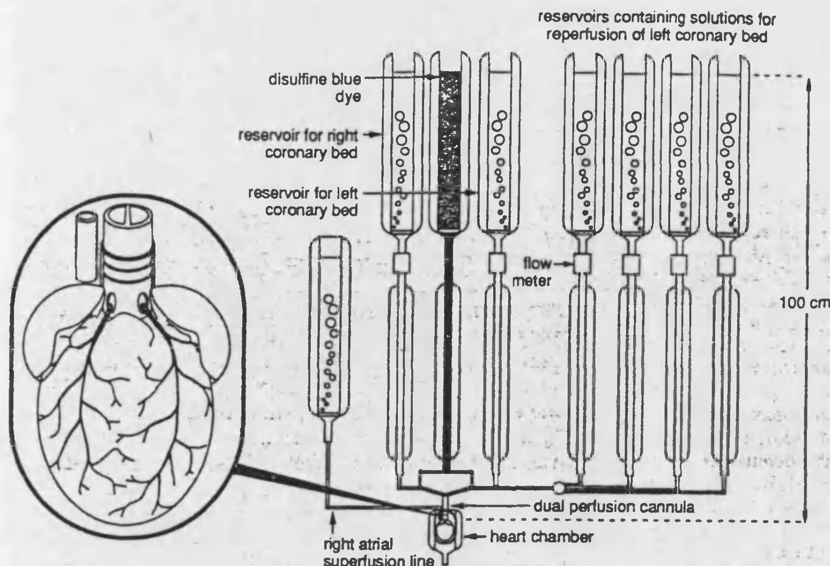

Fig. 1. Schematic illustration of perfusion apparatus. Regional ischemia was induced by stopping flow to left coronary bed. Subsequently, left coronary bed was selectively reperfused with one of 4 reperfusion solutions. Right coronary bed was perfused with standard perfusion solution throughout experimental protocol. *Inset*: details of dual perfusion cannula.

superfused (5 ml/min) with oxygenated perfusion solution at 37°C; this was necessary because the sinus node of the rat heart is not perfused by its coronary arteries (14).

Experimental Protocol

After 15 min of perfusion of both coronary beds with standard perfusion solution, regional ischemia was induced by halting perfusion of the left coronary bed. The right coronary bed continued to be perfused with standard perfusion solution at constant pressure throughout the experiment. Regional ischemia was maintained for 10 min, a duration that has been shown (15) to result in maximum vulnerability to reperfusion-induced VF in the isolated rat heart. At the end of the ischemic period, the hearts (n = 12 hearts/group) were randomly assigned to one of the four study groups (Fig. 2). The left coronary beds were then selectively reperfused with HCO_3^--containing or HCO_3^--free solutions at either pH 6.6 or 7.4, according to the protocol shown in Fig. 2 and as described below.

HCO_3^--containing groups. In these two groups of hearts, the left coronary bed was reperfused with CO_2-HCO_3^--buffered solution. In the control reperfusion group, the left coronary bed received the standard solution at pH 7.4 throughout 5 min of reperfusion. In the acidic reperfusion group, the left coronary bed received solution at pH 6.6 for the first 2 min of reperfusion followed by the standard solution at pH 7.4 from 2 to 5 min of reperfusion.

HCO_3^--free groups. In two further groups, the left coronary bed was reperfused with HCO_3^--free solution, which contained 5.0 mmol/l of *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES) as buffer. Again, in the control reperfusion group the left coronary bed received solution at pH 7.4 throughout 5 min of reperfusion, whereas in the acidic reperfusion group the left coronary bed received solution at pH 6.6 for the first 2 min and solution at pH 7.4 from 2 to 5 min of reperfusion.

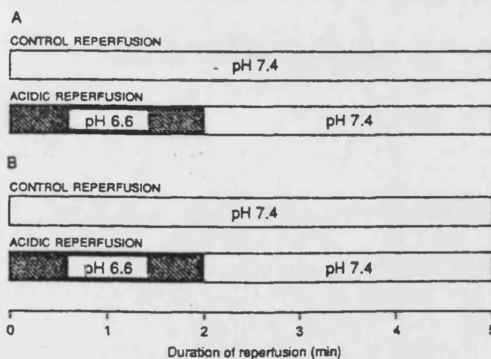

Fig. 2. Reperfusion protocols in 4 study groups (n = 12 hearts/group). After 10 min of regional ischemia (induced by stopping flow to left coronary bed) ischemic bed was selectively reperfused for 5 min with HCO_3^--containing solution (*A*) or HCO_3^--free solution (*B*). Within each subset, one group of hearts received acidic (pH 6.6) solution for the first 2 min of reperfusion.

Composition of Perfusion Solutions

Standard perfusion solution contained (in mmol/l) 118.5 NaCl, 25.0 $NaHCO_3$, 3.2 KCl, 1.2 $MgSO_4$, 1.2 KH_2PO_4, 1.4 $CaCl_2$, and 11.1 glucose. This solution was oxygenated and maintained at pH 7.4 by continuous gassing with 95% O_2-5% CO_2. Acidic (pH 6.6) HCO_3^--containing solution was prepared by lowering the $NaHCO_3$ concentration to 4.0 mmol/l while maintaining the same gassing mixture (95% O_2-5% CO_2).

HCO_3^--free solutions had an identical composition to the HCO_3^--containing solutions except that $NaHCO_3$ was substituted by HEPES (5.0 mmol/l). HCO_3^--free solutions were gassed with 100% O_2, and the pH was adjusted to either pH 7.4 or 6.6 by the addition of small quantities of NaOH. [Na^+] was kept constant (143 ± 2 mmol/l) in all solutions by modifying the NaCl content, as necessary. Before use, all solutions were filtered through a membrane with 5-μm pore size and [Na^+], pH, and Po_2 were measured (Nova Biomedical Statprofile).

Assessment of Buffering Capacity

To assess the relative buffering capacities of the HCO_3^--containing and HCO_3^--free control reperfusion solutions (pH 7.4), titration curves were constructed as follows. The two solutions were prepared as described previously and allowed to equilibrate with the appropriate gassing mixtures (95% O_2-5% CO_2 for the HCO_3^--containing solution and 100% O_2 for the HCO_3^--free HEPES solution). To 1,000 ml of each solution,

cumulative amounts of HCl (1.0 mol/l in 1-ml increments) were then added, and the pH of the solution was measured (Corning 140 pH meter with Ag/AgCl glass combination electrode) at each stage.

Measured Variables

Arrhythmias. The presence of arrhythmias was determined from a unipolar electrogram, which was recorded using a silver electrode attached to the apex of the heart and a reference electrode connected to the aortic cannula. The electrogram was continuously monitored on a digital storage oscilloscope (Gould type 1421) at 100 mm/s sweep speed to enable arrhythmia diagnosis. The electrogram was also recorded on an ink-jet recorder (Gould 2200S) at a chart speed of 2 mm/s. From a few seconds before reperfusion, the recorder chart speed was increased to 50 mm/s to obtain a permanent high-speed recording of the initial phase of reperfusion-induced arrhythmias. The electrogram was retrospectively analyzed in a blinded manner, and the incidence and time-to-onset of ventricular tachycardia (VT) and VF were determined. Arrhythmias were defined in accordance with the Lambeth Conventions (35). Thus VT was defined as a run of four or more consecutive premature beats of ventricular origin, VF as an electrical signal with no recognizable QRS complex for which heart rate could not be determined, and sustained VF as an episode of VF lasting for more than 120 s.

Heart rate and coronary flow. Throughout the experimental protocol, heart rate was determined from the electrogram, and flow to each coronary bed was monitored using the in-line flowmeters attached to each perfusion reservoir.

Size of ischemic zone. At the end of the reperfusion period, the left coronary bed (i.e., the tissue mass that had been subjected to ischemia and reperfusion) was perfused at 100 cmH_2O perfusion pressure for 3 min with a solution containing 0.016% wt/vol disulfine blue dye. The heart was then removed from the perfusion apparatus, the atria and mediastinal tissues were removed, and the stained ventricular tissue was carefully dissected away from the unstained tissue. Both fractions were blotted and weighed, and the weight of the left coronary bed (i.e., the size of the ischemic zone) was expressed as a percent of total ventricular weight.

Exclusion Criteria

Exclusion criteria, based on our previous experience with this model (1, 19), were prospectively defined to minimize interheart variation in heart rate and size of ischemic zone. Hearts were excluded if: *1*) heart rate before ischemia was <280 or >420 beats/min, or *2*) size of ischemic zone exceeded 70% of total ventricular weight. In addition, hearts were excluded if ventricular arrhythmias occurred during the final 3 s of ischemia before reperfusion, as in those hearts it would be impossible to distinguish between reperfusion-induced and ischemia-induced arrhythmias. Hearts were also excluded if there was significant cross-flow between right and left coronary ostia. To test whether this was occurring, the perfusion line to the left coronary bed was clamped for 10 s at the beginning of each experiment. If right coronary flow increased by >10% of the flow in the left coronary bed before clamping, the heart was excluded from the study. This criterion was based on the assumption that in the rat heart collateral flow alone could not have been responsible for such an increase (26).

Of a total of 61 hearts entered into this study, 3 were excluded based on heart rate, 4 on size of ischemic zone, 4 on account of arrhythmias during the final 3 s of ischemia, and 2 on account of cross-flow. When a heart was excluded, it was immediately replaced according to the randomization table.

Data Analysis

The general approach to statistical analysis was based on the guidelines described by Wallenstein et al. (36). Gaussian-distributed variables were expressed as means ± SE and subjected to an analysis of variance. If a difference among mean values was established with one-way analysis of variance, intergroup comparisons were performed using Tukey's test. Variables that were not Gaussian-distributed (such as time-to-onset of VF) were $\log_{10}$ transformed before parametric statistical testing as described above. Binomially distributed variables, such as the incidences of VT and VF, were compared using the chi-squared test for a 2 × 4 table followed by a sequence of 2 × 2 chi-squared tests with Yates' correction. Temporal changes in coronary flow and heart rate were assessed by the paired *t* test. A probability of <0.05 ($P < 0.05$) was considered significant.

RESULTS

The objective of the present study was to assess the effects of various interventions (applied after ischemia) on the incidence of reperfusion-induced arrhythmias. However, arrhythmias were also quantified in the preceding period of ischemia (during which time the composition of the perfusate was identical in all study groups) to confirm that all groups were in fact identical before the onset of reperfusion. This was confirmed by the data shown in Table 1, which illustrate that there were no significant differences among the four study groups in the time-to-onset, incidence, or severity of ischemia-induced arrhythmias.

Reperfusion-Induced Ventricular Arrhythmias

HCO_3^--containing groups. The time course of reperfusion-induced ventricular arrhythmias in individual hearts is illustrated in Fig. 3*A*. Almost immediately on reperfusion all hearts developed VT. In 11 of 12 hearts (92%) in the control group (pH 7.4 throughout reperfusion), VT degenerated into VF within 35 s of reperfusion, and all

Table 1. *Incidence, time-to-onset, and duration of arrhythmias during 10-min period of regional ischemia*

Group	Incidence		Mean Time-to-Onset, s		Mean Duration of Nonsinus Rhythm, s
	Ventricular tachycardia	Ventricular fibrillation	Ventricular tachycardia	Ventricular fibrillation	
HCO_3^- containing					
Control reperfusion	11/12 (92%)	0/12 (0%)	493±19		30±13
Acidic reperfusion	8/12 (67%)	1/12 (8%)	518±13	473*	16±6
HCO_3^- free					
Control reperfusion	7/12 (58%)	0/12 (0%)	522±17		13±4
Acidic reperfusion	9/12 (75%)	0/12 (0%)	524±25		13±4

Values are means ± SE; *n*, 12 hearts/group. * Only 1 heart exhibited ventricular fibrillation.

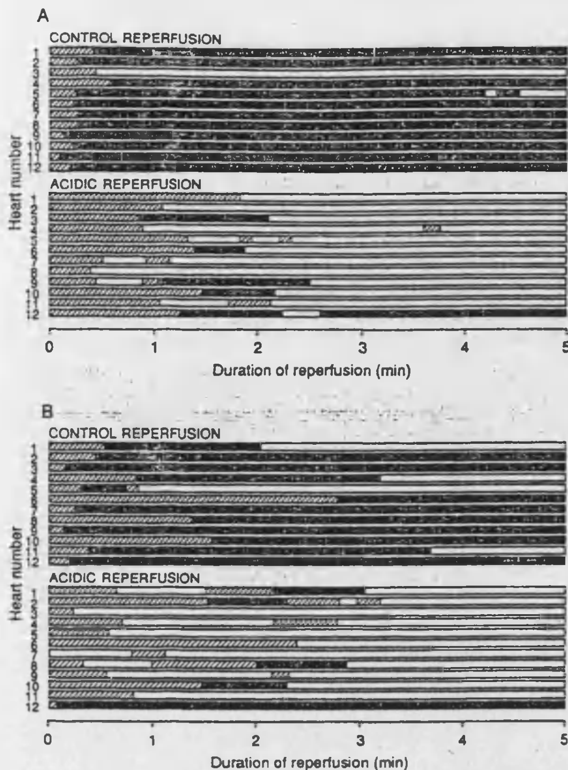

Fig. 3. Time course for arrhythmias during 5 min of reperfusion in groups reperfused with HCO_3^--containing solution (*A*) or HCO_3^--free solution (*B*). Each horizontal bar represents an individual heart (n = 12 hearts/group). Reperfusion was initiated at *time 0*, at which moment all hearts were in sinus rhythm. Hatched areas, ventricular tachycardia; solid areas, VF; open areas, sinus rhythm.

episodes of VF were sustained (>120 s). In the acidic reperfusion group, only five hearts (42%) exhibited VF during reperfusion ($P < 0.05$), and in four of these the arrhythmia reverted to a stable sinus rhythm within 90 s of its initiation. In those hearts that exhibited VF during reperfusion, the time-to-onset in the acidic reperfusion group (72 ± 7 s) was significantly prolonged compared with that in the control reperfusion group (16 ± 2 s).

In the acidic reperfusion group, eight hearts were not in VF at the end of the 2-min period of acidic reperfusion; none of these hearts exhibited de novo episodes of VF following the switch to perfusion with solution at pH 7.4.

HCO_3^--free groups. In the groups that were reperfused with HCO_3^--free solution, the pattern of arrhythmias was similar to that observed in the groups reperfused with HCO_3^--containing solution (Fig. 3*B*). Almost immediately on reperfusion, all of the hearts in the control (pH 7.4) reperfusion group and 92% of the hearts in the acidic reperfusion group developed VT. In the control reperfusion group, 100% of hearts exhibited VF during reperfusion, and in 10 of 12 hearts (83%) this was sustained (>120 s). In contrast, in the acidic reperfusion group, only 5 of 12 hearts (42%) exhibited reperfusion-induced VF ($P < 0.05$), which was sustained in only one heart ($P < 0.05$). The time-to-onset of VF in the control reperfusion group receiving HCO_3^--free solution (45 ± 14 s) tended to be delayed relative to the control reperfusion group receiving HCO_3^--containing solution (16 ± 2 s). Transient acidic reperfusion tended to produce a further delay in the time-to-onset of VF (87 ± 22 s), but this effect did not achieve a level of statistical significance.

In the acidic reperfusion group, only 2 of 9 hearts that were not in VF at the time of switching the perfusion solution from pH 6.6 to 7.4 subsequently exhibited de novo episodes of VF.

Heart Rate, Coronary Flow, and Size of Ischemic Zone

HCO_3^--containing groups. Before ischemia, heart rate was 326 ± 6 beats/min in the control (pH 7.4) reperfusion group and 333 ± 8 beats/min in the acidic reperfusion group (NS). Heart rate did not change significantly during the subsequent period of ischemia, and after 9 min of ischemia the heart rates were 334 ± 11 and 319 ± 13 beats/min in the control and acidic reperfusion groups, respectively. Mean heart rate could not be determined during reperfusion due to the high incidence of VT and VF.

Right and left coronary flows during the experimental protocol are shown in Table 2. Before ischemia, right and left coronary flows were similar in the two groups. Right coronary flow did not change significantly in either group during the 10-min period of regional ischemia in the left

Table 2. *Right and left coronary flows before and during regional ischemia and during reperfusion*

Group	Coronary Bed	Coronary Flow, ml·min⁻¹·g wet wt⁻¹						
		Before ischemia	During ischemia			During reperfusion		
			1 min	5 min	9 min	1 min	3 min	5 min
HCO_3^- containing								
Control reperfusion	Right	10.2±0.7	10.7±0.8	10.2±0.7	10.1±0.8	16.4±1.5†	15.1±1.2†	14.8±1.4†
	Left	11.6±0.6	0	0	0	24.4±2.5†	24.8±2.7†	24.5±2.2†
Acidic reperfusion	Right	11.4±0.9	11.5±0.9	11.0±0.9	11.0±1.3	15.4±1.6†	10.3±0.9*	10.4±1.0*
	Left	12.1±0.6	0	0	0	22.3±1.5†	26.3±0.8†	24.1±0.8†
HCO_3^- free								
Control reperfusion	Right	11.2±0.7	11.7±0.8	10.9±0.8	11.2±1.0	18.4±1.1†	15.3±1.3†	13.4±1.7
	Left	12.4±0.9	0	0	0	24.4±2.0†	23.7±1.7†	24.8±1.7†
Acidic reperfusion	Right	10.8±0.4	11.2±0.5	10.7±0.5	10.8±0.5	13.1±1.1	10.9±1.3*	9.2±1.3*
	Left	11.1±0.4	0	0	0	20.1±2.6†	26.1±1.6†	26.4±0.9†

Values are means ± SE; n, 12 hearts/group. * $P < 0.05$ vs. corresponding control reperfusion group; † $P < 0.05$ vs. before ischemia.

coronary bed. On reperfusion, coronary flow increased to rates significantly greater than the preischemic values in both left and right coronary beds, with the increase in the left being substantially greater than that in the right. Left coronary flow remained significantly elevated throughout the 5 min of reperfusion in both groups. Right coronary flow also remained elevated in the control reperfusion group but returned toward prereperfusion values within 3 min in the acidic reperfusion group.

Total ventricular wet weights were 0.70 ± 0.02 and 0.71 ± 0.02 g, and sizes of the ischemic zone were 54 ± 3 and 53 ± 3% in the control and acidic reperfusion groups, respectively (NS).

HCO_3^--free groups. Before ischemia, heart rate was similar in the control and acidic reperfusion groups (332 ± 8 and 328 ± 9 beats/min, respectively). Regional ischemia had no significant effect on heart rate which, after 9 min of ischemia, was 327 ± 10 beats/min in the control (pH 7.4) reperfusion group and 319 ± 13 beats/min in the acidic reperfusion group. Again, mean heart rate could not be determined during reperfusion due to VT and VF.

Right and left coronary flows were similar in the two groups before ischemia (Table 2). Right coronary flow did not change significantly in either group during regional ischemia. Coronary flow increased significantly in both beds on reperfusion, and left coronary flow remained elevated throughout the 5-min reperfusion period in both groups. Right coronary flow returned to the prereperfusion value within 3 min of reperfusion in the acidic reperfusion group, while it remained elevated in the control reperfusion group.

Total ventricular wet weights were 0.72 ± 0.01 and 0.71 ± 0.02 g in the control and acidic reperfusion groups, respectively (NS); there was no significant difference in size of the ischemic zone between the two groups (50 ± 3 vs. 50 ± 2%).

Buffering Capacity of HCO_3^--Containing and HCO_3^--Free Solutions

Figure 4 shows the titration curves for the HCO_3^--containing and HCO_3^--free solutions, which both had an initial pH of 7.4. As illustrated by the different slopes of the curves, the buffering capacity of the HCO_3^--containing solution was substantially greater than that of the HCO_3^--free solution containing HEPES as buffer.

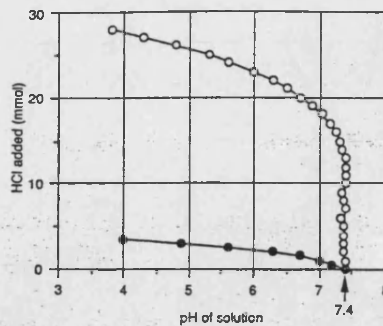

Fig. 4. Titration curves for HCO_3^--containing (open circles) and HCO_3^--free (solid circles) perfusion solutions. Latter contained 5 mmol/l HEPES as buffer. To 1,000 ml of each solution (initially adjusted to pH 7.4, see text) cumulative amounts of HCl were added (1 mol/l in 1-ml increments), and pH was measured at each stage. Both solutions were continuously gassed with appropriate mixture (95% O_2-5% CO_2 for HCO_3^--containing solution and 100% O_2 for HCO_3^--free solution).

DISCUSSION

Two conclusions can be drawn from the data presented in this study. First, reperfusion at pH 7.4 with HCO_3^--free HEPES solution had no significant effect on the severity of reperfusion-induced arrhythmias relative to reperfusion with a HCO_3^--containing solution of the same pH, despite the vastly different buffering capacities of the two solutions. Second, acidic reperfusion resulted in a significant antifibrillatory effect regardless of the presence or absence of HCO_3^-. These observations were made in preparations with no intergroup differences in variables such as heart rate, coronary flow, and size of ischemic zone, any one of which might influence the severity of reperfusion-induced arrhythmias. It appears therefore that extracellular $[H^+]$, rather than $[HCO_3^-]$, plays the pivotal role in the antiarrhythmic effect of transient metabolic acidosis during early reperfusion.

Possible Mechanisms of Antiarrhythmic Effect of Transient Metabolic Acidosis

Role of extracellular buffering capacity. Dennis and colleagues (10) have demonstrated a positive association between the buffering capacity of a reperfusion solution and the duration of reperfusion-induced arrhythmias. They argued that this reflected the effect of extracellular buffering on sarcolemmal Na^+-H^+ exchange. However, our data suggest that in the present model the buffering capacity of the reperfusion solution is not a critical determinant of the severity of reperfusion-induced arrhythmias. Although there is apparent disagreement between the two studies, there are also several potentially important differences in methodology.

First, whereas Dennis et al. (10) used 15 min of global, low-flow ischemia, we subjected our hearts to 10 min of regional, zero-flow ischemia. The severity of ischemia-induced acidosis and thus the consequences of subsequent reperfusion at pH 7.4 are likely to differ under low-flow and zero-flow conditions. In support of this, comparison of the results for hearts from both studies that were subjected to reperfusion with solutions containing HEPES (5 mmol/l, pH 7.4) shows that the mean total duration of nonsinus rhythm (expressed as a percentage of the total duration of reperfusion) was 83 ± 8% in the present study and only 34 ± 4% in the study by Dennis et al. (10), despite the greater mass of ischemic-reperfused tissue in the latter.

The second point to consider is that Dennis et al. (10) did not report the incidences of VT and VF in their various study groups; thus it is not clear which type of arrhythmia was influenced by the buffering capacity of the perfusate. In this context, it is noteworthy that in both our present and our previous (2, 18) studies only the incidence of VF, but not that of VT, was influenced by the pH of the reperfusion solution.

Finally, the higher concentrations of HEPES (15–50 mmol/l) used by Dennis et al. (10) were in excess of those normally used in physiological solutions for isolated heart

studies (e.g., Refs. 22, 28, and 29). The possibility cannot be excluded that at these high concentrations, HEPES might itself have a direct arrhythmogenic effect on the myocardium. Indeed, there is some evidence (3) that HEPES may influence the electrical and mechanical activities of ventricular muscle in a manner that is independent of its properties as a buffer.

Role of Cl^--HCO_3^- exchange. In the presence of CO_2-HCO_3^-, the recovery of intracellular pH from acidosis is mediated not only by Na^+-H^+ exchange but also by a Na^+-dependent, Cl^--HCO_3^- exchange process (9, 24). As proposed earlier, it is possible that the antiarrhythmic effect of transient metabolic acidosis during early reperfusion (18) may be mediated by inhibition of Cl^--HCO_3^- exchange as a consequence of the low extracellular [HCO_3^-]. However, the present study has demonstrated that transient acidic reperfusion is protective regardless of the presence or absence of HCO_3^- in the perfusate, an observation that would argue against such a possibility. Indeed, the high incidence of reperfusion-induced VT and VF in the hearts reperfused with HCO_3^--free solution at pH 7.4 suggests that Cl^--HCO_3^- exchange may be unimportant in the genesis of reperfusion arrhythmias. Although this might appear to contradict the studies of Curtis and colleagues (5, 13) in which the Cl^--HCO_3^- exchange inhibitors SITS and DIDS were shown to inhibit the genesis of reperfusion-induced arrhythmias, the same group subsequently suggested (6) that SITS may exert an antiarrhythmic action through a reduction in ventricular excitability, an effect that might reflect some nonspecific action of the compound rather than its ability to influence Cl^--HCO_3^- exchange. These observations suggest that, at least in the present experimental model, inhibition of Na^+-dependent Cl^--HCO_3^- exchange does not contribute to the suppression of reperfusion-induced arrhythmias by transient metabolic acidosis. Rather, they indicate that the [H^+] of the reperfusion solution is a more likely candidate for the role of primary determinant of the severity of reperfusion-induced arrhythmias.

Role of Na^+-H^+ exchange. Several studies in the past few years, which used pharmacological inhibitors of sarcolemmal Na^+-H^+ exchange, have supported a role for this exchange process in postischemic calcium overload (27, 32), contractile dysfunction (21, 28, 32), and arrhythmias (10). We propose that the inhibition of Na^+-H^+ exchange is also a likely contributor to the antiarrhythmic effect of high extracellular [H^+] during early reperfusion.

Vaughan-Jones and Wu (34) have demonstrated in sheep Purkinje fibers that H^+ extrusion and Na^+ influx via Na^+-H^+ exchange during recovery from intracellular acidosis is inhibited by high extracellular [H^+]. Similar observations have also been made in isolated rat myocytes (37). It is therefore highly probable that sarcolemmal Na^+-H^+ exchange was significantly inhibited during reperfusion at pH 6.6 in the present study. As proposed by Lazdunski et al. (23), during uncontrolled reperfusion the rapid washout of the extracellular H^+ may result in Na^+ influx via Na^+-H^+ exchange with a consequent increase in intracellular [Ca^{2+}] via Na^+-Ca^{2+} exchange. Such an increase in intracellular [Ca^{2+}] ("Ca^{2+} overload") has been proposed as a mediator of reperfusion-induced arrhythmias, probably via the induction of oscillatory release of Ca^{2+} from the sarcoplasmic reticulum and subsequent delayed afterdepolarizations (31). It is conceivable therefore that the antiarrhythmic effect of acidic reperfusion may result from the inhibition of Na^+ influx via Na^+-H^+ exchange and the consequent inhibition of Ca^{2+} overload. It should be noted, however, that the inhibition of Na^+-H^+ exchange would inhibit not only Na^+ influx but also H^+ extrusion, which itself may influence vulnerability to arrhythmias by maintaining intracellular acidosis during early reperfusion.

Role of maintained intracellular and extracellular acidosis. Many cellular mechanisms that regulate ion movements are known to be pH sensitive, and their inhibition by maintained intracellular and extracellular acidosis during the acidic reperfusion period might contribute to the protective effects observed. Such pH-sensitive ion transport mechanisms include the slow inward Ca^{2+} current (20), the fast inward Na^+ current (40), and the inwardly rectifying K^+ current (11). Intracellular acidosis has also been shown (12) to inhibit the release of Ca^{2+} from the sarcoplasmic reticulum and, as previously discussed, oscillatory release of Ca^{2+} from the sarcoplasmic reticulum has been implicated (31) in the genesis of reperfusion-induced arrhythmias. In this context, ryanodine, an inhibitor of Ca^{2+} release from the sarcoplasmic reticulum, has also been shown to suppress reperfusion-induced arrhythmias (33).

Role of coronary flow during reperfusion. In the present study, there was a large increase in left coronary flow on reperfusion that was maintained throughout the 5-min period of reperfusion in all groups. There were no significant differences in the magnitude of the hyperemic response between control and acidic reperfusion groups. However, there were differences between control and acidic reperfusion groups in the temporal profiles for right coronary flow during reperfusion of the left coronary bed, which probably reflected the differences between the groups in the severity and duration of reperfusion-induced arrhythmias. Severe ventricular arrhythmias reduce extravascular compression, which in turn allows coronary flow to increase even in the bed not subjected to ischemia and reperfusion (1). Thus although right coronary flow was significantly elevated in all groups during the first minute of reperfusion (during which all hearts exhibited episodes of VT and/or VF), in acidic reperfusion groups it had fallen to prereperfusion values after 3 min of reperfusion, by which time a significant proportion of the hearts had reverted to sinus rhythm.

Limitations and Advantages of Present Model

The major limitation of the present study may be the use of crystalloid perfusion, which results in coronary flows in excess of those encountered in vivo. Such high flows may result in faster washout during reperfusion of components (such as H^+ and K^+) that have accumulated during the preceding period of ischemia, which in turn may lead to an overestimation of the role of washout in reperfusion arrhythmogenesis. Although our recent studies (19) suggest that the rate of reflow may not be a major

factor in determining vulnerability to reperfusion-induced arrhythmias, it would be of interest to discover the antiarrhythmic efficacy of acidic reperfusion in preparations with more physiological coronary flows.

It may also be argued that the use of the rat heart, which has unusual electrophysiological characteristics (such as a short action potential duration and a high heart rate), may limit the applicability of the present findings to other species. With this acknowledgment, it must be pointed out that in terms of arrhythmogenesis the response of the rat heart to ischemia and reperfusion resembles that of other species (8). In addition, the model employed in the present study offers many advantages (1), such as the consistent generation of regional ischemia and arrhythmias, the ability to use groups of adequate size (due to the low cost of the preparation), and the ability to reperfuse the ischemic zone selectively with modified solutions.

In conclusion, the present study has confirmed that transient (2 min) acidic (pH 6.6) reperfusion results in a significant reduction in the incidence and duration of reperfusion-induced VF. This protective effect occurs regardless of the presence or absence of HCO_3^- in the reperfusion solution, and our results provide evidence to suggest that it is the $[H^+]$ of the reperfusion solution, rather than its buffering capacity, that is the primary determinant of the severity of reperfusion-induced arrhythmias. The likely mechanism of the protective effect of high extracellular $[H^+]$ during early reperfusion appears to be the inhibition of sarcolemmal Na^+-H^+ exchange and the consequent inhibition of Na^+ influx and H^+ efflux, either or both of which may result in a reduced vulnerability to VF.

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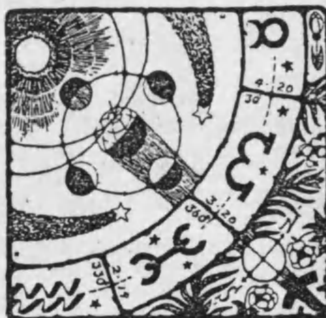

Effects of acidic reperfusion on arrhythmias and Na^+-K^+-ATPase activity in regionally ischemic rat hearts

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Avkiran, Metin, Chikao Ibuki, Yasuyuki Shimada, and Peter S. Haddock. Effects of acidic reperfusion on arrhythmias and Na^+-K^+-ATPase activity in regionally ischemic rat hearts. *Am. J. Physiol.* 270 (*Heart Circ. Physiol.* 39): H957–H964, 1996.—We studied the effects of acidic reperfusion on *1*) the incidence of ventricular fibrillation (VF) and *2*) sarcolemmal Na^+-K^+-adenosinetriphosphatase (ATPase) activity. Isolated rat hearts (n = 12/group) were subjected to independent perfusion (15 min) of left and right coronary beds with pH 7.4 buffer followed by zero-flow ischemia (10 min) of the former bed. This was then reperfused for 5 min, with acidic (pH 6.6) buffer for the first 0 (control), 0.5, 1, 2, or 4 min and with pH 7.4 buffer thereafter. In the control group, 92% of hearts developed VF within 20 s of reperfusion and remained in VF. In the 0.5-, 1-, 2-, and 4-min acidic reperfusion groups, only 17, 17, 42, and 25% of hearts ($P < 0.05$ vs. control for all groups), respectively, exhibited VF during acidic reperfusion. However, on switching to pH 7.4, VF occurred in a further 50, 58, 0, and 0% of hearts, respectively; thus the overall incidences of VF were 67, 75, 42 ($P < 0.05$ vs. control), and 25% ($P < 0.05$ vs. control), respectively. Additional hearts (n = 8/group) were used for cytochemical determination of sarcolemmal Na^+-K^+-ATPase activity in both the ischemic/reperfused left ventricular (LV) and the nonischemic right ventricular (RV) free walls. Ischemia (10 min) reduced LV Na^+-K^+-ATPase activity from 110 ± 8 to 25 ± 3% of the RV value. After 0.5, 1, 2, 3, and 4 min of acidic reperfusion, LV Na^+-K^+-ATPase activity was 24 ± 3, 29 ± 3, 37 ± 5†, 55 ± 6†, and 70 ± 4†, respectively (†$P < 0.05$ vs. 10-min ischemia). No significant recovery of LV Na^+-K^+-ATPase activity occurred following up to 4 min of pH 7.4 reperfusion. In conclusion, *1*) at least 2 min of acidic reperfusion is required to achieve sustained protection against VF and *2*) the protective mechanism may involve enhanced recovery of Na^+-K^+-ATPase activity as well as inhibition of Na^+ influx.

regional ischemia; reperfusion arrhythmias; intracellular sodium; sodium/hydrogen exchange; sodium/bicarbonate symport; sodium-potassium-adenosinetriphosphatase

LAZDUNSKI ET AL. (16) were the first to suggest that the rapid washout of extracellular H^+ on reperfusion of ischemic myocardium may create an intracellular-to-extracellular H^+ gradient, resulting in Na^+ influx coupled to H^+ extrusion via the Na^+/H^+ exchanger. Recent evidence (33) suggests that, during reperfusion, Na^+ influx coupled to H^+ extrusion may occur also through the Na^+/HCO_3^- symporter. Such an influx of Na^+ could result in an increased intracellular Na^+ concentration ($[Na^+]_i$) which, in turn, would favor an increase in $[Ca^{2+}]_i$ via the Na^+/Ca^{2+} exchanger (16, 30). An increase in $[Ca^{2+}]_i$ on reperfusion {that can be ameliorated by the Na^+/H^+-exchange inhibitor amiloride (31) or by acidic reperfusion (21)} has been demonstrated in experimental studies and implicated as a mediator of postischemic contractile dysfunction (31).

Ca^{2+} overload via Na^+/H^+ and Na^+/Ca^{2+} exchange-mediated mechanisms has also been proposed as an important trigger for reperfusion arrhythmogenesis (7). In support of this, modulation of the activity of the Na^+/H^+ exchanger in isolated hearts through the use of solutions of reduced buffering capacity (7) or pharmacological inhibitors {such as amiloride and its analogues (7, 28, 37) or the novel compound HOE 694 (27, 37)} has been shown to significantly attenuate the severity of reperfusion-induced arrhythmias. Our recent studies in the isolated rat heart (3, 12) have shown that limiting the rate of normalization of extracellular pH during early reperfusion, by transient (2 min) acidic reperfusion, significantly suppresses the induction of ventricular fibrillation (VF). The protection afforded by acidic reperfusion was maximal at pH 6.6, and the abrupt restoration of extracellular pH to 7.4 after 2 min of acidic reperfusion did not initiate new episodes of VF (3). However, the minimum duration of acidic reperfusion required to achieve such sustained protection against VF remains unknown.

Intracellular Na^+ homeostasis is maintained largely through Na^+ extrusion by the sarcolemmal Na^+-K^+ pump, the biochemical correlate of which is the sarcolemmal Na^+-K^+-ATPase. In a number of experimental models, Na^+ influx during recovery from intracellular acidosis has been shown to result in the activation of electrogenic Na^+ pumping by Na^+-K^+-ATPase (23, 24) that would oppose an increase in $[Na^+]_i$. However, during recovery from intracellular acidosis in the presence of low extracellular $[K^+]$ or ouabain (both of which inhibit Na^+-K^+-ATPase activity) there is a large increase in $[Na^+]_i$ (1, 19). This is thought to occur via Na^+/H^+ exchange (1) and is associated with an increase in $[Ca^{2+}]_i$ via Na^+/Ca^{2+} exchange (1, 19). It is possible therefore that during reperfusion also the magnitude of any increase in $[Ca^{2+}]_i$ and its unfavorable sequelae would depend largely on sarcolemmal Na^+-K^+-ATPase activity. Indeed, recent studies employing pharmacological inhibitors of Na^+-K^+-ATPase have supported a key role for the activity of this enzyme during reperfusion in determining not only the severity of postischemic contractile dysfunction (18) but also the incidence of reperfusion-induced VF (32).

From the above, we hypothesized that if the induction of VF during uncontrolled reperfusion (3, 12) was initiated by Na^+ influx coupled to H^+ extrusion and consequent Ca^{2+} overload, then the period of regional ischemia employed (10 min) must be sufficient to result in a significant inhibition of sarcolemmal Na^+-K^+-ATPase activity. Furthermore, there must be a progres-

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sive recovery of Na^+-K^+-ATPase activity during acidic reperfusion which, if maintained for a sufficient period, would result in reduced susceptibility to VF even after a subsequent abrupt normalization of extracellular pH, thus providing sustained protection. To determine whether a correlation exists between Na^+-K^+-ATPase activity at the moment of the abrupt increase in extracellular pH and the likelihood of developing VF subsequent to the pH change, we used isolated rat hearts subjected to independent perfusion of the left and right coronary beds together with a cytochemical assay for the in situ measurement of sarcolemmal Na^+-K^+-ATPase activity. In two parallel studies, we subjected hearts to 10 min of regional ischemia and examined *1*) susceptibility to VF during transient (0.5, 1, 2, or 4 min) acidic (pH 6.6) reperfusion and subsequent abrupt normalization of perfusate pH relative to that during normal (pH 7.4) reperfusion throughout and *2*) the changes in sarcolemmal Na^+-K^+-ATPase activity following ischemia and after various durations of acidic (pH 6.6) or normal (pH 7.4) reperfusion.

METHODS

All experiments were conducted in accordance with institutional guidelines and the Home Office "Guidance on the Operation of the Animals (Scientific Procedures) Act 1986."

Animals and Perfusion Technique

Independent dual perfusion of left and right coronary arteries in isolated hearts from male Wistar rats (220–280 g; B & K Universal, Hull, UK) was performed as described in detail by Avkiran and Curtis (2). Perfusion of both coronary beds with oxygenated perfusion solution at 37°C was carried out at a constant perfusion pressure equivalent to 75 mmHg, with left and right coronary flow rates continously monitored through in-line flow detectors (Transonic T206 flowmeter with 1N probes, Transonic Systems, Ithaca, NY). When required, the left coronary bed could be selectively perfused with modified solution (also at 37°C) from a separate reservoir. An additional reservoir held perfusion solution containing disulphine blue dye (0.016% wt/vol) that could be infused unilaterally to delineate the two perfusion beds. To maintain sinus rate, the right atrium was continuously superfused with oxygenated perfusion solution (37°C) at a rate of 5 ml/min (2).

Composition of Perfusion Solutions

Standard perfusion solution contained (in mM) 118.5 NaCl, 25.0 $NaHCO_3$, 3.2 KCl, 1.2 $MgSO_4$, 1.2 KH_2PO_4, 1.4 $CaCl_2$, and 11.1 glucose. This solution was oxygenated and maintained at pH 7.4 by continuous gassing with 95% O_2-5% CO_2. Acidic (pH 6.6) solution was prepared by lowering [$NaHCO_3$] to 4.0 mM while maintaining the same gas mixture containing 5% CO_2. [Na^+] was kept constant (143 ± 2 mM) in all solutions by modifying [NaCl] as necessary. Before use, all solutions were filtered through a membrane with 5-µm pore size.

Experimental Protocols

In all studies, both coronary beds were perfused with standard perfusion solution for an initial 15-min period, after which the left coronary bed was subjected to zero-flow ischemia. The right coronary bed was perfused with standard perfusion solution throughout all experiments.

Arrhythmia study. Regional ischemia was maintained for 10 min, a duration which in the absence of protective interventions results in a high incidence of reperfusion-induced VF in this model (2). At the end of the ischemic period hearts were randomly assigned to one of five groups (n = 12/group), and the left coronary bed was selectively reperfused for 5 min, during which arrhythmias were monitored. In the control group, the left coronary bed received the standard solution at pH 7.4 throughout the reperfusion period. In the acidic reperfusion groups, the left coronary bed received the modified solution at pH 6.6 for the first 0.5, 1, 2, or 4 min of reperfusion and the standard solution at pH 7.4 thereafter. In a parallel study (see below), Na^+-K^+-ATPase activity was determined at the end of a 10-min period of regional ischemia and after 0.5–4 min of acidic or normal reperfusion in separate populations of hearts.

Na^+-K^+-ATPase study. In the acidic reperfusion protocol, hearts were randomly assigned to one of seven groups (n = 8/group) and sectioned at the midventricular level after 15 min of aerobic perfusion, after 10 min of zero-flow ischemia, and after 10 min of zero-flow ischemia followed by 0.5, 1, 2, 3, or 4 min of reperfusion of the left coronary bed with the acidic (pH 6.6) perfusion solution. In the normal reperfusion protocol, hearts were assigned to one of five groups (n = 6/group) and similarly sectioned after 15 min of aerobic perfusion, after 10 min of zero-flow ischemia, and after 10 min of zero-flow ischemia followed by 0.5, 2, or 4 min of reperfusion of the left coronary bed with standard (pH 7.4) perfusion solution. In all cases, the apical portions of the hearts were rapidly frozen by immersion in n-hexane cooled to −70°C. The tissues were stored in liquid N_2 until they were assayed for sarcolemmal Na^+-K^+-ATPase activity (see *Na^+-K^+-ATPase activity*).

Measured Variables

Arrhythmias. Arrhythmias were diagnosed from a unipolar electrocardiogram (ECG) that was obtained using a silver electrode inserted into the free wall of the left ventricle and a reference electrode connected to the aorta. The ECG was continuously monitored on a digital storage oscilloscope (Gould type 1421, Gould Electronics, Ilford, UK) and recorded on an ink-jet recorder (Gould 2200S). The ECG record was retrospectively analyzed (in a blinded manner) for the incidence, time to onset, and duration of ventricular tachycardia (VT) and VF. All analyses were carried out in accordance with the Lambeth Conventions (35). VT was defined as four or more consecutive premature beats of ventricular origin, and VF was defined as a signal in which both rate and amplitude varied from cycle to cycle.

Coronary flow rate and heart rate. In the arrhythmia study, flow rates to each coronary bed (monitored using the in-line flowmeters) and heart rate (determined from the ECG) were noted at selected time points during the protocol.

Ischemic zone size. At the end of each experiment in the arrhythmia study, the left coronary bed was perfused at 75-mmHg perfusion pressure for 3 min with a solution containing 0.016% disulphine blue dye. The heart was then removed from the perfusion apparatus, the atria and mediastinal tissue were excised, and dye-stained tissue (representing ventricular myocardium subjected to ischemia and reperfusion) was carefully dissected away from the remainder. The stained and unstained tissues were lightly blotted and weighed. The size of the ischemic zone, expressed as a percentage of total ventricular weight, was calculated from the equation (wt of stained tissue/total ventricular wt) × 100. The absolute weights obtained also enabled the calculation of flows in left and right coronary beds on the basis of tissue weights supplied by each bed (in ml per min per g).

Na+-K+-ATPase activity. Sequential pairs of midventricular sections (10-μm thick) were obtained from each heart using an automated cryostat (Bright Instrument, Huntingdon, UK). The sections were then flash dried onto glass microscope slides. Ouabain-sensitive Na+-K+-ATPase activity was determined in left and right ventricular (RV) free walls (supplied by the left and right coronary beds, respectively) using the cytochemical assay initially developed by Chayen et al. (5) and subsequently adapted by others to quantify localized Na+-K+-ATPase activity in rat kidney tubules (15) and ventricular myocardium (9). All reagents used were purchased from Sigma (Poole, UK).

Briefly, a Perspex ring was placed around each section to enable submersion of the section in a 40% (wt/vol) solution of Polypep 5115 in 200 mM tris(hydroxymethyl)aminomethane (Tris) buffer (pH 7.5 at 37°C) containing 100 mM potassium acetate. After 10-min exposure to remove excess free inorganic phosphate (that would otherwise produce a strong background coloration at a later stage of the assay), this medium was removed and immediately replaced by a reaction medium consisting of 40% (wt/vol) Polypep 5115 in 200 mM Tris buffer (pH 7.5 at 37°C) containing (in mM) 1.0 sodium acetate, 410 NaCl, 20.0 $MgCl_2$, 37.5 KCl, 16.5 ATP, and 32 mg/ml lead ammonium citrate/acetate complex. On each slide, one of the two sections was submerged in a similar reaction medium containing 1 mM ouabain, which has been shown to produce maximal inhibition of ATPase activity in rat tissue (15), thus enabling the determination of total ATPase activity and ouabain-insensitive ATPase activity in sequential sections.

After incubation for 15 min at 37°C, the medium was removed by aspiration, and the slides were rinsed twice in 200 mM Tris buffer (pH 7.5 at 37°C). The slides were then immersed for 1–2 min in water saturated with hydrogen sulfide and, after being rinsed in distilled water, were allowed to dry at room temperature. The density of the reaction product (lead sulfide) was then quantified at 585 nm using a microdensitometer (model M86, Vickers Instruments, York, UK) with a ×40 objective and a flying spot of 0.5-μm diameter in the plane of the section. At least 10 readings were taken from 10 cells per ventricle, and a mean value was calculated. Readings were corrected for background coloration, standardized against an internal standard, and data were expressed as mean integrated extinction × 100 (MIE × 100). Ouabain-sensitive ATPase (i.e., Na+-K+-ATPase) activity was calculated from the total and ouabain-insensitive ATPase activities measured in sequential sections. The measurement of Na+-K+-ATPase activity in both the left ventricular (LV) free wall (selectively subjected to ischemia and reperfusion) and the RV free wall (perfused with standard perfusion solution throughout) enabled each heart to act as its own control. Determinations of Na+-K+-ATPase activity in the various samples were carried out in a blinded manner.

Exclusion Criteria

Exclusion criteria were the same as those employed in our previous studies with this model (2, 3, 11, 12, 37). The overall exclusion rate was <15%, and the excluded hearts were evenly distributed among the study groups.

Data Analysis

Gaussian-distributed variables were expressed as means ± SE and were subjected to analysis of variance (ANOVA). If a difference among mean values was established with ANOVA, intergroup comparisons were performed using Tukey's test. Binomially distributed variables, such as the incidences of VT and VF, were compared using the χ^2 test for a 2 × 5 table followed by a sequence of 2 × 2 χ^2 tests with Yates' correction. A probability of <0.05 ($P < 0.05$) was considered significant.

RESULTS

Arrhythmia Study

Reperfusion arrhythmias. Consistent with our earlier studies (3, 12), reperfusion after 10 min of regional ischemia resulted in the rapid (within a few beats) induction of VT in 100% of hearts in all groups regardless of the pH of the reperfusion solution. Episodes of reperfusion-induced VT were commonly uninterrupted until either spontaneous reversion to normal sinus rhythm or degeneration into VF. Figure 1 illustrates the time course of episodes of VF in individual hearts in the various study groups in relation to the duration of acidic reperfusion (shaded area). In the control group, VF developed within 20 s of the onset of reperfusion in 92% of hearts; in all hearts that exhibited VF, VF was sustained until the end of the reperfusion period. In the 0.5-, 1-, 2-, and 4-min acidic reperfusion groups, only 17, 17, 42, and 25% of hearts, respectively, exhibited VF during the period of acidic reperfusion (in all cases $P < 0.05$ vs. corresponding period in control group). However, on switching the reperfusate to pH 7.4, VF occurred in a further 50, 58, 0, and 0% of hearts, respectively. Thus the overall incidences of VF were 92% in the control group and 67, 75, 42 ($P < 0.05$), and 25% ($P < 0.05$) in the 0.5-, 1-, 2-, and 4-min acidic reperfusion groups, respectively (Fig. 2). At the end of the 5-min reperfusion period, only 8% of control hearts were in normal sinus rhythm; this incidence was increased to 58, 75 ($P < 0.05$), 92 ($P < 0.05$), and 83% ($P < 0.05$) in the 0.5-, 1-, 2-, and 4-min acidic reperfusion groups, respectively.

Coronary flow rate, heart rate, and ischemic zone size. In control and 0.5-, 1-, 2-, and 4-min acidic reperfusion groups, preischemic flow rates were similar in both the left (14.1 ± 1.0, 13.0 ± 0.4, 12.6 ± 0.6, 12.1 ± 0.6, and 13.0 ± 0.5 $ml \cdot min^{-1} \cdot g^{-1}$, respectively) and the right (15.3 ± 0.7, 14.3 ± 0.7, 14.8 ± 0.5, 14.7 ± 0.9, and 15.7 ± 1.1 $ml \cdot min^{-1} \cdot g^{-1}$, respectively) coronary beds. In all groups, on reperfusion, flow rate increased above its basal value in both coronary beds regardless of perfusate pH, as previously described (3). In the control group, the increased flow rates were maintained throughout reperfusion. In the acidic reperfusion groups, right coronary flow rate returned toward its basal value within 2 min of reperfusion, whereas in the left coronary bed there was a secondary increase in flow rate following the switch to pH 7.4.

Basal heart rate did not differ significantly among control and acidic reperfusion groups (360 ± 11, 356 ± 11, 343 ± 9, 333 ± 8, and 350 ± 12 beats/min in control, 0.5-, 1-, 2-, and 4-min acidic reperfusion groups, respectively). Heart rate did not change significantly in any of the study groups during the period of regional ischemia and could not be measured during early reperfusion due to the rapid onset of ventricular arrhythmias in the majority of hearts. There was no significant difference among groups in ischemic zone size (54 ± 3, 50 ± 3,

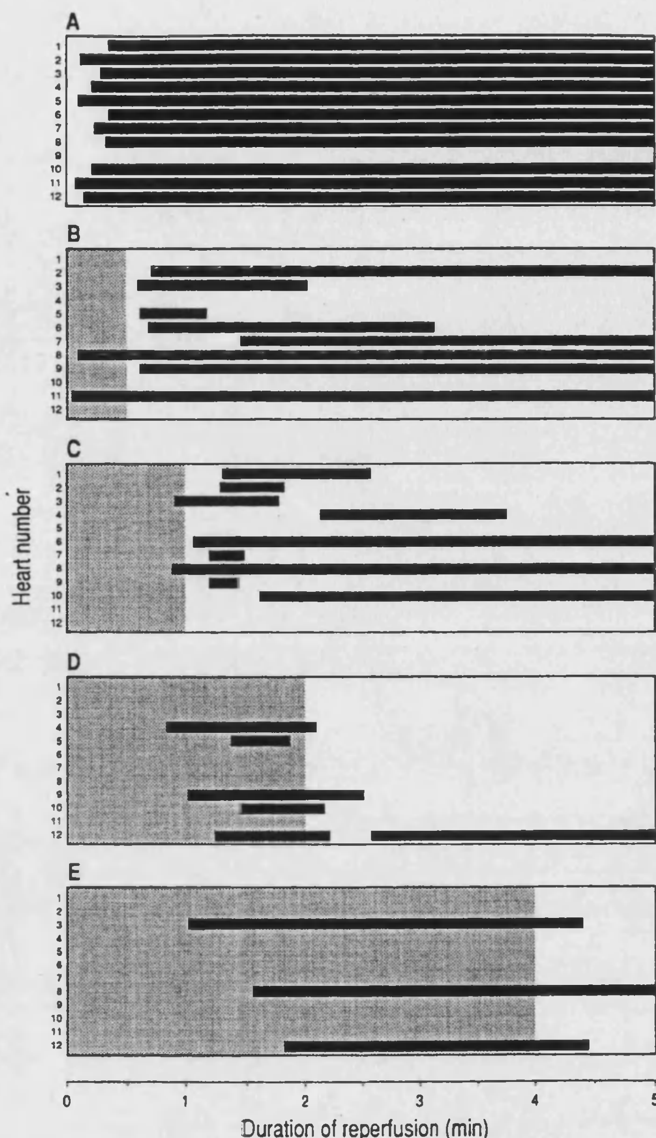

Fig. 1. Time course of reperfusion-induced ventricular fibrillation (VF), indicated by horizontal black bars, in individual hearts (n = 12/group) in control group (*A*) and in groups in which hearts were subjected to acidic reperfusion for 0.5 (*B*), 1 (*C*), 2 (*D*), or 4 min (*E*). Shaded areas indicate period of acidic (pH 6.6) reperfusion. Hearts in each group are arranged in order in which experiments were carried out (study was randomized).

59 ± 2, 53 ± 3, and 55 ± 3% in control, 0.5-, 1-, 2-, and 4-min acidic reperfusion groups, respectively).

Na^+-K^+-ATPase Study

In the acidic reperfusion protocol, Na^+-K^+-ATPase activity in the RV free wall did not differ significantly among the study groups, with values (in MIE × 100) ranging from 10.4 ± 0.9 to 12.5 ± 0.6. Figure 3*A* shows Na^+-K^+-ATPase activity in the LV free wall in nonischemic hearts, those subjected to 10 min of regional ischemia, and those subjected to 10 min of regional ischemia followed by various periods (0.5–4 min) of acidic reperfusion. In nonischemic hearts, Na^+-K^+-ATPase activity in the LV free wall was 110 ± 8% of that in the RV free wall. In contrast, in hearts subjected to 10 min of regional ischemia, Na^+-K^+-ATPase activity in the LV free wall was reduced significantly to 25 ± 3%. Hearts subsequently subjected to 0.5, 1, 2, 3, or 4 min of acidic reperfusion exhibited a progressive recovery of Na^+-K^+-ATPase activity, although values remained significantly depressed relative to nonischemic hearts. Nevertheless, 2, 3, or 4 min of acidic reperfusion resulted in a significant recovery of Na^+-K^+-ATPase activity relative to the value obtained at the end of ischemia.

In the normal reperfusion protocol also, Na^+-K^+-ATPase activity in the RV free wall did not differ significantly among groups [range (in MIE × 100): 12.7 ± 0.6 to 15.2 ± 0.8]. As shown in Fig. 3*B*, Na^+-K^+-ATPase activity in the LV free wall once again

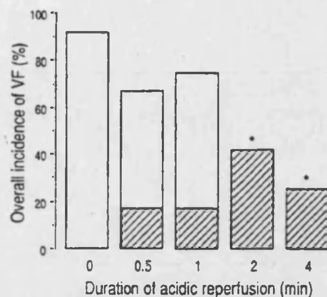

Fig. 2. Overall incidence of reperfusion-induced VF in groups in which hearts were subjected to transient acidic reperfusion for 0 (control), 0.5, 1, 2, or 4 min (n = 12/group). Hatched areas indicate proportion of hearts in which VF occurred during period of acidic (pH 6.6) reperfusion. $*P < 0.05$ vs. control.

declined significantly following 10 min of ischemia from 112 ± 9% in nonischemic hearts to 29 ± 2%. In contrast to the observations made with acidic reperfusion, however, LV Na^+-K^+-ATPase activity did not recover significantly (relative to the end-ischemic value) following up to 4 min of normal reperfusion.

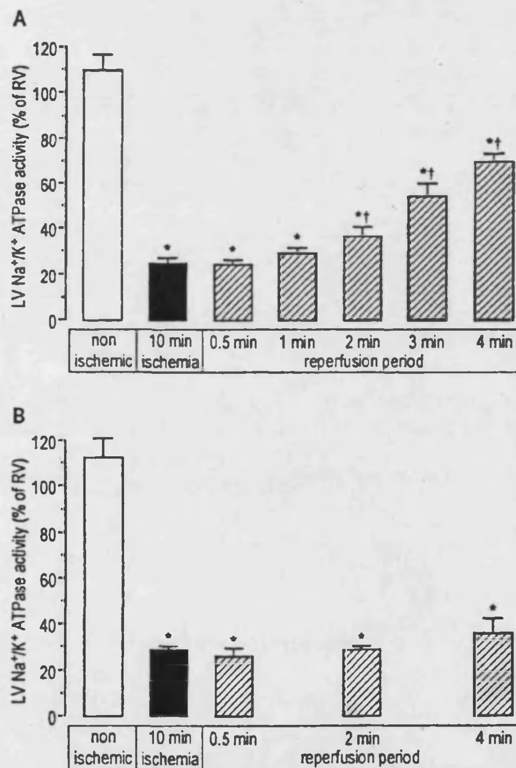

Fig. 3. Na^+-K^+-ATPase activity in the left ventricular (LV) free wall in heart samples collected at various time points during acidic reperfusion (A; n = 8/group) and normal reperfusion (B; n = 6/group) protocols. Values are means ± SE. In each heart, Na^+-K^+-ATPase activity in ischemic/reperfused LV free wall was calculated as a percentage of activity in nonischemic right ventricular (RV) free wall in same heart. Hatched bars, 10-min regional ischemia followed by various periods of acidic (pH 6.6) or normal (pH 7.4) reperfusion. $*P < 0.05$ vs. nonischemic group, $\dagger P < 0.05$ vs. 10-min ischemia group.

DISCUSSION

The present study has confirmed our earlier finding (3) that in isolated rat hearts subjected to 10 min of regional ischemia, selective acidic (pH 6.6) reperfusion of the ischemic zone protects against reperfusion-induced VF. In addition, the present study has shown that, to afford sustained protection, the duration of acidic reperfusion needs to be at least 2 min; with shorter durations of acidic reperfusion, the subsequent switch to pH 7.4 may induce new episodes of VF. Furthermore, the present study has revealed that 10 min of regional ischemia results in marked inhibition of sarcolemmal Na^+-K^+-ATPase activity within the ischemic zone. A progressive recovery of Na^+-K^+-ATPase activity occurred during subsequent acidic reperfusion, which was not observed with normal reperfusion for an identical period. The minimum duration of acidic reperfusion required to achieve a significant recovery of Na^+-K^+-ATPase activity from ischemia-induced inhibition matched closely that required to afford sustained protection against reperfusion-induced VF. This finding is consistent with the hypothesis that the protective mechanism of transient acidic reperfusion may involve a pH-mediated inhibition of Na^+ influx and additionally suggests that enhanced recovery of Na^+-K^+-ATPase activity also may play a contributory role.

Reperfusion-Induced VF: A Role for Loss of Na^+ and Ca^{2+} Homeostasis?

As noted earlier, activation of Na^+/H^+ exchange (and possibly Na^+/HCO_3^- symport) during uncontrolled reperfusion may result in an increased influx of Na^+ and, consequently, Ca^{2+} overload via Na^+/Ca^{2+} exchange. The latter has been proposed (20) as a progenitor of reperfusion-induced severe arrhythmias, probably through a mechanism that involves the oscillatory release of Ca^{2+} from the sarcoplasmic reticulum and the subsequent induction of delayed afterdepolarizations.

The magnitude and potential detrimental consequences of any increase in $[Na^+]_i$ that may occur during reperfusion due to increased Na^+ influx coupled to H^+ extrusion would be expected to depend on the activity of the primary Na^+ extrusion pathway, namely sarcolemmal Na^+-K^+-ATPase. Indeed, in the presence of pharmacological inhibition of Na^+-K^+-ATPase, the abrupt creation of an intracellular-to-extracellular H^+ gradient has been shown in isolated myocytes (19) and whole hearts (1) to result in the rapid intracellular accumulation of Ca^{2+}. Similarly, in the setting of postischemic reperfusion, pharmacological inhibition of Na^+-K^+-ATPase has been shown to result in a greater increase in $[Ca^{2+}]_i$ relative to that observed in the absence of such inhibition (18, 32). Of particular relevance to the present study is the observation that this increase in intracellular Ca^{2+} accumulation was associated with an increased incidence of reperfusion-induced VF (32). Recently, Vandenberg et al. (33) have calculated that, during reperfusion after 10 min of ischemia in the

ferret heart, Na^+ influx coupled to H^+ extrusion may be as great as 5 mM/min. Under the ionic conditions that are likely to prevail at onset of reperfusion, this high rate of influx may only just be countered by Na^+ efflux via Na^+-K^+-ATPase running at full activity (33). However, in the present study, 10 min of ischemia resulted in a 75% inhibition of maximal Na^+-K^+-ATPase activity. This would be expected to favor intracellular Na^+ accumulation following an increased Na^+ influx during subsequent uncontrolled reperfusion. Because extracellular acidosis inhibits Na^+ influx coupled to H^+ extrusion (34), it is likely that the protective effect of acidic reperfusion in our present and previous (3, 12) studies was mediated, at least in part, through an inhibition of Na^+ influx at a time when myocardial capacity to extrude Na^+ was significantly impaired. However, as we have previously noted (3, 37), maintained tissue acidosis during acidic reperfusion is likely also to exert other actions (e.g., inhibition of Ca^{2+} and K^+ channels, Na^+/Ca^{2+} exchange, Ca^{2+} release from the sarcoplasmic reticulum) that might contribute to an antifibrillatory effect. In this regard, it is important to stress that the protective effect of acidic reperfusion appears to be a property of the low pH rather than the low bicarbonate content of the perfusate (12).

Nuclear magnetic resonance (NMR) spectroscopic studies (22) that have failed to show a large increase in $[Na^+]_i$ during uncontrolled reperfusion question an important role for intracellular Na^+ accumulation in reperfusion-induced arrhythmogenesis. It should be noted, however, that there is substantial evidence to suggest that there may be a subsarcolemmal "fuzzy space" where Na^+ diffusion is limited and where increased Na^+ influx may result in a sufficient increase in $[Na^+]$ to stimulate Ca^{2+} influx through the Na^+/Ca^{2+} exchanger [see recent review by Carmeliet (4)]. Therefore, it is feasible that an increased influx of Na^+ during reperfusion may result in a transient increase in $[Na^+]$ in this compartment (thus modulating Na^+/Ca^{2+} exchanger activity) without an increase in bulk cytosolic $[Na^+]$; such a change in $[Na^+]_i$ may not be detectable by currently available techniques, such as NMR spectroscopy (4). Indeed, even a modest increase in $[Na^+]$ in the relevant intracellular space would be expected to alter significantly the reversal potential of the Na^+/Ca^{2+} exchanger, favoring intracellular Ca^{2+} accumulation (17).

It may be argued that maintained intracellular acidosis during acidic reperfusion would be expected to result in an increased $[Ca^{2+}]_i$ due to altered Ca^{2+} buffering and extrusion (14) and thereby in the exacerbation of reperfusion-induced arrhythmias. However, reperfusion-induced arrhythmias are believed to occur not as a result of an increased $[Ca^{2+}]_i$ per se but through a mechanism that involves the oscillatory release of Ca^{2+} from the sarcoplasmic reticulum and the subsequent induction of delayed afterdepolarizations (20). Because oscillatory Ca^{2+} release from the sarcoplasmic reticulum and delayed afterdepolarizations are inhibited by low pH (20), this might counteract any proarrhythmic effect of an acidosis-induced increase in $[Ca^{2+}]_i$.

Ischemia-Induced Inhibition of Sarcolemmal Na^+-K^+-ATPase

Previous studies that have used conventional biochemical techniques involving tissue homogenization and membrane purification have provided conflicting results regarding the effects of ischemia on sarcolemmal Na^+-K^+-ATPase activity. Kim et al. (13) failed to show a reduction in Na^+-K^+-ATPase activity after as long as 6 h of ischemia in isolated guinea pig hearts. In contrast, in isolated rat hearts, Daly et al. (6) found a 21–24% reduction in Na^+-K^+-ATPase activity after 60 min of global ischemia, with no further change in activity following a subsequent 15-min period of reperfusion. In porcine myocarium, Winston et al. (36) showed that up to 45 min of ischemia did not affect Na^+-K^+-ATPase activity when measured in tissue homogenates; however, using a qualitative histochemical method to detect in situ Na^+-K^+-ATPase activity, these investigators were able to demonstrate a marked loss of activity after shorter periods of ischemia. This observation is in agreement with the findings of the present study, obtained using a similar (but quantitative) in situ assay, which have shown an ~75% reduction in LV Na^+-K^+-ATPase activity after only 10 min of ischemia. It is possible therefore that factors responsible for inhibiting Na^+-K^+-ATPase activity in situ may be partially lost during tissue homogenization and membrane purification, rendering such methods inappropriate for use in the study of ischemia-induced changes in enzyme activity. Furthermore, loss of Na^+-K^+-ATPase during tissue processing, the extent of which may vary depending on the nature of the tissue (i.e., control vs. pathological), may complicate the interpretation of results obtained using these methods (26). The cytochemical method used in the present study, which enables the assessment of Na^+-K^+-ATPase activity in situ, avoids the problems associated with enzyme loss during tissue homogenization and purification.

In the present study, Na^+-K^+-ATPase activity in the tissue sections was measured under conditions of substrate saturation, optimal pH, and abundant ATP; thus the activity measured reflects the maximal activity (V_{max}) of the enzyme. A reduction in V_{max}, as observed after ischemia in the present study, may occur due to *1*) a reduction in tissue Na^+-K^+-ATPase content and/or *2*) a reduction in the maximal activity of individual enzyme units. Although the present study does not allow delineation of the precise mechanism(s) involved, previous studies have shown that neither the number of [^{3}H]ouabain binding sites (26) nor the distribution and content of immunoreactive Na^+-K^+-ATPase (36) is altered during ischemia, suggesting that the former mechanism may not be of primary importance. With regard to the latter mechanism, it is possible that Na^+-K^+-ATPase activity may be inhibited during ischemia as a consequence of changes in cellular glutathione status (10) or in the enzyme's lipid environment (8, 29). Thus the observed recovery of Na^+-K^+-ATPase activity during acidic reperfusion may have been mediated by the reversal of such ischemia-induced changes.

In this regard, it is interesting to note that, unlike with acidic reperfusion, no significant recovery of Na^+-K^+-ATPase activity was observed, following up to 4 min of reperfusion with solution at pH 7.4. The mechanisms that are responsible for the apparent pH dependency of Na^+-K^+-ATPase recovery from ischemia-induced inhibition are unclear. However, with regard to the potential modulation of Na^+-K^+-ATPase activity during ischemia and reperfusion by reversible changes in myocardial glutathione status (10), it is worth noting that certain thiol-disulfide interchange reactions involving glutathione exhibit pH sensitivity in vitro (25).

Role of Differences in Coronary Flow Profiles

The possibility that pH-mediated differences in left and right coronary flow profiles during reperfusion may have mediated the intergroup differences in susceptibility to arrhythmias needs to be considered. The increase in right coronary flow rate, which occurred in all groups on reperfusion, was maintained throughout reperfusion in the control group but normalized rapidly in the transient acidic reperfusion groups. However, the sustained increase in right coronary flow rate in the control group was probably a consequence (rather than the cause) of the greater severity of reperfusion-induced arrhythmias and may have occurred due to reduced extravascular compression (3, 12). In all groups subjected to transient acidic reperfusion, there was a secondary increase in left coronary flow rate following the switch to pH 7.4; this occurred regardless of whether the antifibrillatory protection was sustained, suggesting that it was not causally related to the induction of new episodes of VF.

Limitations of Present Study

Our data have revealed a temporal correlation between the recovery of Na^+-K^+-ATPase activity during acidic reperfusion in one population of hearts and the decline in susceptibility to VF following a subsequent increase in perfusate pH in another population of hearts. This study design was necessitated because the cytochemical assay used allows only a single measurement of Na^+-K^+-ATPase activity to be made in each heart. Thus it was impossible to correlate Na^+-K^+-ATPase activity at the moment of the abrupt change in extracellular pH with susceptibility to VF following a subsequent increase in perfusate pH on a heart-by-heart basis. Therefore, although our findings are consistent with the hypothesis that inhibition of intracellular Na^+ (and consequently Ca^{2+}) accumulation may be involved in the protective actions of acidic reperfusion, it does not prove a "cause and effect" relationship between Na^+-K^+-ATPase activity and susceptibility to VF. Indeed, it should be noted that the activity/inactivity of various ion transporting pathways (e.g., Na^+/H^+ and Na^+/Ca^{2+} exchangers) during reperfusion are presumed, based on published data, rather than measured directly in the present study. In the absence of continuous ion measurements simultaneously with ECG monitoring, it cannot be deduced whether the protection afforded by acidic reperfusion was mediated by reduced intracellular accumulation of Na^+ and/or Ca^{2+} during reperfusion.

A further limitation of the present study may be the use of an erythrocyte-free perfusate, which results in coronary flows in excess of those encountered in vivo. Such high flows may result in faster washout during reperfusion of components (such as H^+) that have accumulated during the preceding period of ischemia, which in turn may lead to an overestimation of the role of washout phenomena in reperfusion arrhythmogenesis. However, it is worth noting that in the present model even a 90% reduction in the rate of reflow is unable to suppress reperfusion-induced VF (11).

Despite the above limitations, the model employed in the present study offers many advantages in the study of the pathophysiological determinants of arrhythmogenesis (2). Of particular advantage in the present study was the reproducible coronary distribution afforded by the model (2), which enabled the measurement in individual hearts of Na^+-K^+-ATPase activity in myocardium selectively exposed to ischemia and reperfusion as well as in "control" myocardium that was not exposed to any such intervention.

In conclusion, the present study has shown that, in isolated rat hearts subjected to 10 min of regional ischemia, a minimum of 2 min of acidic reperfusion is required to achieve sustained protection against reperfusion-induced VF. Furthermore, the present study has revealed for the first time that 10 min of regional ischemia results in a substantial and significant inhibition of the maximal activity of sarcolemmal Na^+-K^+-ATPase within the ischemic zone. This inhibition has been shown to be reversible, with a progressive recovery of Na^+-K^+-ATPase activity occurring during 4 min of acidic reperfusion but not during an identical period of normal reperfusion. There was a close similarity in the durations of acidic reperfusion required to achieve *1*) sustained protection against reperfusion-induced VF and *2*) significant recovery of maximal Na^+-K^+-ATPase activity from ischemia-induced inhibition. This is consistent with the hypothesis that the protective mechanism of transient acidic reperfusion may involve the maintenance of cellular Na^+ and Ca^{2+} homeostasis. Furthermore, it suggests that this may occur not only through a suppression of Na^+ influx coupled to H^+ extrusion, but also via an enhanced recovery of the primary Na^+ efflux mechanism, the Na^+-K^+-ATPase.

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Na^+/H^+ exchange and reperfusion arrhythmias: protection by intracoronary infusion of a novel inhibitor

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Yasutake, Masahiro, Chikao Ibuki, David J. Hearse, and Metin Avkiran. Na^+/H^+ exchange and reperfusion arrhythmias: protection by intracoronary infusion of a novel inhibitor. *Am. J. Physiol.* 267 (*Heart Circ. Physiol.* 36): H2430–H2440, 1994.—Activation of sarcolemmal Na^+/H^+ exchange has been proposed as a causal factor in reperfusion arrhythmogenesis. To test this hypothesis, we determined the antiarrhythmic efficacy of two structurally distinct but equipotent Na^+/H^+ exchange inhibitors, 5-(*N*-ethyl-*N*-isopropyl)amiloride (EIPA) and the novel drug, 3-methylsulfonyl-4-piperidinobenzoyl guanidine (HOE-694), in isolated rat hearts (n = 12/group) subjected to independent dual coronary perfusion. After 15 min of aerobic perfusion of both beds, flow to the left coronary bed (LCB) was terminated for 10 min; this was followed by 5 min of reperfusion. Various concentrations of each drug were selectively infused into the LCB either during the 5-min period preceding ischemia plus during reperfusion or during reperfusion alone. With the former protocol, 0.01, 0.1, 1, and 10 μM EIPA reduced the incidence of reperfusion-induced ventricular fibrillation (VF) from 92% in controls to 83, 83, 50, and 0% ($P < 0.05$); the number of hearts in sinus rhythm at the end of reperfusion was increased from 17 to 42, 25, 83 ($P < 0.05$), and 100% ($P < 0.05$). HOE-694, at the same concentrations, reduced VF incidence from 92% in control to 83, 58, 50, and 8% ($P < 0.05$); 25, 67, 75 ($P < 0.05$), and 100% ($P < 0.05$) of hearts were in sinus rhythm, compared with 17% of controls, at the end of reperfusion. Even when infused during reperfusion alone, both drugs afforded significant protection against reperfusion-induced VF, which did not differ significantly from that observed when the drugs were also given before ischemia. The similar antiarrhythmic efficacy of EIPA and HOE-694 is consistent with an arrhythmogenic role for activation of Na^+/H^+ exchange during early reperfusion.

regional ischemia; dual coronary perfusion; 5-(*N*-ethyl-*N*-isopropyl)amiloride; 3-methylsulfonyl-4-piperidinobenzoyl guanidine; rat heart

RECENT STUDIES in our laboratory (2, 11) have shown that the incidence of reperfusion-induced ventricular fibrillation (VF) can be strikingly reduced by using transient acidic reperfusion to limit the rate at which extracellular H^+ is washed out. Because the sarcolemmal Na^+/H^+ exchanger is inhibited by extracellular acidosis (32), we proposed that inhibition of the exchanger was the most likely mechanism underlying the protection afforded by acidic reperfusion (2, 11). Indeed, pharmacological inhibitors of the Na^+/H^+ exchanger, such as amiloride and its analogues ethylisopropylamiloride, dimethylamiloride, and hexamethyleneamiloride, also have been shown (6, 7, 19, 25) to afford protection against reperfusion-induced arrhythmias.

Despite considerable evidence suggesting that activation of the Na^+/H^+ exchanger may be an important progenitor of reperfusion-induced arrhythmias, the interpretation of many studies is confounded by a number of factors. First, in some studies (7, 25), Na^+/H^+ exchange inhibitors were administered before the onset of ischemia, thus making it impossible to deduce whether the protective mechanisms were operative primarily during ischemia or during reperfusion. A second concern is that the chemical structures of the Na^+/H^+ exchange inhibitors used in many previous studies (6, 7, 19, 25) are based on amiloride, which is known to inhibit a number of other ion transport processes (e.g., Na^+, Ca^{2+}, and K^+ channels; Na^+/Ca^{2+} exchange) (13). Although amiloride analogues, in which the 5-amino nitrogen atom bears one or two substituents, can exhibit increased potency as inhibitors of the Na^+/H^+ exchanger (13), the possibility cannot be excluded that nonselective actions might contribute to their protective effects. A final concern is that, although acidic reperfusion (2, 11) is likely to inhibit the Na^+/H^+ exchanger (32), it is likely also to exert other actions (e.g., inhibition of Ca^{2+} and K^+ channels, Na^+/Ca^{2+} exchange, and Ca^{2+} release from the sarcoplasmic reticulum) that may contribute to an antiarrhythmic effect (2).

The present study was designed with the primary objective of obtaining stronger evidence for a specific involvement of the Na^+/H^+ exchanger in the induction of reperfusion arrhythmias by a mechanism that is operative primarily during the reperfusion phase. To achieve this, we used as pharmacological tools an amiloride analogue that is a potent inhibitor of the Na^+/H^+ exchanger, 5-(*N*-ethyl-*N*-isopropyl)amiloride (EIPA), and compared its effects with those of a recently developed inhibitor, 3-methylsulfonyl-4-piperidinobenzoyl guanidine (HOE-694) (24), which has a chemical structure distinct from that of amiloride and its analogues (Fig. 1). To obtain a detailed assessment of the potency and efficacy of these drugs and to determine their primary phase of action, we used four concentrations of each agent and two treatment protocols, one in which the drug was given before ischemia and during reperfusion and the other in which it was given during reperfusion alone. Another novel aspect of the present study was our decision to administer selectively the drugs only to the zone that was subjected to ischemia and reperfusion; this was made possible by our recent development (1) of a unique cannula that allows the independent perfusion of left and right coronary beds in small mammalian hearts. In further studies using Langendorff-perfused hearts, we have also investigated the effects of EIPA and HOE-694 on contractile function and electrocardiographic parameters under aerobic conditions.

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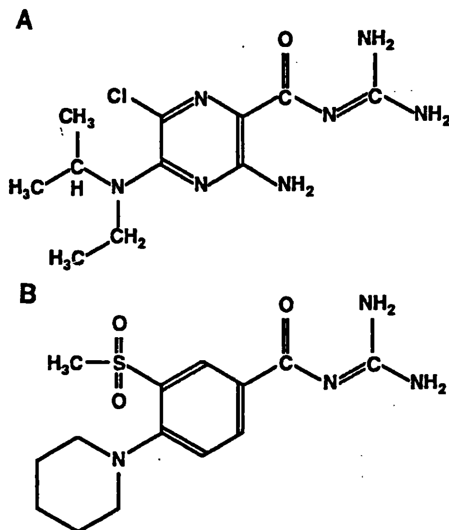

Fig. 1. Chemical structures of 5-(*N*-ethyl-*N*-isopropyl)amiloride (EIPA; *A*) and 3-methylsulfonyl-4-piperidinobenzoyl guanidine (HOE-694; *B*).

METHODS

This investigation was performed in accordance with the Home Office Guidance on the Operation of the Animals (Scientific Procedures) Act 1986, published by Her Majesty's Stationery Office, London, UK.

Arrhythmia Study

Dual coronary perfusion of isolated rat hearts. Isolated hearts from male Wistar rats (Bantin and Kingman, North Humberside, UK) were subjected to independent perfusion of left and right coronary arteries as described in detail by Avkiran and Curtis (1). Each coronary bed was initially perfused at a constant perfusion pressure equivalent to 75 mmHg with oxygenated perfusion solution from a temperature-regulated reservoir (37°C). The perfusion solution was of the following composition (in mM): 118.5 NaCl, 25.0 $NaHCO_3$, 3.2 KCl, 1.2 $MgSO_4$, 1.2 KH_2PO_4, 1.4 $CaCl_2$, and 11.0 glucose. The solution was filtered (pore size, 5 μm) before use and bubbled continuously with 95% O_2-5% CO_2 (pH 7.4 at 37°C). Flow to each coronary bed was monitored using in-line flow detectors (Transonic T206 Animal Research Flowmeter with 1N probes, Transonic Systems, Ithaca, NY) with a linear detection range of 0.05–30 ml/min. After 15 min of perfusion of both coronary beds, basal flow rate in the left coronary bed was recorded, and perfusion of this bed was switched from constant pressure to constant flow at the basal flow rate (supplied by a Gilson Minipuls 3 roller pump). This enabled the infusion of drug solutions or vehicle into the left coronary bed, at a known percentage of the total flow supplied to that bed (see *Drug administration and study protocol*). The right coronary bed was perfused at constant pressure throughout the experiment. The heart was housed in a temperature-regulated chamber at 37°C throughout the experiment, and the right atrium was continuously superfused with oxygenated perfusion solution (37°C) at 8 ml/min to maintain sinus rate (1).

Drug administration and study protocol. HOE-694 has limited solubility in physiological buffer solutions. Therefore, both drugs were dissolved in deionized water at concentrations of 0.143, 1.43, 14.3, and 143 μM. When required, these solutions were infused selectively into the perfusion line supplying the left coronary bed at 7% of the total flow rate delivered to that bed; this resulted in final perfusate drug concentrations of 0.01, 0.1, 1, and 10 μM. Control hearts received vehicle (deionized water) at the same infusion rate.

Each concentration of EIPA or HOE-694 was infused into the left coronary bed either for 5 min immediately before ischemia plus throughout reperfusion or throughout reperfusion alone. All hearts (n = 12/group) were subjected to 10 min of regional ischemia (this was achieved by terminating flow to the left coronary bed) followed by 5 min of reperfusion. Experiments were carried out in a prospectively randomized manner.

Measured variables. ARRHYTHMIAS. Arrhythmias were diagnosed from a unipolar electrogram (ECG) that was obtained through a silver electrode inserted into the free wall of the left ventricle and a reference electrode connected to the aorta. The ECG was continuously monitored on a digital storage oscilloscope (model 1421, Gould Electronic, Ilford, UK) and was recorded on an ink-jet recorder (model 2200S, Gould). Chart speed was set at 50 mm/s a few seconds before reperfusion so as to obtain a permanent high-speed record of the changes in the ECG during early reperfusion. The ECG was retrospectively analyzed, in a blinded manner, for the incidence, time to onset, and duration of ventricular tachycardia (VT) and VF. All analyses were carried out in accordance with the Lambeth Conventions (30). VT was defined as four or more consecutive premature beats of ventricular origin and VF as a signal in which individual QRS deflections could no longer be distinguished from one another and for which rate could not be determined.

VT CYCLE LENGTH. VT cycle length was determined 10 s after the onset of reperfusion, since at this time point a large proportion of the hearts (n = 10–12) were in VT in most groups, enabling intergroup statistical comparison. The only exception was the group that received 10 μM EIPA before ischemia plus during reperfusion; none of the hearts in this group exhibited VT (see RESULTS). VT cycle length was calculated from the number of QRS deflections over a 2-s interval, using the high-speed ECG trace (2).

CORONARY FLOW AND HEART RATE. Throughout the experimental protocol, coronary flow was monitored using the in-line flow detectors. Heart rate was determined at selected intervals from the ECG trace.

SIZE OF ISCHEMIC ZONE. At the end of each experiment, the left coronary bed was perfused for 30 s with a solution containing 0.02% disulphine blue dye at a perfusion pressure of 75 mmHg. The heart was then removed from the perfusion apparatus, the atria and mediastinal tissue were removed, and the dye-stained tissue (representing ventricular myocardium subjected to ischemia and reperfusion) was carefully dissected out. The stained and unstained tissues were lightly blotted and weighed. The size of the ischemic zone, expressed as a percentage of total ventricular weight, was calculated from the equation: (weight of stained tissue/total ventricular weight) × 100. The absolute weights obtained also enabled the calculation of flows in left and right coronary beds on the basis of tissue weights supplied by each bed ($ml \cdot min^{-1} \cdot g^{-1}$).

Exclusion criteria. These criteria, selected to minimize variations in heart rate and size of ischemic zone (due to atypical coronary anatomy) among the hearts, were as previously described (1, 2, 10, 11). Hearts were also excluded if there was cross-flow between right and left coronary ostia, due to a mismatch between aorta size and cannula diameter (1, 2, 10, 11). In addition, hearts that exhibited ventricular arrhythmias during the final 3 s of ischemia before reperfusion were not included in the analysis of reperfusion-induced arrhyth-

mias, because in those hearts it would have been impossible to differentiate arrhythmias induced by reperfusion from those induced by ischemia. Of 230 hearts entered into the arrhythmia study, 5 were excluded on the basis of heart rate, 1 on the basis of size of ischemic zone, 4 on account of cross flow, and 4 on account of arrhythmias during the final 3 s of ischemia. The overall exclusion rate was 6%.

Contractile Function Study

Langendorff perfusion of isolated rat hearts. Hearts were excised from male Wistar rats as described in an earlier study (1) and were subjected to Langendorff perfusion through a conventional single-lumen cannula at a constant perfusion pressure equivalent to 75 mmHg. The left atrium was excised, and an ultrathin balloon, specially constructed to match the dimensions of the ventricular cavity, was inserted into the left ventricle. The intraventricular balloon was inflated to give a left ventricular end-diastolic pressure (LVEDP) of 6–8 mmHg, and the balloon volume was kept constant thereafter. The perfusion solution was identical to that used for dual coronary perfusion. After 15 min of perfusion at constant pressure, the basal total coronary flow rate was noted, and perfusion was switched to a constant-flow system at the basal flow rate. Again, 7% of the total flow supplied was from a sidearm through which drug solutions or vehicle could be administered.

Drug administration and study protocol. For the first 10 min of constant-flow perfusion, hearts (n = 6/group) received vehicle (deionized water) through the sidearm. Subsequently, drug solutions were administered, in a cumulative manner, to give final perfusate concentrations of 0.01, 0.1, 1, and 10 μM, and perfusion with each drug concentration was maintained for 10 min. Hearts in the control group continued to receive vehicle (deionized water), at the same infusion rate, throughout the protocol.

Measured variables. CONTRACTILE FUNCTION. LVEDP and left ventricular developed pressure (LVDP), obtained from a pressure transducer attached to the intraventricular balloon through a fluid-filled catheter, were noted at 2-min intervals during perfusion with each drug concentration.

CORONARY VASCULAR RESISTANCE (CVR) AND HEART RATE. CVR was determined at 2-min intervals during perfusion with each drug concentration, from the total coronary flow (which was constant) and the perfusion pressure (which was monitored through a sidearm of the aortic cannula). Heart rate was determined at the same intervals from the ECG trace (see below).

ECG PARAMETERS. At the end of the 10-min perfusion period with each drug concentration, chart speed was increased to 250 mm/s to obtain a permanent high-speed ECG recording. These recordings were utilized to measure the interval from the beginning of the P wave to the beginning of the ventricular complex (P-R interval) and the width of ventricular complex. As a separate T wave is not seen in the rat ECG, the width of the ventricular complex was measured at 90% repolarization (with the maximum positive deflection of the ventricular complex defined as the point of 0% repolarization) and defined as $QRST_{90}$, as previously described (21).

EXCLUSION CRITERIA. These prospectively defined criteria demanded that hearts were excluded if basal heart rate was <280 or >420 beats/min or if basal total coronary flow rate was <9 ml/min. Of 18 hearts entered into the contractile function study, none were excluded.

Statistical Analysis

Statistical analyses were based on the guidelines described by Wallenstein et al. (31). Gaussian-distributed variables were expressed as means ± SE and were subjected to one-way analysis of variance. If a difference among mean values was established, comparison with controls was performed using Dunnett's test. Temporal changes in heart rate, developed pressure, and coronary vascular resistance were analyzed using analysis of variance for repeated measurements. Binomially distributed variables, such as the incidence of VT or VF, were compared using the chi-squared test for a 2 × n table. If a significant difference was revealed, each drug-treated group was then compared with the control group using the Fisher exact test, with the Bonferroni correction for multiple comparisons. A value of $P < 0.05$ was considered significant.

RESULTS

Arrhythmia Study

Reperfusion-induced arrhythmias. Consistent with our earlier studies (2, 11), reperfusion after 10 min of regional ischemia frequently resulted in the rapid (within a few beats) induction of VT. Reperfusion-induced VT was generally polymorphic in nature, and episodes of VT were usually uninterrupted until either spontaneous reversion to normal sinus rhythm or degeneration into VF.

DRUG ADMINISTRATION BEFORE ISCHEMIA PLUS DURING REPERFUSION. Figure 2 shows the overall incidence of reperfusion-induced VF in control hearts and hearts that received EIPA or HOE-694 before ischemia plus during reperfusion. Both drugs suppressed the incidence of VF in a concentration-dependent manner, the reduction reaching a level of statistical significance at a

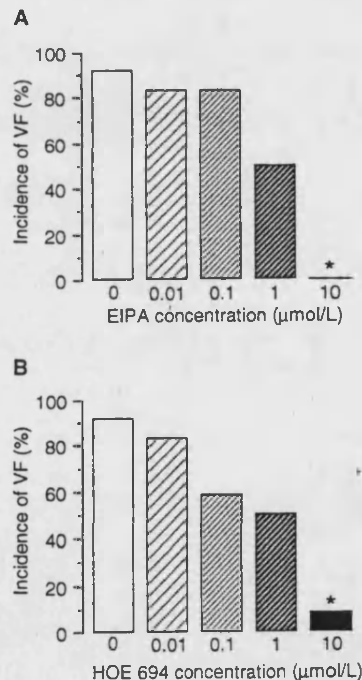

Fig. 2. Effects of EIPA (*A*) and HOE-694 (*B*) when given before ischemia plus during reperfusion on overall incidence of reperfusion-induced ventricular fibrillation (VF). *$P < 0.05$ vs. 0 μM (control).

concentration of 10 μM in both cases. Figure 3 illustrates the incidence of VF in each of the study groups during 0.5-min intervals throughout the 5-min reperfusion period. The time-to-onset of reperfusion-induced VF was not altered by either drug, with the induction of VF occurring during the first 0.5 min of reperfusion in the majority (67–100%) of hearts that exhibited VF in the various study groups.

Although the overall incidence of VF was reduced only by the highest concentration (10 μM) of each drug, in the groups that received 1 μM EIPA or 0.1 μM HOE-694 there were significant reductions in the incidence of VF during some of the later time intervals during reperfusion (Fig. 3). This probably reflected the ability of the drugs to enhance spontaneous defibrillation at these lower concentrations. The concentration-response characteristics of EIPA and HOE-694 with respect to their antifibrillatory actions appeared to be similar (Figs. 2 and 3), with half-maximal protection obtained within the 0.1–1 μM concentration range in both cases. The maximal protection afforded was also similar for both drugs, indicating comparable efficacy. The incidence of reperfusion-induced VT was 100% in all control and drug-treated groups, except in the group that received 10 μM EIPA in which reperfusion-induced VT or VF was not detected. In both control groups, only 17% of

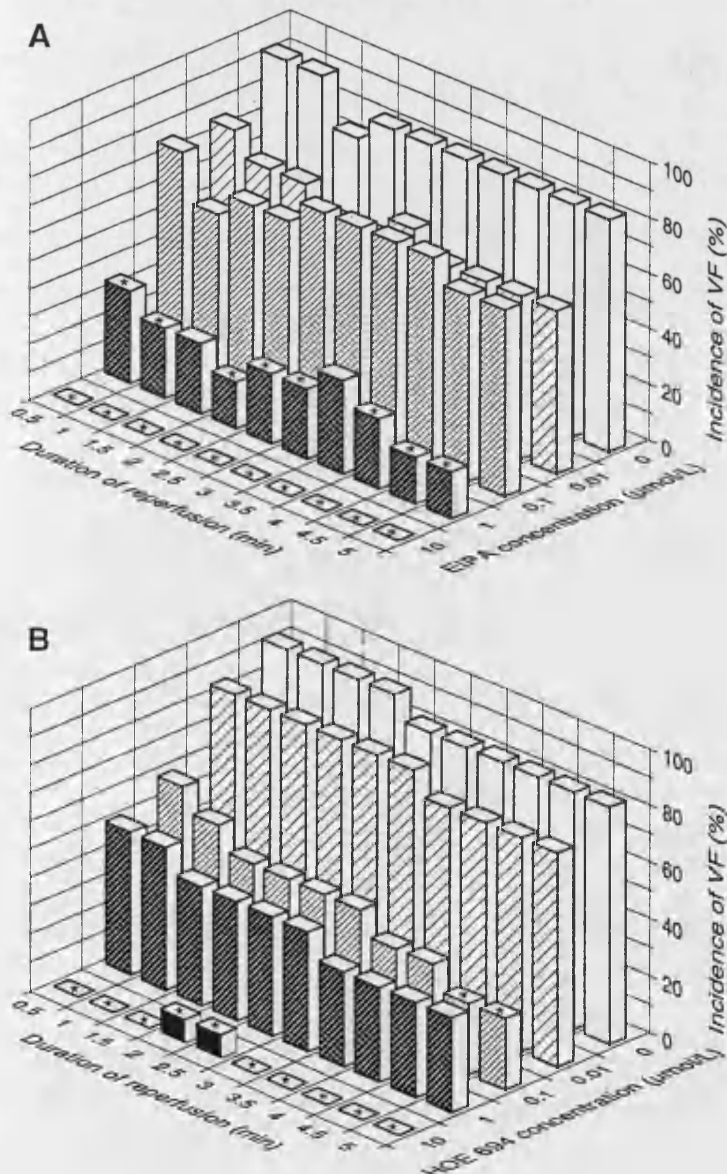

Fig. 3. Effects of EIPA (*A*) and HOE-694 (*B*) when given before ischemia plus throughout reperfusion on time course of reperfusion-induced VF. Incidence of VF was noted during 0.5-min intervals throughout the 5-min reperfusion period. *$P < 0.05$ vs. 0 μM (control) during corresponding interval.

hearts were in normal sinus rhythm at the end of the reperfusion period. In the groups that received 0.01, 0.1, 1, and 10 μM EIPA before ischemia and during reperfusion, 42, 25, 83 ($P < 0.05$), and 100% ($P < 0.05$) of hearts, respectively, were in sinus rhythm at the end of reperfusion. The corresponding values in the groups that received HOE-694 were 25, 67, 75 ($P < 0.05$), and 100% ($P < 0.05$), respectively.

DRUG ADMINISTRATION DURING REPERFUSION ALONE. Figure 4 shows the time course of reperfusion-induced VF in the groups that received EIPA and HOE-694 during reperfusion alone and in the contemporary control groups. Both drugs again suppressed the incidence of VF and enhanced spontaneous reversion to normal sinus rhythm in a concentration-dependent manner. The reductions in the overall incidence of VF, relative to the control values of 92%, reached statistical significance at the concentration of 10 μM for both HOE-694 (25%) and EIPA (33%). HOE-694 at 0.01 μM tended to enhance spontaneous defibrillation, as indicated by the significant reductions in the incidence of VF during some of the later time intervals during reperfusion (Fig. 4); however, the overall incidence of VF was not significantly reduced by this concentration. The concentration-response profiles for the drugs (Fig. 4) suggest that HOE 694 may exhibit greater potency than EIPA when given

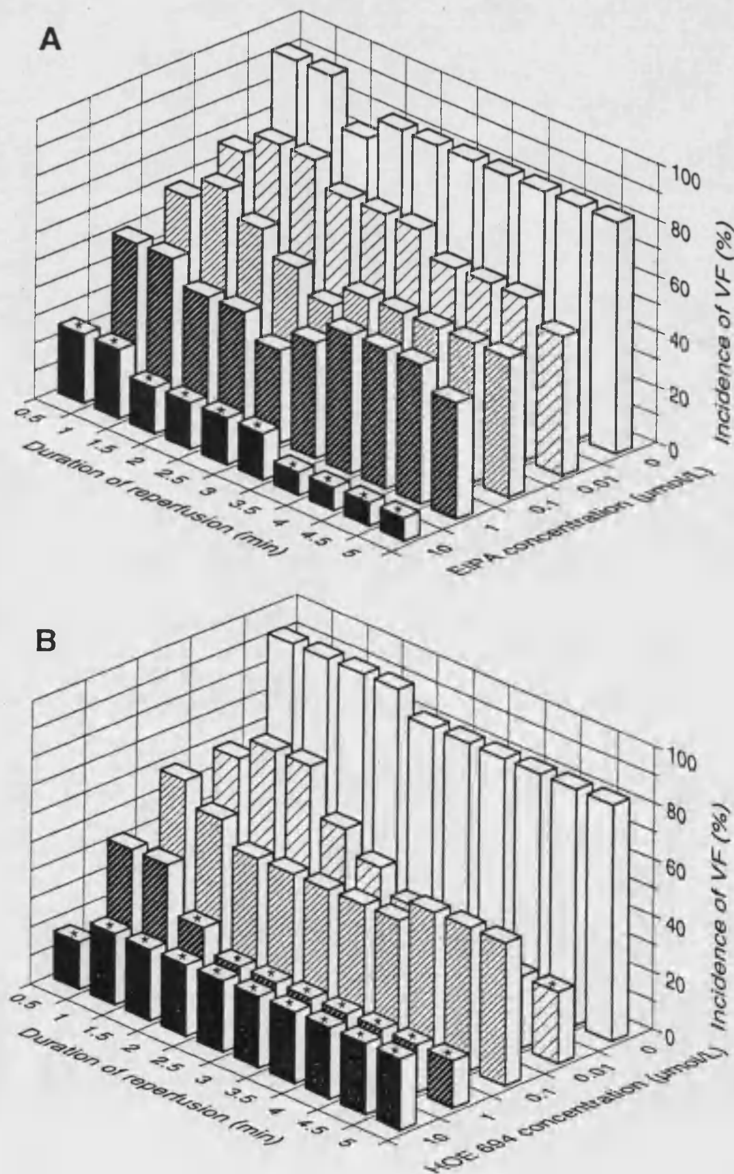

Fig. 4. Effects of EIPA (*A*) and HOE-694 (*B*) when given during reperfusion alone on time course of reperfusion-induced VF. Incidence of VF was noted during 0.5-min intervals throughout 5-min reperfusion period. *$P < 0.05$ vs. 0 μM (control) during corresponding interval.

during reperfusion alone; however, the efficacy of the drugs appeared comparable, with similar protection afforded by the highest concentration (10 μM) of each agent. The incidence of VT was 100% in all groups, regardless of the concentration or identity of the drug. In both control groups, only 17% of hearts were in normal sinus rhythm at the end of reperfusion. In the groups that received 0.01, 0.1, 1, and 10 μM of EIPA during reperfusion, this incidence was increased to 50, 50, 50, and 83% ($P < 0.05$), respectively. The corresponding values in the groups that received identical concentrations of HOE-694 during reperfusion were 75 ($P < 0.05$), 50, 92 ($P < 0.05$), and 92% ($P < 0.05$), respectively.

VT cycle length. As shown in Fig. 5, when administered before ischemia plus during reperfusion, both drugs produced a concentration-dependent prolongation of VT cycle length, when measured 10 s after the initiation of reperfusion. In the group that received 10 μM of EIPA before ischemia plus during reperfusion, VT cycle length could not be measured because none of the hearts exhibited reperfusion-induced VT. VT cycle length during early reperfusion was not significantly different from control when EIPA and HOE-694 were administered during reperfusion alone, regardless of drug concentration.

Coronary flow, heart rate, and ischemic zone size. As shown in Table 1, there was no significant difference

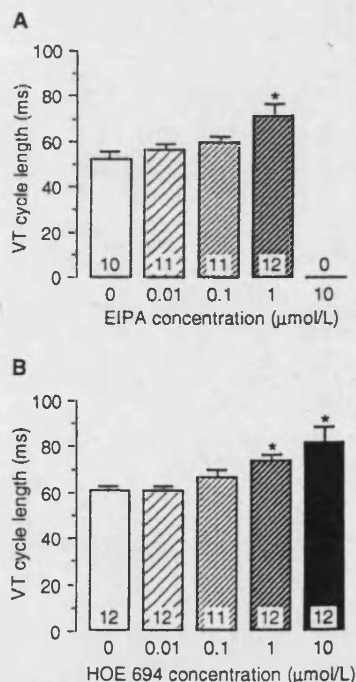

Fig. 5. Effects of EIPA (*A*) and HOE-694 (*B*) when given before ischemia plus during reperfusion on cycle length of ventricular tachycardia (VT) determined 10 s after onset of reperfusion (see METHODS). Nos. in columns indicate group size (0 hearts exhibited VT in group that received 10 μM EIPA). $^*P < 0.05$ vs. 0 μM (control).

Table 1. *Basal coronary flow, heart rate, and ischemic zone size in the 18 groups included in the arrhythmia study*

Concentration, μM	Treatment Protocol	Coronary Flow Rate, $ml \cdot min^{-1} \cdot g^{-1}$		Heart Rate, beats/min	Ischemic Zone Size, %
		LCB	RCB		
EIPA					
0 (control)		13.6 ± 0.5	14.0 ± 0.7	350 ± 11	60 ± 2
0.01	pre-I + R	14.7 ± 1.1	16.8 ± 1.0	330 ± 5	57 ± 2
0.1	pre-I + R	13.3 ± 0.7	17.1 ± 1.3	345 ± 9	60 ± 3
1	pre-I + R	13.1 ± 0.8	16.2 ± 1.1	352 ± 10	60 ± 2
10	pre-I + R	14.3 ± 0.9	16.4 ± 0.7	356 ± 12	56 ± 3
0.01	R	13.9 ± 0.7	16.4 ± 1.3	333 ± 10	56 ± 3
0.1	R	13.0 ± 0.9	15.6 ± 1.1	368 ± 15	55 ± 2
1	R	13.7 ± 0.6	16.6 ± 1.2	346 ± 14	61 ± 3
10	R	13.7 ± 0.8	16.8 ± 0.6	340 ± 11	60 ± 3
HOE-694					
0 (control)		11.6 ± 0.3	14.6 ± 0.6	322 ± 7	61 ± 2
0.01	pre-I + R	11.0 ± 0.4	15.9 ± 0.8	334 ± 9	62 ± 3
0.1	pre-I + R	11.7 ± 0.5	16.2 ± 0.6	338 ± 6	63 ± 2
1	pre-I + R	10.3 ± 0.5	14.3 ± 0.7	323 ± 9	63 ± 2
10	pre-I + R	13.1 ± 0.4	15.2 ± 0.7	352 ± 8	56 ± 2
0.01	R	11.1 ± 0.4	13.9 ± 0.7	321 ± 7	64 ± 2
0.1	R	12.1 ± 0.7	15.6 ± 1.1	339 ± 8	61 ± 2
1	R	10.9 ± 0.6	14.5 ± 0.9	336 ± 7	59 ± 2
10	R	13.1 ± 0.6	15.3 ± 0.8	338 ± 7	56 ± 2

Values are means ± SE; n = 12 rat hearts/group. LCB and RCB, left and right coronary beds, respectively; pre-I, before ischemia; R, during reperfusion; EIPA, 5-(*N*-ethyl-*N*-isopropyl)amiloride; HOE-694, 3-methylsulfonyl-4-piperidinobenzoyl guanidine.

between control and drug-treated groups in basal left and right coronary flow rates, when measured at the end of the initial 15-min period of perfusion at constant pressure. Thereafter, left coronary flow rate was held constant at the basal value; therefore, there were no significant intergroup differences in left coronary flow rate for the rest of the experimental protocol. Flow rate in the right coronary bed (which was perfused at constant pressure throughout) did not change significantly during the period of zero-flow ischemia in the left coronary bed. Right coronary flow rate increased during reperfusion commensurate with the severity of reperfusion arrhythmias; this was probably due to reduced extravascular compression, as previously described (2, 11).

Basal heart rate also did not differ significantly between control and drug-treated groups (Table 1). The infusion of EIPA or HOE-694 into the left coronary bed for 5 min immediately before the onset of ischemia had no effect on heart rate, regardless of drug concentration. Heart rate did not change significantly in any of the study groups during the period of regional ischemia and could not be measured during early reperfusion due to the rapid onset of ventricular arrhythmias in the majority of hearts. There was no difference between control and drug-treated groups in the size of the ischemic zone (Table 1).

Contractile Function Study

Contractile function, CVR, and heart rate. Throughout the study, coronary flows in the control, EIPA, and

HOE-694 groups were kept constant at their basal values of 16.2 ± 0.8, 16.4 ± 0.6, and 16.7 ± 0.5 $ml \cdot min^{-1} \cdot g^{-1}$, respectively [not significant (NS)]. Basal values (measured after 10 min of constant pressure perfusion) for LVEDP were 7.0 ± 0.1, 7.0 ± 0.2, and 7.0 ± 0.2 mmHg in the control, EIPA, and HOE-694 groups, respectively (NS). The corresponding values for LVDP were 106 ± 3, 105 ± 3, and 119 ± 3 mmHg, respectively (NS). LVEDP did not change significantly during the remainder of the experimental protocol in any of the study groups. The temporal changes in LVDP are shown in Fig. 6. There was a small decline in LVDP with time in the control group (Fig. 6*A*). There was also a small decline in LVDP with increasing concentration of HOE-694 (Fig. 6*C*); however, the similarity with the control group suggests that this was a time-dependent rather than a drug-induced effect. In contrast, EIPA resulted in a steeper decline in LVDP during infusion of the highest concentration (Fig. 6*B*) such that by the end of the experimental protocol LVDP in this group was only 48% of its basal value.

Basal values for CVR were 6.8 ± 0.2, 6.5 ± 0.2, and 6.3 ± 0.1 $mmHg \cdot ml^{-1} \cdot min^{-1}$ in the control, EIPA, and HOE-694 groups, respectively (NS). There was a small increase in CVR during the first 20 min of constant flow perfusion in the control group; thereafter, CVR remained stable (Fig. 6*A*). The temporal changes in CVR in the HOE-694 group resembled those in the control group (Fig. 6*C*). In the EIPA group, however, CVR increased further with increasing concentration of drug such that by the end of drug infusion CVR was 170% of its basal value (Fig. 6*B*).

Basal values for heart rate were also similar in the control, EIPA, and HOE-694 groups at 323 ± 17, 330 ± 12, and 315 ± 9 beats/min, respectively (NS). Heart rate did not change thereafter in the control (Fig. 6*A*) and HOE-694 (Fig. 6*C*) groups but started to decline during the infusion of 1 μM EIPA and reached 75% of its basal value following the infusion of 10 μM EIPA (Fig. 6*B*).

ECG parameters. In control, EIPA, and HOE-694 groups, basal values for P-R interval were 39 ± 3, 41 ± 3, and 37 ± 2 ms, respectively (NS), and the corresponding values for $QRST_{90}$ were 57 ± 3, 58 ± 3, and 62 ± 3 ms, respectively (NS). P-R interval and $QRST_{90}$ did not change significantly throughout the protocol in control and HOE-694 groups. During the infusion of 0.01, 0.1, and 1 μM EIPA, these parameters also did not change significantly; however, after exposure to 10 μM EIPA, P-R interval was prolonged to 73 ± 5 ms ($P < 0.05$) and $QRST_{90}$ to 126 ± 12 ms ($P < 0.05$). One of the six hearts that received EIPA developed second-degree (2 to 1) atrioventricular (AV) block.

DISCUSSION

The present study has demonstrated that, in isolated rat hearts, the selective intracoronary administration of EIPA or HOE-694 into the zone subjected to ischemia and reperfusion affords substantial protection against reperfusion-induced VF. EIPA and HOE-694 exhibit both antifibrillatory and defibrillatory properties, with similar potency and efficacy. These effects are observed

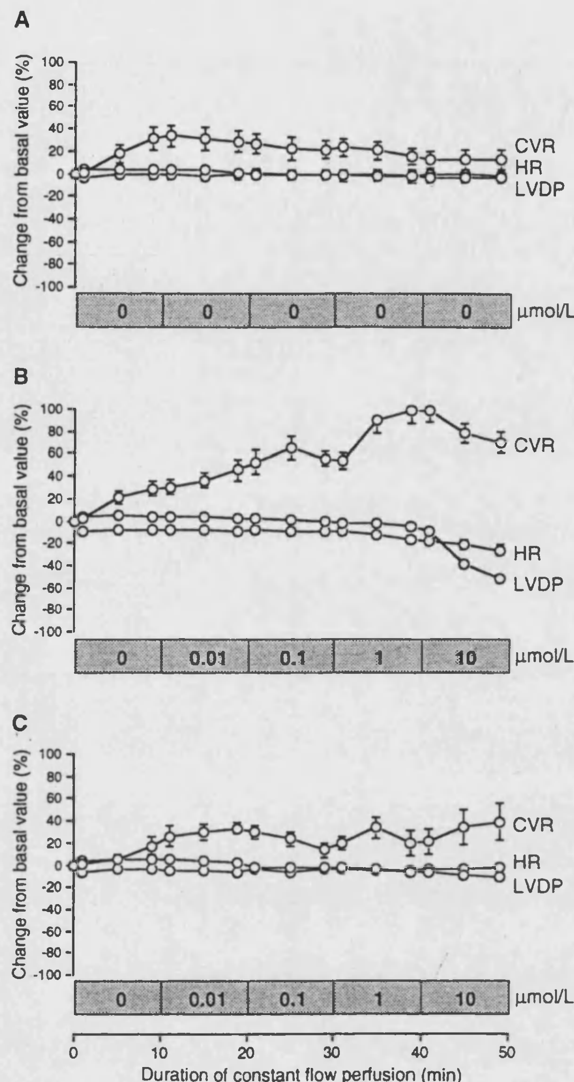

Fig. 6. Temporal changes in left ventricular developed pressure (LVDP), coronary vascular resistance (CVR), and heart rate (HR) during infusion of vehicle (control; *A*), EIPA (*B*), or HOE-694 (*C*). Stippled sections at bottom of *A-C* indicate period of constant-flow perfusion. Nos. in stippled sections indicate drug concentration (μM), with 0 denoting vehicle infusion.

even when the agents are administered during reperfusion alone, thus indicating the involvement of a mechanism that is operative primarily during the reperfusion phase.

Possible Mechanism(s) of Action of EIPA and HOE-694

Na^+/H^+ exchange inhibition. Activation of Na^+/H^+ exchange during early reperfusion has been proposed to result in the intracellular accumulation of Na^+ and consequently an increase in intracellular Ca^{2+} concentration ($[Ca^{2+}]$) via inhibition or reversal of the sarcolem-

mal Na^+/Ca^{2+} exchanger (15). There is indeed evidence that in the rat heart increased cellular Ca^{2+} uptake occurs during reperfusion, and this can be attenuated by pharmacological inhibition of the Na^+/H^+ exchanger (28). Such a disturbance of Ca^{2+} homeostasis has been proposed as a progenitor of reperfusion-induced arrhythmias, probably through a mechanism that involves the oscillatory release of Ca^{2+} from the sarcoplasmic reticulum and the subsequent induction of delayed afterdepolarizations (17). Indeed, in the light of their activation mapping studies in the feline heart, Pogwizd and Corr (20) have suggested that the induction of VF during reperfusion may be mediated by a nonreentrant mechanism that involves Ca^{2+} overload-mediated afterdepolarizations and triggered activity. In support of such a mechanism, the recent studies of Kihara and Morgan (12) in aequorin-loaded ferret hearts have provided evidence that reperfusion after 20 min of ischemia may result in an increase in intracellular [Ca^{2+}], which is associated with the transition to VF. Thandroyen et al. (29), using isolated spontaneously beating ventricular myocytes, have also suggested that increased intracellular [Ca^{2+}] may be a causal factor in the degeneration of VT into VF. In addition to its proposed role in the induction of VF, there is evidence to suggest that increased intracellular [Ca^{2+}] may be important also for the maintenance of VF (12, 29). In the light of the above, the inhibition of Na^+ influx through Na^+/H^+ exchange and subsequent Ca^{2+} overload through Na^+/Ca^{2+} exchange could have mediated both the antifibrillatory and the defibrillatory effects of EIPA and HOE-694 observed in the present study.

PROTECTION BY NA+/H+ EXCHANGE INHIBITION DURING REPERFUSION. It has been suggested (24, 25) that the preischemic administration of Na^+/H^+ exchange inhibitors could attenuate tissue injury by inhibiting Na^+ influx during the early minutes of ischemia [before the development of significant extracellular acidosis, which itself would be expected to inhibit the Na^+/H^+ exchanger (32)]. Therefore, it could be argued that under conditions in which Na^+/H^+ exchange inhibitors are administered before ischemia, inhibition of reperfusion-induced arrhythmias may occur, at least in part, as a consequence of reduced ischemic injury. In the present study, EIPA and HOE-694 suppressed reperfusion-induced VF not only when they were administered before ischemia and during reperfusion but also when they were given during reperfusion alone. This observation provides strong evidence to suggest that the protective effect was mediated primarily by drug action during the reperfusion phase and supports the hypothesis that the activity of the Na^+/H^+ exchanger during early reperfusion is an important determinant of the severity of reperfusion-induced arrhythmias. Indeed, our studies with transient acidic reperfusion (2, 11) suggest that, in this model, inhibition of Na^+/H^+ exchange for the first 2 min of reperfusion may be sufficient to provide sustained protection against VF.

STRUCTURE-ACTIVITY AND SPECIFICITY CONSIDERATIONS. In several cell types, EIPA and HOE-694 have been shown to exhibit similar potency as inhibitors of Na^+/H^+ exchange (23, 24). The similar concentration-response profiles of the two agents observed in the present study are consistent with the hypothesis that Na^+/H^+ exchange inhibition is the primary and common mechanism underlying their ability to protect against reperfusion-induced VF. Although HOE-694 is not an amiloride analogue, it contains an acyl guanidine moiety in common with both amiloride and EIPA. Structure-activity studies with amiloride analogues in fibroblasts (14) and cardiac myocytes (15) have revealed that this moiety is important for Na^+/H^+ exchange inhibitory activity, with derivatives that contain a substituted guanidino group (e.g., benzamil and dichlorobenzamil) exhibiting much reduced potency. The acyl guanidine moiety is also present in other agents that interfere with Na^+ transport, such as tetrodotoxin, and it has been suggested that this moiety may facilitate drug interaction with a site involved in Na^+ transport (15).

Recent cloning and sequencing studies (18, 33) have shown that there are at least four isoforms of the plasma membrane Na^+/H^+ exchanger (NHE-1, NHE-2, NHE-3, and NHE-4), which may exhibit differing responsiveness to inhibition by amiloride and its analogues (4). Only the ubiquitous NHE-1 isoform (18) and, to a lesser extent, the NHE-2 (33) and NHE-3 (18) isoforms appear to be expressed in the intact adult rat heart. Within the context of the present study, it should be noted that HOE-694 has been shown recently (4) to exhibit significantly greater selectivity than amiloride and its 5-amino-substituted derivatives for NHE-1, the predominant Na^+/H^+ exchanger isoform expressed in the rat heart.

VT cycle length. In our earlier acidic reperfusion study (2), we reported a pH-dependent prolongation of VT cycle length during early reperfusion and proposed that slowing of VT may be a mechanism through which acidic reperfusion exerts its antifibrillatory action (by enhancing spontaneous reversion of VT to sinus rhythm and inhibiting its deterioration to VF). In the present study, the administration of EIPA and HOE-694 before ischemia plus during reperfusion resulted in a concentration-dependent prolongation of VT cycle length, when measured 10 s after the onset of reperfusion. This prolongation of VT cycle length correlated well with the antifibrillatory effects of both drugs, suggesting a causal role. However, the administration of EIPA or HOE-694 during reperfusion alone did not affect VT cycle length but nonetheless had a protective effect comparable to that achieved when the drugs were also given before ischemia. This would suggest that prolongation of VT cycle length during early reperfusion is unlikely to be the primary mechanism by which these Na^+/H^+ exchange inhibitors exert their antifibrillatory effects.

Nonselective effects. In the present study, exposure of the hearts to cumulatively increasing concentrations of EIPA resulted in negative inotropic and chronotropic effects and increased vascular resistance; this is consistent with previous observations with EIPA in the guinea pig heart (19). During infusion of the 10 μM concentration, EIPA also prolonged the P-R interval and $QRST_{90}$, indicative of abnormalities in AV nodal conduction (in one heart AV block was observed) and ventricular

depolarization and/or repolarization. It is well established that amiloride (and to a lesser extent its analogues such as EIPA) inhibit a number of other ion transport processes (e.g., Na^+, Ca^{2+}, and K^+ channels, Na^+/Ca^{2+} exchange) (13) and can have multiple electrophysiological effects (8). In this regard, previous in vitro studies have shown that amiloride can slow AV nodal conduction without affecting intraventricular conduction (34), have a negative chronotropic effect on the sinoatrial node (22), and prolong the action potential duration in Purkinje fibers (16). Interestingly, the pronounced effect on repolarization in Purkinje fibers was manifest only after prolonged exposure to amiloride, suggesting that this effect may be mediated by the intracellular accumulation of the drug (16). In a similar manner, the cardiodepressant and electrocardiographic effects of EIPA observed in the present study were evident only during infusion of the 10 μM concentration, following prolonged (30 min) exposure to the lower concentrations of the drug. Therefore, nonselective actions (perhaps mediated by intracellular drug accumulation) may have contributed to the protective effects of high concentrations of EIPA observed in the present study, when the drug was administered before ischemia (thus allowing extended exposure to the drug trapped in the ischemic bed). Indeed, with this treatment protocol, 10 μM EIPA totally abolished reperfusion-induced arrhythmias (including VT), a property not shared by HOE-694. However, nonselective effects are less likely to have played a major role in the protective effects of EIPA against reperfusion-induced arrhythmias when the drug was infused during reperfusion alone, where the duration of exposure to the drug was limited.

Although detailed electrophysiological data on HOE-694 are not currently available, some observations from the present study suggest that relative to EIPA, HOE-694 may exhibit fewer nonselective effects. Thus, unlike EIPA, prolonged exposure to increasing concentrations of HOE-694 (up to 10 μM) in the absence of ischemia and reperfusion did not affect heart rate, contractile function, or ECG parameters. This would be the expected profile of a selective Na^+/H^+ exchange inhibitor which, in bicarbonate-buffered medium under control conditions, should not affect intracellular pH or other ion transport mechanisms.

Relevance to the Mechanism of Reperfusion-Induced Arrhythmias

The results of the present study indicate that the activation of Na^+/H^+ exchange during reperfusion is an important arrhythmogenic factor; they do not, however, preclude a significant role for other factors [e.g., washout of extracellular K^+ (5), generation of oxygen free radicals (3)] in reperfusion-induced arrhythmogenesis. Indeed, whereas EIPA and HOE-694 significantly inhibited reperfusion-induced VF in a concentration-dependent manner, the incidence of reperfusion-induced VT remained at 100% in most groups, thus supporting the argument that multiple factors are involved. The only exception was the group that received 10 μM EIPA before ischemia plus during reperfusion, in which arrhythmias were not detected; however, the nonselective effects of EIPA (discussed in *Nonselective effects*) may have contributed to this phenomenon. It is also possible that some factors associated with reperfusion, such as free radical-induced oxidant stress, may act in a synergistic manner with activation of Na^+/H^+ exchange to disrupt Ca^{2+} homeostasis (9).

Myocardial ischemia results in the neuronal release and extracellular accumulation of catecholamines within the ischemic zone, through locally mediated mechanisms (26). Previous studies (27) have shown that pharmacological inhibition of the Na^+/H^+ exchanger markedly suppresses this ischemia-induced catecholamine release. In the light of the established arrhythmogenic potential of adrenergic stimulation, it is probable that inhibition of ischemia-induced catecholamine release may have contributed to the protective effects of EIPA and HOE-694 in the present study, particularly when the drugs were given before ischemia.

Limitations of the Study

The present study utilized the known pharmacological characteristics of two structurally distinct drugs to investigate the role of the Na^+/H^+ exchanger in reperfusion arrhythmogenesis. Because EIPA and HOE-694 have in common the ability to inhibit the Na^+/H^+ exchanger in an equipotent manner, it is reasonable to ascribe their similar protective effects against reperfusion-induced arrhythmias in the present study to inhibition of the exchanger. However, on the basis of this study, it cannot be deduced whether the protection was mediated by reduced intracellular accumulation of Na^+ and/or Ca^{2+} during reperfusion.

One limitation of the present study may be the use of an erythrocyte-free perfusion medium, which results in coronary flows in excess of those encountered in vivo; such high flows may result in faster washout during reperfusion of components (such as H^+) that have accumulated during the preceding period of ischemia, which in turn may lead to an overestimation of the role of washout phenomena in reperfusion arrhythmogenesis. However, it is worth noting that in the present model even a 90% reduction in the rate of reflow is unable to suppress reperfusion-induced VF (10).

It may be argued also that the use of the rat heart, which has unusual electrophysiological characteristics (such as a short action potential duration and a high heart rate), may limit the applicability of the present findings to other species. Although this is acknowledged, it must be pointed out that the model used in the present study offers many advantages in the study of the pathophysiological determinants of arrhythmogenesis (1), such as the consistent generation of regional ischemia and arrhythmias, the ability to use multiple drug concentrations and groups of adequate size (due to the low cost of the preparation), and the ability to infuse drugs selectively into the ischemic-reperfused zone.

Concluding Comments

The present study has shown that in isolated rat hearts subjected to regional ischemia, the selective infusion into the ischemic-reperfused zone of EIPA or HOE-694, two structurally distinct but equipotent inhibitors of the Na^+/H^+ exchanger, significantly suppresses the induction of VF during reperfusion and promotes spontaneous reversion to normal sinus rhythm. The drugs exhibit similar potency and efficacy and are effective even when given during reperfusion alone. The protective effects are observed in the absence of intergroup differences in the size of the ischemic zone, heart rate before and during ischemia, the rate of reflow, and oxygen tension of the perfusate. These findings are consistent with an arrhythmogenic role for activation of Na^+/H^+ exchange during the early moments of reperfusion.

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Exacerbation of reperfusion arrhythmias by α_1 adrenergic stimulation: a potential role for receptor mediated activation of sarcolemmal sodium-hydrogen exchange

Masahiro Yasutake and Metin Avkiran

Objective: Stimulation of myocardial α_1 adrenoceptors causes (1) exacerbation of reperfusion induced arrhythmias, and (2) stimulation of sarcolemmal Na^+/H^+ exchange. The aims of this study were to identify the α_1 adrenoceptor subtype involved in the former effect and to determine whether stimulation of the Na^+/H^+ exchanger may play a role in this phenomenon. **Methods:** Isolated rat hearts were subjected to independent perfusion of the left and right coronary beds. After 15 min of aerobic perfusion of both beds, the α_1 adrenoceptor agonist phenylephrine (0.1, 1, or 10 μM) was infused *selectively* into the left coronary bed for 2 min. The left coronary bed was then subjected to 7 min of zero flow ischaemia and 5 min of reperfusion. **Results:** The incidence of reperfusion induced ventricular fibrillation was increased from 0% in controls to 8%, 42%*, and 75%* with 0.1, 1, and 10 μM phenylephrine (*$P<0.05$); this dose dependent effect occurred in the absence of significant intergroup differences in vascular resistance or heart rate. Similar infusion of methoxamine at 10 μM also increased the incidence of reperfusion induced ventricular fibrillation from 13% to 88%*. Infusion of 10 μM phenylephrine during reperfusion alone did not affect the incidence of reperfusion induced ventricular fibrillation. Infusion of the selective α_{1A} adrenoceptor antagonist WB4101 at 0.1, 1, or 10 μM for 2 min immediately before ischaemia (concomitantly with 10 μM phenylephrine) reduced the incidence of reperfusion induced ventricular fibrillation from 83% to 75%, 25%*, and 0%*. Similar infusion of the selective α_{1B} adrenoceptor antagonist chloroethylclonidine (0.1 or 1 μM) or the selective β_1 adrenoceptor antagonist atenolol (0.1 or 1 μM) did not reduce the incidence of reperfusion induced ventricular fibrillation. The novel NHE-1 selective Na^+/H^+ exchange inhibitor HOE694 (10 μM), when infused into the left coronary bed before ischaemia (concomitantly with 10 μM phenylephrine) and throughout reperfusion, reduced the incidence of reperfusion induced ventricular fibrillation from 83% to 25%*. In hearts that received 10 μM phenylephrine before ischaemia, HOE694 (10 μM) was partially effective when infused during reperfusion alone (ventricular fibrillation incidence reduced from 83% to 42%). **Conclusions:** (1) the exacerbation of reperfusion induced arrhythmias by α_1 adrenergic stimulation during ischaemia is mediated by the α_{1A} adrenoceptor subtype, and (2) increased Na^+/H^+ exchanger activity during ischaemia and reperfusion may play a causal role in this phenomenon.
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The α_1 adrenergic contributions to arrhythmogenesis during myocardial ischaemia are well documented.[1] Although α_1 adrenoceptors appear also to play a role in modulating the severity of *reperfusion induced* arrhythmias,[2] the receptor subtypes and cellular mechanisms involved remain unclear. There is substantial evidence to suggest that reperfusion induced arrhythmias may be mediated by an increase in intracellular calcium (calcium overload) and subsequent delayed afterdepolarisations and triggered activity (for review, see Opie and Coetzee[3]). In this context, it is worth noting that in both isolated cardiac myocytes[4] and Purkinje fibres[5] exposure to an α_1 adrenoceptor agonist during *simulated* ischaemia has been shown to result in the induction of delayed afterdepolarisations and triggered activity upon subsequent "reperfusion". In the Purkinje fibre, this proarrhythmic effect of α_1 adrenoceptor stimulation appears to be mediated by the WB4101 sensitive α_{1A} adrenoceptor subtype[5]; however, there is only limited evidence[6] to suggest that this receptor subtype may modulate reperfusion arrhythmogenesis in the intact heart.

Recent observations in our laboratory[7,8] have suggested that the rapid washout of extracellular H^+ may be a major progenitor of reperfusion induced arrhythmias, probably through the activation or disinhibition of the Na^+/H^+ exchanger. Such an increase in Na^+/H^+ exchanger activity during reperfusion may result in an increased intracellular sodium and consequently in calcium overload, via inhibition of calcium efflux or induction of calcium influx through the Na^+/Ca^{2+} exchanger.[9,10] Dennis *et al*[11] were the first to provide experimental evidence in favour of a role for this mechanism in reperfusion arrhythmogenesis. This hypothesis has been supported by the antiarrhythmic efficacy during reperfusion of pharmacological inhibitors of the Na^+/H^+ exchanger, such as amiloride and its analogues[11-14] and the novel drug 3-methylsulphonyl-4-piperidinobenzoyl guanidine (HOE694).[15,16] In the light of the evidence in favour of an

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important role for the Na^+/H^+ exchanger in the induction of arrhythmias during reperfusion, it is interesting to note that α_1 adrenergic stimulation has been shown by several investigators to stimulate this exchanger in cardiac myocytes.[17–21] The possibility exists therefore that activation of the Na^+/H^+ exchanger may play a causal role in the exacerbation of reperfusion induced arrhythmias by α_1 adrenergic stimulation. Indeed, Gambassi *et al*[22] have shown recently that α_1 adrenergic stimulation could induce aftercontractions (indicative of calcium overload) during recovery of cardiac myocytes from acidosis; this proarrhythmic effect could be blocked not only by the selective α_{1A} adrenoceptor antagonist WB4101 but also by the Na^+/H^+ exchange inhibitor ethylisopropylamiloride, implicating a causal role for increased exchanger activity.

The primary objectives of the present study were: (1) to confirm in the intact heart the identity of the adrenoceptor subtype involved in the exacerbation of reperfusion induced arrhythmias by α_1 adrenergic stimulation, and (2) to determine whether stimulation of the Na^+/H^+ exchanger could play a causal role in this phenomenon. In order to achieve these objectives we used as pharmacological tools selective antagonists of α_{1A}, α_{1B}, and β_1 adrenoceptors (WB4101, chloroethylclonidine, and atenolol, respectively) and a selective inhibitor (HOE694) of the predominant Na^+/H^+ exchanger isoform expressed in the heart. In addition, we used isolated rat hearts subjected to independent perfusion of the left and right coronary beds,[23] which allowed the use of regional ischaemia and reperfusion without mechanical damage to myocardial or vascular tissue and the administration of the drugs under study *selectively* into the involved zone. Our results show that the WB4101 sensitive α_{1A} adrenoceptor subtype mediates the proarrhythmic effects of α_1 adrenoceptor stimulation and suggest that stimulation of the Na^+/H^+ exchanger may play a causal role in this phenomenon.

Methods

This investigation was performed in accordance with the Home Office *Guidance on the operation of the Animals (Scientific Procedures) Act 1986*, published by HMSO, London.

Dual coronary perfusion of isolated rat hearts

Isolated rat hearts were subjected to independent perfusion of left and right coronary arteries, as described in detail by Avkiran and Curtis.[23] In brief, male Wistar rats were anaesthetised by inhalation of diethyl ether, and 100 IU of sodium heparin was injected into a femoral vein. The heart was then quickly excised and immersed in perfusion solution at 4°C. Within 30 s of excision, the dual perfusion cannula was inserted into the ascending aorta and secured in position with a braided silk suture. Alignment of the coronary ostia with the orifices of the cannula was achieved using in-line monitoring of left and right coronary flow as a guide, the aorta being rotated on the cannula until flow in each perfusion bed reached a maximum. The pulmonary artery was then cut near its origin and a stainless steel needle was inserted into the left ventricle through the apex, to allow adequate drainage of coronary and Thebesian venous effluent.

In all experimental protocols, each coronary bed was initially supplied with oxygenated perfusion solution from a temperature regulated reservoir (37°C), at a constant perfusion pressure equivalent to 75 mm Hg. The perfusion solution was of the following composition (in mmol·litre^{-1}): NaCl 118.5, $NaHCO_3$ 25.0, KCl 3.2, $MgSO_4$ 1.2, KH_2PO_4 1.2, $CaCl_2$ 1.4, glucose 11.0. The solution was filtered (pore size, 5 μm) before use and bubbled continuously with 95% O_2 plus 5% CO_2 (pH 7.4 at 37°C). Flow to each coronary bed was monitored using in-line flow detectors with a linear detection range of 0.05-30 ml·min^{-1}. In some experimental protocols (see later), basal flow rate in the left coronary bed was noted after 15 min of perfusion at constant perfusion pressure and perfusion of this bed was switched to constant flow at the basal flow rate, via a roller pump. During constant flow perfusion, 7% of the total flow supplied to the left coronary bed was from a side arm, through which drug solutions or vehicle could be administered. This technique, which has been described previously,[16] allowed the effective administration of adrenoceptor antagonists and HOE694 (which has limited solubility in physiological buffer solutions) concomitantly with phenylephrine, at the required concentrations. The right coronary bed was perfused at constant pressure throughout the experiment in all protocols. The heart was housed in a temperature regulated chamber at 37°C and the right atrium was continuously superfused with oxygenated perfusion solution (37°C) at a constant rate of 8 ml·min^{-1} to maintain sinus rate.[23]

Experimental protocols

DETERMINATION OF DURATION OF REGIONAL ISCHAEMIA

The severity of reperfusion induced arrhythmias is highly dependent on the duration of the preceding period of ischaemia. In studies testing potential *anti*arrhythmic interventions in isolated rat hearts, a 10 min duration of regional ischaemia is commonly employed, since this results in a high incidence of reperfusion induced ventricular fibrillation.[7 8] For the purpose of demonstrating an α_1 adrenoceptor mediated *pro*arrhythmic effect, it was first necessary to determine an appropriate duration of ischaemia that would result in a low incidence of ventricular fibrillation in the control group. To achieve this objective, hearts (n = 6 per group) were subjected to 6, 7, or 8 min of regional ischaemia by stopping flow to the left coronary bed, and arrhythmias were analysed during a subsequent 5 min period of reperfusion at constant pressure (75 mm Hg).

EFFECTS OF PHENYLEPHRINE AND METHOXAMINE ON REPERFUSION ARRHYTHMIAS

This study was designed to test the effects of α_1 adrenoceptor stimulation during ischaemia on reperfusion induced arrhythmias. Phenylephrine (a non-selective α_1 adrenoceptor agonist) was dissolved in perfusion solution at concentrations of 0.1, 1, and 10 μM. These solutions were supplied selectively to the left coronary bed at constant perfusion pressure (75 mm Hg) for 2 min immediately before the induction of regional ischaemia by stopping flow to this bed. Control hearts received drug-free perfusion solution for an identical period (n = 12 per group). Following 7 min of ischaemia (a duration determined from the protocol described above; see Results), the left coronary bed was reperfused with drug-free solution for 5 min, at constant pressure (75 mm Hg). In additional studies, the effects of 10 μM methoxamine given before ischaemia (n = 8 per group) and 10 μM phenylephrine given during reperfusion alone (n = 6 per group) were also investigated.

ROLE OF α_{1A} ADRENOCEPTORS

This study was designed to determine the role of the WB4101 sensitive α_{1A} adrenoceptor subtype in any proarrhythmic effect of phenylephrine. After 15 min perfusion of both coronary beds at constant pressure, perfusion of the left coronary bed was switched to a constant flow system, as described earlier. For the 2 min immediately before the induction of regional ischaemia, the left coronary bed received perfusion solution containing 10 μM phenylephrine. During this period, solutions containing WB4101 were infused selectively into the perfusion line supplying the left coronary bed at 7% of the total flow rate to that bed, to give a final WB4101 concentration of 0.1, 1, or 10 μM. Control hearts received vehicle (deionised water) at the same infusion rate. Subsequently, all hearts (n = 12 per group) were subjected to 7 min of regional ischaemia (by stopping flow to the left coronary bed) and 5 min of reperfusion with drug-free solution at constant flow.

ROLE OF α_{1B} AND β ADRENOCEPTORS

This study was designed to determine the role of α_{1B} and β_1 adrenoceptor mediated mechanisms in any proarrhythmic effect of phenylephrine. The protocol was as described above except that, instead of WB4101, the left coronary bed received either chloroethylclonidine (α_{1B} adrenoceptor antagonist) or atenolol (β_1 adrenoceptor antagonist) at 0.1 or 1 μM, concomitantly with 10 μM phenylephrine (n = 6 per group).

ROLE OF Na^+/H^+ EXCHANGER

This study was designed to determine whether stimulation of the Na^+/H^+ exchanger could play a causal role in any proarrhythmic effect of phenylephrine. The protocol was as described above except that the left coronary bed received 10 μM HOE694 (a novel Na^+/H^+ exchange inhibitor[15 24 25]) *either* for 2 min immediately before ischaemia (concomitantly with 10 μM phenylephrine) plus throughout reperfusion *or* throughout reperfusion alone (n = 12 per group). The concentration of HOE694 was chosen on the basis of its protective efficacy against reperfusion induced arrhythmias in isolated rat hearts in the absence of exogenous α_1 adrenoceptor stimulation.[15 16]

Measured variables

Arrhythmias – Arrhythmias were diagnosed from a unipolar electrogram (ECG) that was obtained through a silver electrode inserted into the free wall of the left ventricle and a reference electrode connected to the aorta. The ECG was continuously monitored on a digital storage oscilloscope and recorded on an ink jet recorder. Chart speed was set at 50 mm·s^{-1} a few seconds before reperfusion so as to obtain a

permanent high speed record of the changes in the ECG during early reperfusion. The ECG record obtained during the reperfusion period was retrospectively analysed, in a blinded manner, for the incidence, time to onset, and duration of ventricular tachycardia and ventricular fibrillation. All analyses were carried out in accordance with the Lambeth Conventions.[26] Ventricular tachycardia was defined as four or more consecutive premature beats of ventricular origin and ventricular fibrillation as a signal in which both rate and amplitude varied from cycle to cycle.

Coronary flow and heart rate – Throughout the experimental protocol, coronary flow was monitored using the in-line flow detectors. Heart rate was determined at selected intervals from the ECG trace.

Size of ischaemic zone – At the end of each experiment, the left coronary bed was perfused for 30 s with a solution containing 0.016% disulphine blue dye, at 75 mm Hg perfusion pressure. The heart was then removed from the perfusion apparatus, the atria and mediastinal tissue were removed, and dye stained tissue, representing ventricular myocardium subjected to ischaemia and reperfusion, was carefully dissected away from the remainder. The stained and unstained tissues were lightly blotted and weighed. The size of the ischaemic zone, expressed as a percentage of total ventricular weight, was calculated from the equation: (weight of stained tissue/total ventricular weight) × 100. The absolute weights obtained also enabled the calculation of flows in left and right coronary beds on the basis of tissue weights supplied by each bed ($ml \cdot min^{-1} \cdot g^{-1}$).

Drug sources

Phenylephrine, methoxamine, WB4101, and atenolol were purchased from Sigma. Chloroethylclonidine was purchased from SEMAT (SEMAT Technical Ltd, St Albans, UK). HOE694 was a kind gift from Hoechst (Hoechst AG, Frankfurt, Germany).

Exclusion criteria

These criteria, selected to minimise variations in heart rate and size of ischaemic zone (due to atypical coronary anatomy) among the hearts, were as previously described.[7,8,23] Hearts were also excluded if there was cross flow between right and left coronary ostia.[7,8,23] In addition, hearts with ventricular arrhythmias during the final 3 s of ischaemia before reperfusion were not included in the analysis of reperfusion induced arrhythmias, because in those hearts it would have been impossible to differentiate arrhythmias induced by reperfusion from those induced by ischaemia. Of a total of 222 hearts entered into this study, three were excluded on the basis of heart rate, three on the basis of size of ischaemic zone, four on account of cross flow, and four on account of arrhythmias during the final 3 s of ischaemia. Thus the overall exclusion rate was 6%.

Statistical analysis

All experiments were carried out in a prospectively randomised manner. Gaussian distributed variables were expressed as mean(SEM) and were subjected to one way analysis of variance. If a difference among mean values was established, comparison with controls was performed using Dunnett's test. Binominally distributed variables, such as the incidence of ventricular tachycardia or fibrillation, were compared using the χ^2 test for a 2 × n table. If a significant difference was revealed, each drug treated group was then compared with the control group using the Fisher exact test, with the Bonferroni correction for multiple comparisons. A P value of <0.05 was considered significant.

Results

Reperfusion induced arrhythmias

DETERMINATION OF DURATION OF REGIONAL ISCHAEMIA

Figure 1 shows the incidence of reperfusion induced ventricular tachycardia and ventricular fibrillation in hearts subjected to 6, 7, or 8 min of regional ischaemia. On the basis of these results, 7 min of regional ischaemia, which resulted in a 67% incidence of reperfusion induced ventricular tachycardia and a 17% incidence of reperfusion induced ventricular fibrillation, was chosen for use in subsequent protocols designed to test the proarrhythmic effects of α_1 adrenoceptor stimulation and to determine the mechanisms of any such effect.

EFFECTS OF PHENYLEPHRINE AND METHOXAMINE ON REPERFUSION ARRHYTHMIAS

Figure 2 shows the time course of reperfusion induced arrhythmias in the control group and in the groups in which the left coronary bed received 0.1, 1, or 10 μM phenylephrine for 2 min immediately before 7 min of ischaemia. Phenylephrine tended to increase the incidence of reperfusion induced ventricular tachycardia from 58% in the control group to 67%, 100%, and 100% at concentrations of 0.1, 1, and 10 μM, respectively; however, these increases were not statistically significant. At the same concentrations, phenylephrine also increased the incidence of ventricular fibrillation from 0% in the control group to 8% (NS), 42% (NS), and 75% ($P<0.05$), respectively. By the end of the reperfusion period, 100% of the control hearts were in normal sinus rhythm; phenylephrine reduced this incidence to 92% (NS), 58% (NS), and 50% ($P<0.05$) at concentrations of 0.1, 1, and 10 μM, respectively. Methoxamine (10 μM) had a similar effect to phenylephrine, increasing the incidence of reperfusion induced ventricular fibrillation from 13% to 88% ($P<0.05$) and decreasing the proportion of hearts in normal sinus rhythm at the end of reperfusion from 88% to 13% ($P<0.05$). Phenylephrine at 10 μM, when given during reperfusion alone, did not significantly affect the incidence of reperfusion induced ventricular tachycardia and ventricular fibrillation relative to a contemporary control group.

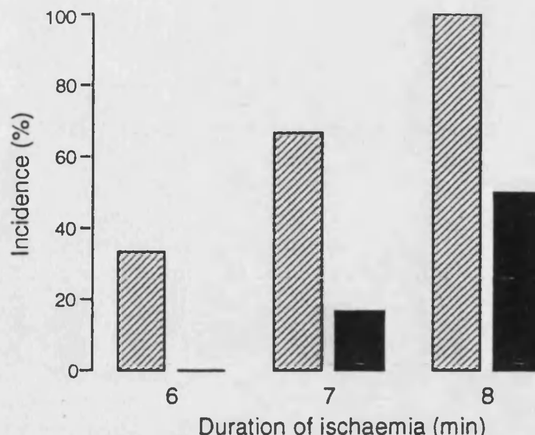

Figure 1 *The incidence of reperfusion induced ventricular tachycardia (hatched bars) and ventricular fibrillation (solid bars) in hearts subjected to 6, 7, or 8 min of regional ischaemia (n = 6 per group).*

ROLE OF α_{1A} ADRENOCEPTORS

Figure 3 shows the time course of reperfusion induced arrhythmias in four study groups in which the left coronary bed received 10 μM phenylephrine for 2 min immediately before the onset of ischaemia, either in the absence (control) or presence of WB4101 (0.1, 1, or 10 μM). WB4101 suppressed the proarrhythmic effects of 10 μM phenylephrine in a dose dependent manner, such that the incidence of reperfusion induced ventricular fibrillation was reduced from 83% in the control group to 75% (NS), 25% ($P<0.05$), and 0% ($P<0.05$) at concentrations of 0.1, 1, and 10 μM, respectively. The incidence of reperfusion induced ventricular tachycardia was 100% in the control group, and 100% (NS), 92% (NS), and 0% ($P<0.05$) in the groups that received 0.1, 1, and 10 μM WB4101, respectively. In the control group, only 33% of hearts were in normal sinus rhythm at the end of the reperfusion period. The infusion of WB4101 at concentrations of 0.1, 1, or 10 μM increased this incidence to 50% (NS), 75% (NS), and 100% ($P<0.05$), respectively.

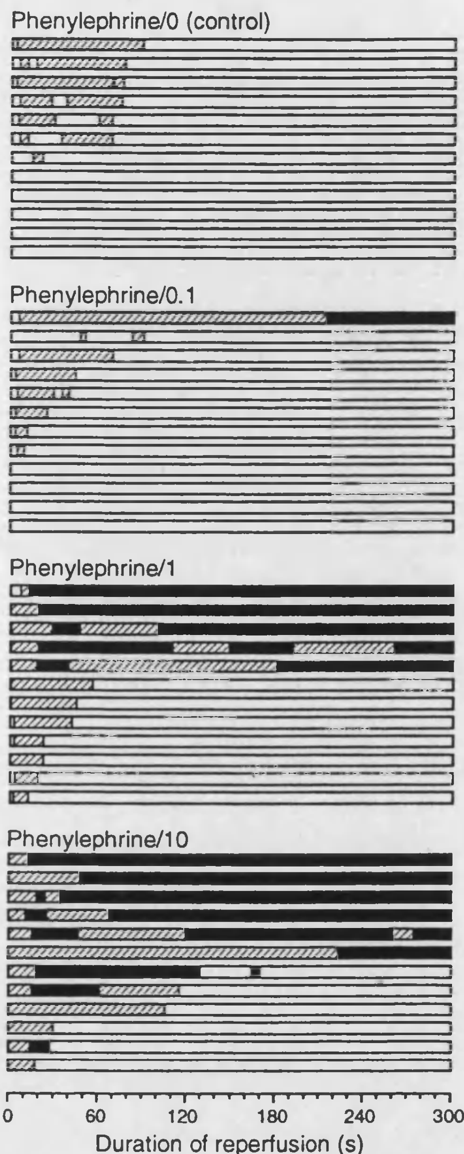

Figure 2 Time course of reperfusion induced ventricular arrhythmias in individual hearts in the various study groups comprising the phenylephrine dose-response study. The numbers given above each panel indicate the concentration of phenylephrine in μmol·litre^{-1}. Each horizontal bar represents an individual heart; open sections represent normal sinus rhythm, hatched sections ventricular tachycardia, and filled sections ventricular fibrillation. For clarity, the hearts in each group (n = 12) are arranged in relation to the time of reversion to stable sinus rhythm and not in relation to the order in which the experiments were carried out (the study was randomised).

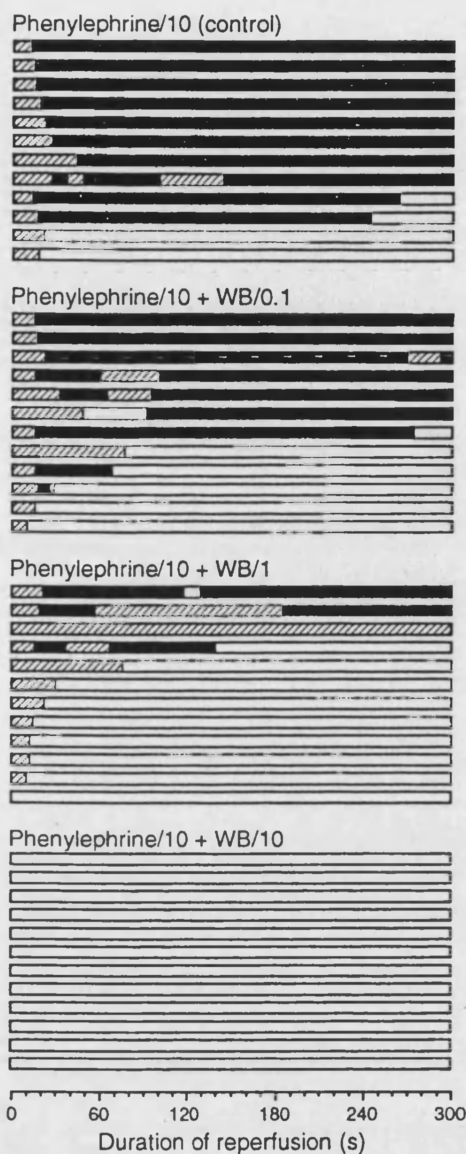

Figure 3 Time course of reperfusion induced ventricular arrhythmias in individual hearts in the various study groups that received 10 μM phenylephrine either alone or in combination with various concentrations of WB4101 (WB). The numbers given above each panel indicate the concentrations of phenylephrine and WB4101 in μmol·litre^{-1}. Each horizontal bar represents an individual heart; open sections represent normal sinus rhythm, hatched sections ventricular tachycardia, and filled sections ventricular fibrillation. For clarity, the hearts in each group (n = 12) are arranged in relation to the time of reversion to stable sinus rhythm and not in relation to the order in which the experiments were carried out (the study was randomised).

ROLE OF α_{1B} AND β ADRENOCEPTORS

Figure 4 shows the effects of concomitant infusion of chloroethylclonidine (α_{1B} adrenoceptor antagonist) or atenolol (β_1 adrenoceptor antagonist) on the proarrhythmic effects of 10 μM phenylephrine. Neither drug produced a significant change in the incidence of reperfusion induced ventricular tachycardia or ventricular fibrillation, relative to the control group that received phenylephrine alone.

ROLE OF Na^+/H^+ EXCHANGE

Figure 5 shows the time course of reperfusion induced arrhythmias in the control group in which the left coronary bed received 10 μM phenylephrine for 2 min immediately before ischaemia and in the two treatment groups in which 10 μM HOE694 was also given, *either* concomitantly with phenylephrine plus during reperfusion *or* during reperfusion

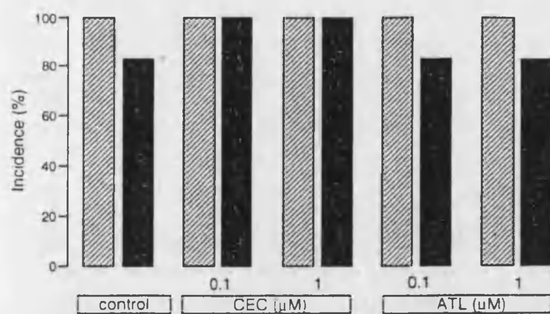

Figure 4 *The effects of chloroethylclonidine (CEC) and atenolol (ATL) on the incidence of reperfusion induced ventricular tachycardia (hatched bars) and ventricular fibrillation (solid bars), when administered concomitantly with 10 μM phenylephrine. Control hearts received 10 μM phenylephrine alone (n = 6 per group).*

alone. The incidence of reperfusion induced ventricular tachycardia was 100% in all three groups. When HOE694 was infused before ischaemia (concomitantly with 10 μM phenylephrine) plus throughout reperfusion, the incidence of ventricular fibrillation was reduced from 83% in the control group to 25% ($P < 0.05$). In addition, 100% of hearts were in normal sinus rhythm at the end of reperfusion, relative to only 42% in the control group ($P < 0.05$). The infusion of HOE694 during reperfusion alone was partially effective, reducing the incidence of ventricular fibrillation from 83% in the control group to 42% (NS) and increasing the number of hearts in normal sinus rhythm at the end of reperfusion from 42% in the control group to 92% ($P < 0.05$).

Ischaemic zone size, coronary flow, and heart rate

There were no significant differences between the groups within each protocol in ischaemic zone size [range: 52(SEM 2) to 63(6)%] and basal values for coronary flow rate [range: 10.0(0.2) to 13.1(1.4) $ml{\cdot}min^{-1}{\cdot}g^{-1}$ in the left coronary bed, 10.6(0.9) to 14.8(0.8) $ml{\cdot}min^{-1}{\cdot}g^{-1}$ in the right coronary bed] and heart rate [range: 310(4) to 349(10) $beats{\cdot}min^{-1}$]. In the protocol designed to test the effects of phenylephrine on reperfusion induced arrhythmias, the effects on vascular resistance were determined by continuous monitoring of the coronary flow rate during the preischaemic perfusion of phenylephrine at constant perfusion pressure. Flow in the right coronary bed was not affected during the selective infusion of phenylephrine into the left coronary bed. The lowest concentration of phenylephrine (0.1 μM) produced a transient vasodilatation in the left coronary bed, whereas 1 and 10 μM phenylephrine had no significant effect; by the end of the 2 min period of perfusion with control or phenylephrine containing solution, there was no significant difference in left coronary flow rate between the four study groups. Methoxamine (10 μM) also did not have a significant effect on either left or right coronary flow rate. During the period of zero flow ischaemia in the left coronary bed, flow rate in the right coronary bed did not change significantly. During reperfusion, flow rate increased in both coronary beds commensurate with the severity of reperfusion induced arrhythmias, as previously described.[7 8] The absence of a significant coronary vasoconstrictor effect with phenylephrine at concentrations of up to 10 μM allowed the infusion of phenylephrine into the left coronary bed at constant flow without an increase in perfusion pressure in subsequent studies. This made it possible to give other agents

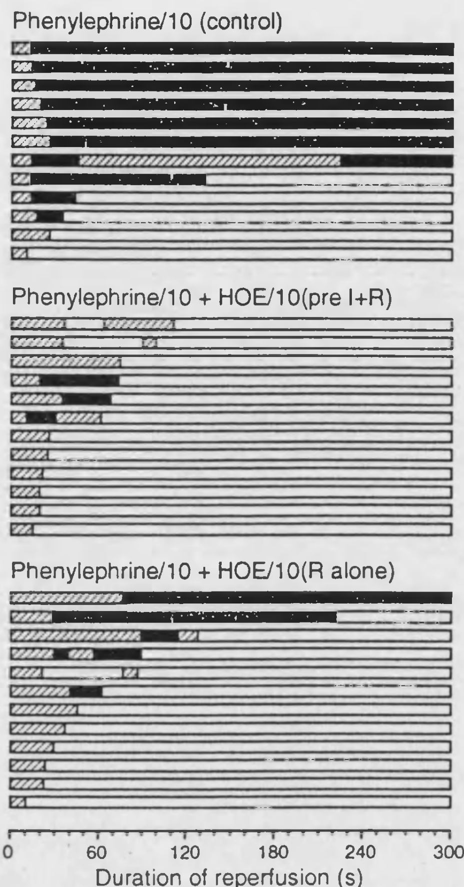

Figure 5 *Time course of reperfusion induced arrhythmias in individual hearts in the various study groups that received 10 μM phenylephrine, with or without HOE694 (HOE). HOE694 was infused* either *before ischaemia (concomitant with phenylephrine) and throughout reperfusion (pre I + R)* or *during reperfusion alone (R alone). The numbers given above each panel indicate the concentrations of phenylephrine and HOE694 in* $\mu mol{\cdot}litre^{-1}$*. Each horizontal bar represents an individual heart; white sections represent normal sinus rhythm, hatched sections ventricular tachycardia, and black sections ventricular fibrillation. For clarity, the hearts in each group (n = 12) are arranged in relation to the time of reversion to stable sinus rhythm and not in relation to the order in which the experiments were carried out (the study was randomised).*

into the left coronary bed concomitantly with phenylephrine through a side arm, as described in Methods.

The selective administration of phenylephrine or methoxamine to the left coronary bed for 2 min immediately prior to the onset of ischaemia, either alone or concomitantly with any of the other agents, did not affect heart rate. Heart rate did not change significantly during the 7 min period of regional ischaemia in any of the study groups and could not be measured during early reperfusion due to the rapid onset of ventricular arrhythmias in the majority of hearts.

Discussion

This study has shown that, in isolated rat hearts subjected to regional ischaemia and reperfusion, selective exposure of

the ischaemic myocardium to an exogenous, non-selective α_1 adrenoceptor agonist (phenylephrine) exacerbates reperfusion induced arrhythmias in a dose dependent manner. This proarrhythmic effect is mimicked by another nonselective α_1 adrenoceptor agonist (methoxamine) and can be inhibited by an antagonist selective for α_{1A} adrenoceptors but not by antagonists selective for either α_{1B} or β_1 adrenoceptors, suggesting that the effect is mediated by a specific adrenoceptor subtype. Furthermore, the present study provides the first evidence in intact hearts that receptor mediated activation of the sarcolemmal Na^+/H^+ exchanger may be involved in the downstream cellular mechanisms of this proarrhythmic effect.

Adrenoceptor subtype(s) mediating the effects of phenylephrine
Mammalian postsynaptic α_1 adrenoceptors have been divided into two pharmacologically distinct receptor subtypes, termed α_{1A} and α_{1B}.[27] The α_{1A} subtype exhibits high affinity for WB4101, a competitive antagonist, and relative insensitivity to chloroethylclonidine, an alkylating agent. In contrast, the α_{1B} subtype exhibits low affinity for WB4101 and is inactivated irreversibly by chloroethylclonidine. Radioligand binding studies[28] have shown both adrenoceptor subtypes to be present in rat ventricular myocardium, with an α_{1A}:α_{1B} ratio of 30:70. Although molecular cloning studies[29] suggest the existence of additional α_1 adrenoceptor subtypes, their expression and functional role in ventricular myocardium have not been fully characterised.

The observation in the present study that WB4101 could inhibit (in a dose dependent manner) the proarrhythmic effect of phenylephrine suggests that this effect was mediated via α_{1A} adrenoceptors. This possibility is further supported by the apparent inability of α_{1B} and β_1 adrenoceptor blockade (by chloroethylclonidine and atenolol, respectively) to inhibit the proarrhythmic effect of phenylephrine. These results are consistent with previous observations in canine Purkinje fibres subjected to *simulated* ischaemia and reperfusion[5] and are supportive of the preliminary observations of Brittain-Valenti *et al*[6] that α_{1A} adrenoceptor stimulation may exacerbate reperfusion induced arrhythmias in vivo. It should be noted that, at 10 μmol·litre^{-1}, WB4101 not only reversed the profibrillatory effect of phenylephrine but abolished all reperfusion induced arrhythmias. This effect may have occurred through inhibition of α_{1A} adrenoceptor stimulation by endogenous catecholamines or non-specific antiarrhythmic effects of WB4101 at high concentration.

Cellular proarrhythmic mechanisms downstream of receptor activation
As noted earlier, there is increasing evidence[7 8 11-16] that activation of the Na^+/H^+ exchanger may be a major progenitor of reperfusion induced arrhythmias. It is reasonable to propose therefore that modulation of exchanger activity through α_1 adrenoceptor mediated mechanisms may have a significant impact on the severity of reperfusion induced arrhythmias.

Receptor mediated modulation of Na^+/H^+ exchanger activity – Recent cloning and sequencing studies[30 31] have shown that there are at least four different isoforms of the Na^+/H^+ exchanger (NHE-1, NHE-2, NHE-3, and NHE-4), which have distinct regulatory and kinetic properties. In the normal adult rat heart (as used in the present study), only the ubiquitous NHE-1 isoform of the exchanger family appears to be expressed to a significant extent.[30 31] Available evidence suggests that the activity of this isoform is increased not only by intracellular acidosis but also by a variety of growth factors and neurohormonal agents, through receptor mediated mechanisms.[32] Of direct relevance to the present study, recent studies[17-21] in isolated cardiac myocytes have shown that stimulation of α_1 adrenoceptors results in increased sarcolemmal Na^+/H^+ exchanger activity. There is evidence[33] to suggest that, at least in the rat, such activation of the exchanger may be mediated by the pharmacologically identifiable α_{1A} adrenoceptor.

Figure 6 depicts the cellular signalling pathways that may be involved in α_{1A} adrenoceptor mediated activation of the sarcolemmal Na^+/H^+ exchanger. Recent studies[28] have shown that stimulation of myocardial α_{1A} adrenoceptors results in phospholipase C activation and phosphoinositide hydrolysis. In a variety of tissues and cell types, the consequent production of diacylglycerol and release of Ca^{2+} from intracellular stores results in the activation of protein kinase C and its translocation to the cell membrane. Of particular relevance to the present study, translocation of protein kinase C to the sarcolemma has been shown to occur in cardiac myocardium, following exposure to phenylephrine.[34] It is well established that agents that activate protein kinase C are capable of activating NHE-1,[35] although this is unlikely to occur via direct phosphorylation of the exchanger by protein kinase C.[36] Instead, it has been proposed[35] that the signalling pathways downstream of receptor activation may involve activation of other kinases, possibly including a specific NHE kinase. In the present context, α_1 adrenoceptor mediated stimulation of Na^+/H^+ exchanger activity in ventricular myocytes has been shown by several investigators[17-19] to be inhibited by pharmacological inhibition or downregulation of protein kinase C (although this has been disputed by Pucéat and colleagues[20]). Furthermore, myocardial Na^+/H^+ exchanger activity is increased by phorbol esters which directly activate protein kinase C.[37] There is very recent evidence[38] suggesting that receptor mediated activation of NHE-1 can also occur via an additional mechanism, possibly involving a regulatory factor, which does not require phosphorylation of the exchanger. The relevance of this mechanism to α_1 adrenoceptor mediated stimulation of Na^+/H^+ exchanger activity in cardiac myocytes remains to be determined.

Na^+/H^+ exchanger activity in ischaemia versus reperfusion – In the present study, the proarrhythmic effect of phenylephrine was significantly reversed by the administration of

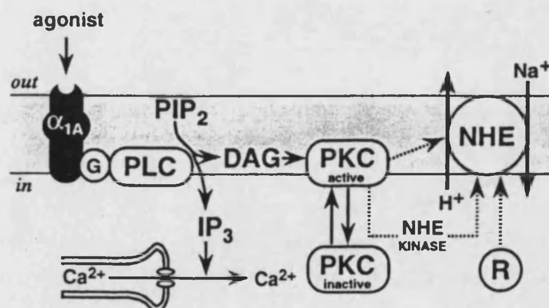

Figure 6 *Schematic diagram of cellular signalling pathways that may be involved in α_{1A} adrenoceptor mediated activation of sarcolemmal Na^+/H^+ exchanger (NHE) activity. G = G protein; PLC = phospholipase C; PIP_2 = phosphatidylinositol 4,5-bisphosphate; IP_3 = inositol 1,4,5-trisphosphate; DAG = diacylglycerol; PKC = protein kinase C; R = regulatory factor (see text for details).*

HOE694 (a selective inhibitor of NHE-1[24]), concomitantly with phenylephrine and during reperfusion. Even when given during reperfusion alone, HOE694 provided partial protection against the proarrhythmic effect of phenylephrine. These observations suggest that under the experimental conditions employed reperfusion-induced arrhythmias were modulated by Na^+/H^+ exchanger activity not only during ischaemia but also during reperfusion. Contrary to this hypothesis, however, phenylephrine (which, in the absence of α_1 adrenoceptor antagonists should increase Na^+/H^+ exchanger activity) did not affect the severity of reperfusion induced arrhythmias when given during reperfusion alone. Our recent studies in the same model[39] suggest the modulation of Na^+/H^+ exchanger activity during reperfusion may only affect the incidence of severe arrhythmias if such modulation occurs during the first two minutes of reperfusion (before recovery of the sarcolemmal Na^+/K^+ pump from ischaemia induced inhibition). It is probable therefore that the lack of effect of phenylephrine when given during reperfusion alone may have been due to inadequate activation of the Na^+/H^+ exchanger during this limited vulnerable period. In this regard, in isolated ventricular myocytes (where drug access to myocardial receptor sites should occur more readily than in intact hearts perfused through the coronary vasculature), maximum stimulation of exchanger activity by phenylephrine has been shown to occur after three to four minutes of exposure to the agonist.[21]

The sarcolemmal Na^+/H^+ exchanger has been shown to be inhibited by extracellular acidosis[40] and hypoxia.[41] These conditions both occur during myocardial ischaemia and would be expected to inhibit the Na^+/H^+ exchanger, thus allowing little scope for pharmacological inhibition of the exchanger during the ischaemic period. Our observation that HOE694 provides greater protection when given before ischaemia *and* during reperfusion, relative to when given during reperfusion alone, may provide indirect evidence that the Na^+/H^+ exchanger retains some activity during the period of ischaemia employed, at least in the presence of phenylephrine. Indeed, even in the absence of exogenous α_1 adrenergic stimulation, recent studies[42] have shown that 5-(*N*-ethyl-*N*-isopropyl) amiloride (EIPA) can inhibit the intracellular accumulation of Na^+ during ischaemia, presumably through inhibition of an active Na^+/H^+ exchanger.

Is calcium the key arrhythmogenic mediator? – As noted earlier, intracellular calcium overload has been proposed as the key mediator of reperfusion induced ventricular arrhythmias.[3] Within the context of the present study, there is evidence[43] that stimulation of myocardial α_1 adrenoceptors may promote calcium overload during reperfusion. Although the present study does not provide any direct evidence regarding cellular ionic mechanisms, the possibility that the proarrhythmic action of phenylephrine may involve receptor mediated stimulation of Na^+/H^+ exchanger activity is consistent with a key role for intracellular calcium in reperfusion arrhythmogenesis. As noted earlier, it has been proposed that calcium overload during reperfusion may occur, at least in part, through mechanisms involving the sarcolemmal Na^+/H^+ and Na^+/Ca^{2+} exchangers (for recent reviews, see Tani[10] and Karmazyn and Moffat[37]). Since pharmacological inhibitors of the Na^+/H^+ exchanger have been shown to *attenuate* both calcium overload[44] and arrhythmias[11–16] during reperfusion, agents that stimulate the Na^+/H^+ exchanger would be expected to *exacerbate* calcium overload and arrhythmias under comparable conditions.

Modulation of reperfusion arrhythmias by endogenous catecholamines

In the present study, we have investigated the potential mechanisms of the proarrhythmic effect of an exogenous α_1 adrenergic agonist. There is substantial evidence that endogenous catecholamines, which are known to be released during ischaemia,[45] may also modulate ventricular arrhythmias induced by ischaemia and reperfusion, through α_1 adrenoceptor mediated mechanisms (for review, see Corr *et al*[1]). Thus the potential proarrhythmic mechanisms (of phenylephrine) identified in the present study may also play a role in modulating reperfusion arrhythmogenesis in the absence of exogenous agonists. Indeed, as noted earlier, there is preliminary evidence[6] that reperfusion induced arrhythmias in vivo may be exacerbated by α_{1A} adrenoceptor stimulation. Furthermore, there is evidence[46] that activation of *neuronal* Na^+/H^+ exchange may enhance the release of endogenous catecholamines during ischaemia; inhibition of this process may provide an additional mechanism by which pharmacological inhibition of the Na^+/H^+ exchanger during ischaemia might be beneficial.

Potential limitations of the study

The conclusions of the present study are based on the selective pharmacological properties of the agents used. Although WB4101, chloroethylclonidine, and atenolol are well characterised as selective inhibitors of α_{1A}, α_{1B}, and β_1 adrenoceptors, respectively, the Na^+/H^+ exchange inhibitor HOE694 is a relatively new compound where pharmacological characterisation may not be complete. Since amiloride analogues (which also inhibit the Na^+/H^+ exchanger and with which HOE694 shares certain structural similarities[25]) have been shown[47] to possess some α_1 adrenoceptor antagonistic activity, such a mechanism might have mediated the reversal of the proarrhythmic effects of phenylephrine by HOE694. In contrast, however, radioligand binding studies have shown that HOE694 does not bind to α_1 adrenoceptors of rat forebrain synaptosomes (W Scholz, personal communication). Furthermore, HOE694 has recently been shown[24] to have greater selectivity than amiloride analogues for NHE-1, the predominant Na^+/H^+ exchanger isoform expressed in the rat heart. In addition, although amiloride and its analogues have negative inotropic effects because of their secondary pharmacological properties,[48] recent studies in our laboratory[16] have shown that HOE694 does not affect cardiac function under normal aerobic conditions. These observations suggest that HOE694 may be more selective than amiloride and its analogues as an inhibitor of sarcolemmal Na^+/H^+ exchange.

It should be noted that there are significant interspecies differences in myocardial α_1 adrenoceptor content, with rat ventricular myocardium possessing a five- to eightfold greater receptor density than guinea pig, mouse, pig, calf, and man.[49] It is possible therefore that any proarrhythmic effect of α_1 adrenoceptor stimulation might be exaggerated in the rat. In the light of this, extrapolation of the findings of the present study to other species should be undertaken with caution.

Concluding comments

Through the application of subtype selective adrenoceptor antagonists and a selective inhibitor of the predominant Na^+/H^+ exchanger isoform expressed in the heart, the present study has (1) shown that the exacerbation of reperfusion induced arrhythmias by an exogenous α_1 adrenoceptor agonist is mediated by the α_{1A} adrenoceptor subtype, and

(2) suggested that receptor mediated stimulation of sarcolemmal Na^+/H^+ exchanger activity may be involved in the downstream cellular mechanisms of this proarrhythmic effect. Further work is required to determine the relevant signal transduction mechanisms and to confirm whether similar receptor mediated mechanisms are involved in the modulation of reperfusion induced arrhythmias by endogenous catecholamines.

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Key terms: α_1 Adrenoceptors; ischaemia; reperfusion induced arrhythmias; dual coronary perfusion; Na^+/H^+ exchange; rat heart.

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CHAPTER 9

Role of the Sarcolemmal Na+/H+ Exchanger in Arrhythmogenesis During Reperfusion of Ischemic Myocardium

Metin Avkiran and Masahiro Yasutake

A. INTRODUCTION

In many experimental studies, reperfusion of myocardium subjected to a brief period of ischemia has been shown to result in the rapid induction of severe ventricular arrhythmias (for a review, see Manning and Hearse[1]). Commonly, polymorphic ventricular tachycardia (VT) develops within a few beats after the onset of reperfusion, and in the absence of protective interventions, this frequently degenerates into ventricular fibrillation (VF).[2] Observations made in patients with Prinzmetal's angina[3] and silent myocardial ischemia[4] suggest that a similar pattern of reperfusion-induced arrhythmogenesis may occur also in man.

In several species, a "bell-shaped" relationship has been demonstrated between the severity of reperfusion-induced arrhythmias and the duration of the preceding period of ischemia.[1] The duration that results in maximum susceptibility to reperfusion-induced VF is relatively short: 5-10 minutes in the anesthetized rat,[1] 10-15 minutes in the isolated rat heart,[1,5] 20-25 minutes in the isolated rabbit heart,[6] and 20-30 minutes in the anesthetized dog.[1] With more prolonged ischemia, susceptibility to reperfusion-induced VF declines with increasing duration. Although, for obvious ethical reasons, the presence

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of a bell-shaped relationship (between ischemia duration and susceptibility to reperfusion-induced arrhythmias) has not been directly tested in man, there is circumstantial evidence to suggest that such a relationship may exist. Thus, reperfusion during cardiac surgery after relatively short (< 60 min.) periods of ischemia has been shown to result in a 50% incidence of ventricular fibrillation.[7] In contrast, as discussed previously,[8] serious reperfusion-induced ventricular arrhythmias were uncommon in large scale clinical trials with intravenously administered thrombolytic agents. A contributory factor to this phenomenon may have been the fact that thrombolytic therapy was started hours, rather than minutes, after the onset of symptoms. Indeed, a survey of clinical trials with intracoronary thrombolysis has revealed a decreasing incidence of severe reperfusion-induced ventricular arrhythmias with increasing duration of ischemia.[9] The malignant nature of the arrhythmias observed during reperfusion in experimental studies (polymorphic VT and/or VF) has led several investigators to suggest that such arrhythmias may underlie some cases of sudden cardiac death in man.[10,11]

The cellular mechanisms of reperfusion-induced arrhythmias are likely to differ from those responsible for ischemia-induced arrhythmias.[12,13] Although the fundamental question of whether the arrhythmogenic process is initiated by the washout of substances accumulated during ischemia or by the resupply of substances absent during ischemia remains to be resolved,[14] several factors have been implicated in reperfusion arrhythmogenesis. Among these putative arrhythmogenic factors, recent evidence appears to favor a causal involvement for free oxygen radical generation (and associated oxidant stress) and, in particular, sarcolemmal Na^+/H^+ exchanger activation.

B. AN INVOLVEMENT OF FREE OXYGEN RADICALS?

A potential link between the generation of free oxygen radicals, upon the reintroduction of oxygen during the early moments of reperfusion, and the induction of arrhythmias first became evident when scavengers of such radicals were shown to inhibit reperfusion-induced arrhythmias.[15] This association was supported by subsequent studies, which not only confirmed that reperfusion arrhythmias could be inhibited by free oxygen radical scavengers but also showed that they could be exacerbated by radical generating systems.[16] Further support came from studies which showed that a burst of free radical production occurs during early reperfusion[17] and that reactive oxygen intermediates are sufficient to generate ventricular arrhythmias in the absence of ischemia and reperfusion.[18,19] However, the results of studies with radical scavenging and generating systems have not been unequivocal,[20] and some investigators have argued that free oxygen radicals are unlikely to be the prime cause of ventricular arrhythmias during early reperfusion.[20,21] In this context, Yamada et al[22] have shown that *anoxic* reperfusion delays the time-to-onset but does not significantly alter

the incidence of reperfusion-induced VF. This observation led these investigators to conclude that re-establishment of flow and readmission of oxygen were independent determinants of reperfusion-induced arrhythmias and that the latter was not a prerequisite for arrhythmogenesis. It appears, therefore, that although free oxygen radicals may modulate reperfusion-induced arrhythmias significantly, their generation may not be the dominant progenitor of these arrhythmias.

C. EVIDENCE FOR A KEY ROLE FOR THE Na^+/H^+ EXCHANGER

C.1 MECHANISM OF ACTIVATION AND CONSEQUENCES ON IONIC HOMEOSTASIS

Myocardial ischemia is known to result in acidosis, due to a retention of H^+ from glycolytic adenosine triphosphate (ATP) turnover, carbon dioxide accumulation and net ATP breakdown.[23] Intracellular acidosis during early ischemia would be expected to stimulate H^+ extrusion pathways, including the Na^+/H^+ exchanger.[23] With maintained ischemia, however, extracellular acidosis has been shown to exceed intracellular acidosis, with reversal of the transmembrane H^+ gradient occurring within 10 minutes in rabbit papillary muscle.[24] Since the sarcolemmal Na^+/H^+ exchanger is inhibited by extracellular acidosis,[25,26] such an effect on extracellular pH of prolonged ischemia would be expected to result in a secondary inhibition of the exchanger.

The potential consequences of postischemic reperfusion on sarcolemmal Na^+/H^+ exchanger activity and intracellular ionic homeostasis are schematically summarized in Figure 9.1. Lazdunski et al[27] were the first to suggest that the rapid washout of extracellular H^+ upon reperfusion could reactivate the Na^+/H^+ exchanger (by alleviating the inhibition by extracellular acidosis) and create an intracellular-to-extracellular H^+ gradient, resulting in a significant influx of Na^+ via this route. Such an influx of Na^+, in the presence of Na^+/K^+ pump inhibition caused by the preceding ischemia, could then result in an increase in intracellular Na^+. Although there is controversy over whether a large increase in intracellular Na^+ concentration occurs during early reperfusion, this may be due partly to the inability of current techniques (e.g., ion-selective microelectrodes, fluorescent dyes and nuclear magnetic resonance) to measure Na^+ concentration in the relevant cellular compartment, the subsarcolemmal "fuzzy space."[28] Even a modest increase in intracellular Na^+ concentration would be expected to alter significantly the reversal potential of the Na^+/Ca^{2+} exchanger (which, under normal conditions, operates primarily to extrude Ca^{2+} from the cell), favoring intracellular Ca^{2+} accumulation.[29] The resulting disturbance of Ca^{2+} homeostasis has been proposed as a progenitor of reperfusion-induced arrhythmias,[30] probably through a mechanism that involves the oscillatory release of Ca^{2+} from the sarcoplasmic reticulum (SR) and the subsequent induction of delayed afterdepolarizations,[30,31] as discussed below.

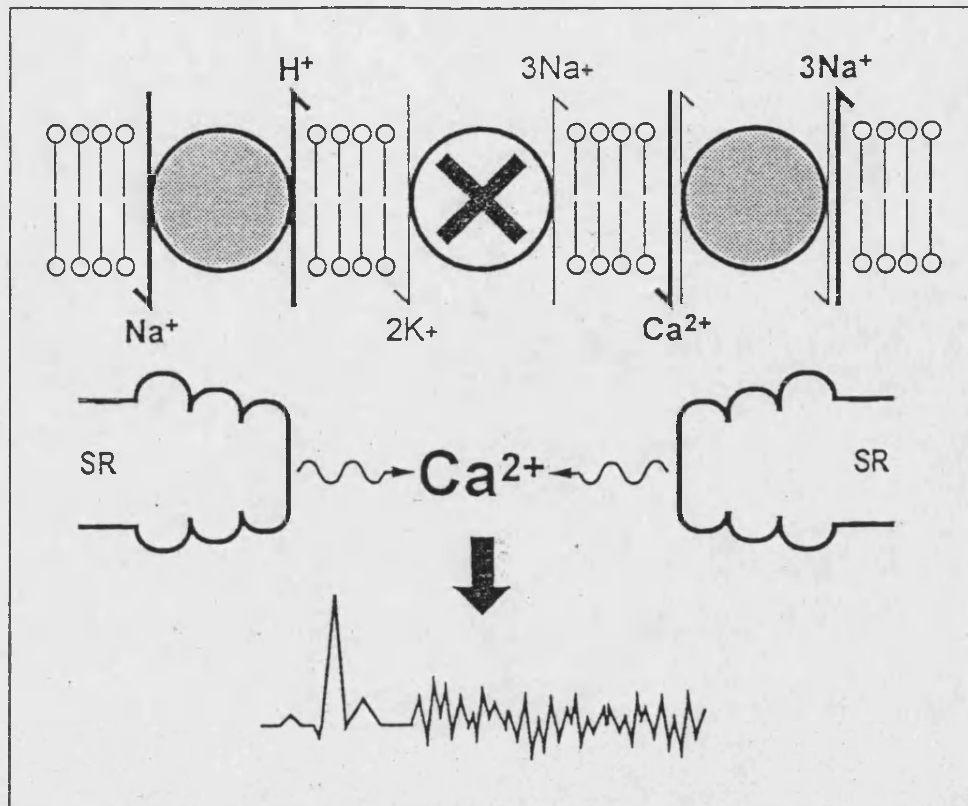

Fig. 9.1. Schematic depiction of the potential consequences of reperfusion on Na^+/H^+ exchanger activity, ionic homeostasis and cardiac rhythm. According to this scheme, an influx of Na^+ through the Na^+/H^+ exchanger results in an increased intracellular Na^+ concentration (due to inhibition of the Na^+/K^+ pump during preceding ischemia) and thereby an increased Ca^{2+} concentration (Ca^{2+} overload) via the Na^+/Ca^{2+} exchanger. The resultant oscillatory release of Ca^{2+} from the sarcoplasmic reticulum (SR) and induction of afterdepolarizations may precipitate ventricular fibrillation (see text for details).

C.2 Evidence for an Arrhythmogenic Role

Dennis et al[32] provided the first experimental evidence to suggest that activation of the Na^+/H^+ exchanger, as proposed by Lazdunski et al,[27] may contribute to arrhythmogenesis during reperfusion. This hypothesis has been supported further by studies employing acidic reperfusion in the authors' laboratory,[2,33] which have shown that slowing the rate at which extracellular pH is restored to its normal physiological value during early reperfusion inhibits the induction of VF and promotes spontaneous reversion from VT to normal sinus rhythm. This effect occurred in a pH-dependent manner,[2] with significant protection against VF observed at pH values (pH 6.6 and 6.4) that would be expected to inhibit significantly the Na^+/H^+ exchanger.[25,26] This is

consistent with a major arrhythmogenic role for the rapid washout of extracellular H^+ during uncontrolled reperfusion, the most likely mechanism being activation of the Na^+/H^+ exchanger.[27] Furthermore, a recent study in rat hearts by the authors' group (using a novel dual coronary perfusion technique[34]) has shown that selective administration of two structurally distinct pharmacological inhibitors of the Na^+/H^+ exchanger (ethyl-isopropyl amiloride and HOE694) into the ischemic/reperfused zone reduces, in a concentration-dependent manner, the incidence of reperfusion-induced VF.[35] Interestingly, both drugs were effective not only when given before ischemia and during reperfusion, but also when given *only* during reperfusion, suggesting that the protective action of Na^+/H^+ exchanger inhibition was operative, at least in part, during the reperfusion phase. Observations consistent with these results have been obtained also with the global administration of a range of Na^+/H^+ exchanger inhibitors, such as amiloride and its analogs[32,36-38] and the newer compounds HOE694[38-40] and HOE642.[41]

C.3 Interaction with the Na^+/K^+ Pump

As discussed earlier and depicted in Figure 9.1, the magnitude of any increase in intracellular Na^+ concentration that occurs during early reperfusion as a consequence of Na^+ influx through the Na^+/H^+ exchanger may depend to a large extent on the activity of the primary Na^+ extrusion pathway, namely the sarcolemmal Na^+/K^+ pump. Even under normal conditions, activation of the Na^+/H^+ exchanger by the abrupt creation of an intracellular to extracellular H^+ gradient can result in increased intracellular Na^+ concentration and stimulate electrogenic Na^+ extrusion via the Na^+/K^+ pump.[42,43] In the presence of pharmacological inhibition of the Na^+/K^+ pump, the creation of such a H^+ gradient has been shown, in both isolated myocytes[44] and whole hearts,[45] to result in the rapid intracellular accumulation of Ca^{2+}, most probably through the Na^+/Ca^{2+} exchanger. In a similar manner, the activity of the Na^+/K^+ pump also may modulate intracellular Ca^{2+} accumulation during reperfusion.[27,46] In support of this, it has been shown that with pharmacological inhibition of the Na^+/K^+ pump reperfusion results in a greater increase in intracellular Ca^{2+} concentration relative to that observed in the absence of such inhibition.[47,48] This increase in intracellular Ca^{2+} accumulation during reperfusion has been associated with an exacerbation of contractile dysfunction,[47] as well as an increased incidence of VF.[48]

In this context, the histochemical studies of Winston et al[49] have shown that the activity of Na^+/K^+ ATPase (the biochemical correlate of the Na^+/K^+ pump) is depressed significantly during acute ischemia, while the tissue enzyme content remains unaffected. It is reasonable to propose, therefore, that the protective effect against arrhythmias of Na^+/H^+ exchanger inhibition during early reperfusion, either by

pharmacological agents or by acidic reperfusion, may be mediated through inhibition of excessive Na^+ influx at a time when myocardial capacity to extrude Na^+ is significantly impaired. Interestingly, a recent study with transient acidic reperfusion in the authors' laboratory has revealed a close similarity in the duration of acidic reperfusion required to achieve: (i) a sustained protection against reperfusion-induced VF; and (ii) a significant recovery of maximal Na^+/K^+ ATPase activity from ischemia-induced inhibition.[50] This suggests that the protective mechanism of transient acidic reperfusion may involve not only a suppression of Na^+ influx coupled to H^+ extrusion via the Na^+/H^+ exchanger but also an enhanced recovery of Na^+ efflux via the Na^+/K^+ pump.

C.4 Selectivity of Pharmacological Inhibitors

Conventional inhibitors of the Na^+/H^+ exchanger, such as amiloride and its 5-amino substituted derivatives, can interact with a number of other cation transporting proteins,[51] which may limit their value as selective pharmacological tools in delineating the arrhythmogenic role of the Na^+/H^+ exchanger in ischemia and reperfusion. Indeed, recent work by Pierce et al[52] has shown that, in the rat, amiloride and several of its derivatives may exhibit cardiodepressant effects and produce changes in action potential characteristics (particularly at high concentration and following prolonged exposure), via actions unrelated to Na^+/H^+ exchanger inhibition. Such actions may preclude attribution of the antiarrhythmic effects of these drugs to Na^+/H^+ exchanger inhibition. Indeed, it has been suggested[53] that the antiarrhythmic action of amiloride may be due partly to an inhibition of the inwardly rectifying K^+ current (I_{K1}).

Despite the above, amiloride derivatives still may be of value as tools in investigating the role of the Na^+/H^+ exchanger in ischemia and reperfusion, provided drug concentration and period of drug exposure are carefully controlled.[52] Furthermore, cardiodepressant actions have not been observed with Na^+/H^+ exchanger inhibitory concentrations of newer inhibitors such as HOE694,[35] which also may exhibit an enhanced selectivity for the cardiac isoform of the exchanger.[54] Nevertheless, HOE694 shares the protective efficacy of other amiloride-based exchanger inhibitors against reperfusion-induced arrhythmias.[35,38-40] Thus, it is likely that the common protection afforded by amiloride and its derivatives[32,35-38] and by the newer benzoyl guanidine compounds (such as HOE694[35,38-40] and its structural congener HOE642[41]) against reperfusion-induced arrhythmias is mediated by these agents' common pharmacological action, that is Na^+/H^+ exchanger inhibition.

C.5 Impact of Acute Neurohormonal Modulation

Activity of the sarcolemmal Na^+/H^+ exchanger in mammalian cardiac myocytes is increased substantially by a variety of neurohormonal agents of cardiovascular relevance, including α_1-adrenergic agonists,[55-59]

endothelin[60] and angiotensin II,[61] apparently through receptor-mediated mechanisms. The Na^+/H^+ exchanger-stimulatory actions of the former two agents appear to be suppressed by putative protein kinase C (PKC) inhibitors[55,56,59,60] and mimicked by PKC activators (such as phorbol esters[55,59]), although these findings have been disputed.[57]

In light of the above, it is interesting to note that exposure of isolated rabbit hearts to phorbol esters has been shown[62] to increase the incidence of VF during hypoxia and reoxygenation. In a similar manner, α_1-adrenoceptor stimulation has been shown by the authors' studies[63] to exacerbate reperfusion-induced VF in isolated rat hearts subjected to regional ischemia. The profibrillatory effect of α_1-adrenoceptor stimulation was reversed by Na^+/H^+ exchanger inhibition, implicating the exchanger in the downstream mechanism(s).[63] Since there is evidence of both norepinephrine release[64] and α_1-adrenoceptor upregulation[65] during myocardial ischemia and reperfusion, regulation of sarcolemmal Na^+/H^+ exchanger activity via endogenous catecholamines could play a significant role in modulating the severity of arrhythmias in this setting.

Another endogenous mediator that could play a role in modulation of Na^+/H^+ exchanger activity in myocardial ischemia and reperfusion is thrombin. Thrombin is a multifunctional protease which, in addition to its established role in thrombus formation, induces a variety of cellular responses through the recently cloned thrombin receptor (for review, see Coughlin[66]). Exposure to thrombin has been shown to activate the plasma membrane Na^+/H^+ exchanger in a number of cell types, including platelets,[67] endothelial cells[68] and smooth muscle cells,[69] through (at least in part) a protein kinase C-mediated pathway. In this regard, recent studies in the authors' laboratory[70] have suggested that a functional thrombin receptor may be expressed also in adult rat ventricular myocytes and may be involved in the regulation of sarcolemmal Na^+/H^+ exchanger activity. Such a regulatory mechanism could have a significant impact on susceptibility to arrhythmias in the setting of ischemia and reperfusion. Indeed, Goldstein et al[71] have shown that the incidence of malignant ventricular arrhythmias during acute ischemia is greater following thrombotic coronary occlusion than nonthrombotic balloon occlusion, implicating an arrhythmogenic role for factors (such as thrombin) that are associated with thrombus formation. Furthermore, the same group has suggested that during myocardial ischemia activation of the thrombin receptor may contribute to arrhythmogenesis by inducing an increase in intracellular Na^+ concentration,[72] an observation which is consistent with sarcolemmal Na^+/H^+ exchanger activation. Nevertheless, the impact of thrombin receptor activation on the severity of ischemia and reperfusion-induced arrhythmias remains to be determined. An arrhythmogenic role for thrombin during ischemia and/or reperfusion via Na^+/H^+ exchanger activation could have direct clinical implications, since intracoronary thrombosis is the commonest cause of acute ischemia in patients with coronary artery disease.

C.6 Potential Impact of Chronic Regulation

There is considerable experimental evidence that the severity of reperfusion-induced arrhythmias is exacerbated in hearts with pressure overload hypertrophy,[73-76] although the cellular mechanisms that are primarily responsible for this increased susceptibility are unclear. With regard to the putative arrhythmogenic scheme depicted in Figure 9.1, it is interesting to note that there is evidence of altered expression and activity of both the Na^+/K^+ pump[77,78] and the Na^+/Ca^{2+} exchanger[79,80] in left ventricular hypertrophy. In this context, mRNA expression of the ubiquitous NHE1 isoform of the Na^+/H^+ exchanger has been shown recently to be upregulated in hypertrophied rabbit myocardium.[81] If such an increase in the steady-state mRNA level is reflected by an increased NHE1 protein expression and sarcolemmal Na^+/H^+ exchanger activity (and is applicable to other species and models of hypertrophy), then this could provide a mechanism for the increased susceptibility of hypertrophied myocardium to reperfusion-induced arrhythmias.

D. CALCIUM—THE COMMON ARRHYTHMOGENIC MEDIATOR

It is apparent from the evidence presented above that the sarcolemmal Na^+/H^+ exchanger may be a key mediator of severe ventricular arrhythmias induced by reperfusion. The question that arises is how this relates to other putative arrhythmogenic processes (e.g., free oxygen radical generation and associated oxidant stress) that have been implicated in reperfusion arrhythmogenesis. The activation mapping studies of Pogwizd and Corr[82] have supported the hypothesis that the induction of VF during reperfusion is mediated by a nonreentrant mechanism that involves Ca^{2+} overload-mediated afterdepolarizations and triggered activity. Indeed, the studies of Kihara and Morgan[83] in the intact ferret heart have shown that spontaneous transitions to VF do not occur unless a state of Ca^{2+} overload is present and that diastolic Ca^{2+} oscillations precede such transitions. More recently, Brooks et al[84] from the same group have demonstrated a potentially causal association between elevated perfusate Ca^{2+} concentration, loss of intracellular Ca^{2+} homeostasis and increased susceptibility to reperfusion-induced VF in isolated rat hearts. That Ca^{2+} overload, and subsequent oscillatory Ca^{2+} release from the SR, may be a primary mechanism underlying VF induction during reperfusion is supported also by the observation that ryanodine (which inhibits Ca^{2+} release from the SR) prevents the degeneration of VT into VF.[85]

As discussed above, activation of the sarcolemmal Na^+/H^+ exchanger during early reperfusion is likely to favor Ca^{2+} overload through the Na^+/Ca^{2+} exchanger. In this regard, Ca^{2+} overload may represent a common arrhythmogenic mechanism that is precipitated by several components associated with reperfusion. Indeed, it has been suggested that the induction of oxidant stress and the activation of the Na^+/H^+ exchanger during early reperfusion may act in a synergistic manner to

disrupt intracellular Ca^{2+} homeostasis.[86] Consistent with this hypothesis and the putative arrhythmogenic scheme depicted in Figure 9.1, oxidant stress has been shown to inhibit the electrogenic Na^+/K^+ pump,[87] stimulate the sarcolemmal Na^+/Ca^{2+} exchanger[88] and increase the open probability of the SR Ca^{2+} release channel.[89] Furthermore, recent studies have shown that reactive oxygen intermediates can induce arrhythmogenic oscillations in membrane potential, indicative of intracellular Ca^{2+} overload, in both isolated ventricular muscles[90] and isolated myocytes.[91] Thus, in the setting of reperfusion, the synergistic action of free oxygen radical generation and sarcolemmal Na^+/H^+ exchanger activation may result in a loss of intracellular Ca^{2+} homeostasis and thereby the induction of severe ventricular arrhythmias.

E. CONCLUDING COMMENTS

Many experimental studies have shown that reperfusion of transiently ischemic myocardium can result in the induction of severe ventricular arrhythmias, such as VF. Although incidences of such arrhythmias have been documented in man, their true clinical relevance and potential role in sudden cardiac death remain to be proven conclusively. Nevertheless, available experimental evidence suggests that during reperfusion of ischemic myocardium the intracellular accumulation of Ca^{2+} (Ca^{2+} overload) may be the final trigger responsible for the induction of VF. Ca^{2+} overload itself may arise through the synergistic interaction of more than one component associated with reperfusion, with activation of the sarcolemmal Na^+/H^+ exchanger playing a dominant role.

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Impact of extracellular buffer composition on cardioprotective efficacy of Na^+/H^+ exchanger inhibitors

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Shimada, Yasuyuki, David J. Hearse, and Metin Avkiran. Impact of extracellular buffer composition on cardioprotective efficacy of Na^+/H^+ exchanger inhibitors. *Am. J. Physiol.* 270 (*Heart Circ. Physiol.* 39): H692–H700, 1996.—There is controversy over whether the cardioprotective effects of Na^+/H^+ exchanger inhibitors are exerted primarily during ischemia or during subsequent reperfusion, possibly because of interstudy differences in experimental conditions. We studied the impact of perfusate buffer composition on the relative degree of protection afforded by Na^+/H^+ exchanger inhibition during ischemia vs. reperfusion. Isolated rat hearts (n = 8/group) were perfused (37°C, 75 mmHg) with bicarbonate- or *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid-buffered medium and subjected to 20 min of global zero-flow ischemia and 45 min of reperfusion. One of two structurally distinct Na^+/H^+ exchanger inhibitors [5-(*N,N*-dimethyl)amiloride (DMA) or (3-methylsulfonyl-4-piperidinobenzoyl)guanidine methanesulfonate (HOE-694), 10 µmol/l] was transiently (5 min) infused *1*) immediately before ischemia, *2*) during initial reperfusion, or *3*) during both of these periods. With bicarbonate-buffered medium, neither drug improved the postischemic recovery of left ventricular developed pressure (LVDP) when given only during reperfusion. In contrast, HOE-694 improved the postischemic recovery of LVDP from 39 ± 5% in control to 66 ± 6% ($P < 0.05$) when given before ischemia and from 33 ± 4% in control to 65 ± 4% ($P < 0.05$) when given before ischemia plus during reperfusion. With the latter protocol, the cardioprotective effect of HOE-694 occurred in a dose-dependent manner at 0.1–10 µmol/l. In contrast to the results with bicarbonate-buffered medium, in the presence of *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid-buffered medium, DMA and HOE-694 significantly improved recovery of LVDP (from 34 ± 5% in controls to 56 ± 3 and 71 ± 8%, both $P < 0.05$) when given only during reperfusion. They also provided significant protection when given before ischemia or before ischemia plus during reperfusion; with the latter protocol, HOE-694 produced an almost complete recovery of LVDP (88 ± 9 vs. 30 ± 7% in controls, $P < 0.05$). In conclusion, our results suggest that the influence of Na^+/H^+ exchanger activity during reperfusion on the extent of functional recovery is modulated significantly by perfusate buffer composition. As a consequence, the cardioprotective efficacy of Na^+/H^+ exchanger inhibitors may be overestimated under bicarbonate-free conditions.

HOE-694; dimethylamiloride; bicarbonate; contractile function; rat heart

THERE IS SUBSTANTIAL EVIDENCE that the sarcolemmal Na^+/H^+ exchanger may be a key mediator of injury during myocardial ischemia and reperfusion (for recent reviews, see Refs. 11 and 26). However, despite general agreement among different investigators (6, 8, 10, 16, 24, 29) that pharmacological inhibition of the exchanger can significantly alleviate postischemic contractile dysfunction, it is unclear whether the protective mechanism is operative primarily during ischemia or during reperfusion. In this connection, there is considerable discrepancy between studies on the efficacy of Na^+/H^+ exchange inhibitors when given only at the time of reperfusion, with reports of full protection (6, 14–16), partial protection (8, 29), and lack of effect (10, 18, 20). A number of factors might have contributed to the apparently contradictory conclusions of the above studies.

First, in the majority of studies, a variety of amiloride analogues were used to inhibit the Na^+/H^+ exchanger. However, these agents have variable selectivity for the Na^+/H^+ exchanger (12) and may produce cardiodepressant effects [particularly at high concentration and after prolonged exposure (23, 32)] that in some cases might counteract any benefit arising from Na^+/H^+ exchanger inhibition. Second, some studies in isolated hearts have been carried out in the presence of bicarbonate buffer (6, 10, 17–20, 29), whereas others have used perfusion media containing the zwitterionic buffer *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES) (14–17, 24). It is probable that in the latter instance, where Na^+/H^+ exchange is the primary mechanism of recovery from intracellular acidosis (13), the contribution of reperfusion-induced activation of the Na^+/H^+ exchanger to contractile dysfunction (and hence the protective efficacy of pharmacological inhibition of the exchanger during reperfusion) may be overestimated.

The primary objective of the present study was to determine whether the relative degree of protection afforded by Na^+/H^+ exchanger inhibition during ischemia vs. during reperfusion is influenced by perfusate buffer composition. To achieve this, we used isolated rat hearts subjected to zero-flow global ischemia, with transient infusion of an Na^+/H^+ exchanger inhibitor into the coronary circulation *1*) immediately before ischemia, *2*) during the initial phase of reperfusion, or *3*) during both of these periods. To distinguish the effects of Na^+/H^+ exchanger inhibition from any secondary effects of amiloride analogues, we used two structurally distinct Na^+/H^+ exchanger inhibitors: *1*) 5-(*N,N*-dimethyl)amiloride (DMA), a potent inhibitor of the Na^+/H^+ exchanger (4, 12), which has been used previously in studies of myocardial ischemia and reperfusion (9, 16, 17), and *2*) (3-methylsulfonyl-4-piperidinobenzoyl)guanidine methanesulfonate (HOE-694), a recently developed agent that has an Na^+/H^+ exchanger inhibitory potency comparable to those of 5-amino-substituted amiloride derivatives (4, 28) but does not appear to share the cardiodepressant actions of such compounds (32).

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MATERIAL AND METHODS

This investigation was performed in accordance with the Home Office Guidance on the Operation of the Animals (Scientific Procedures) Act 1986 (Her Majesty's Stationery Office, London).

Langendorff Perfusion of Isolated Rat Hearts

Adult male Wistar rats (B & K Universal, Hull, UK) were anesthetized by inhalation of diethyl ether. Heparin (200 IU) was then injected into a femoral vein, and the heart was quickly excised and immersed in perfusion solution at 4°C. Within 30 s of excision, hearts were subjected to Langendorff perfusion at a constant perfusion pressure equivalent to 75 mmHg. The pulmonary artery was then cut near its origin to facilitate the drainage of coronary effluent. The left atrium was excised, and an ultrathin balloon specially constructed to match the dimensions of the ventricular cavity (1) was inserted into the left ventricle. The intraventricular balloon was inflated to give a left ventricular end-diastolic pressure (LVEDP) of 5–7 mmHg, and the balloon volume was kept constant thereafter. Two different perfusion solutions were used: one was buffered with CO_2-HCO_3^- (bicarbonate), and the other contained the zwitterionic buffer HEPES. The bicarbonate solution was of the following composition (in mmol/l): 118.5 NaCl, 4.8 KCl, 1.2 $MgSO_4$, 1.2 KH_2PO_4, 1.4 $CaCl_2$, 11.0 glucose, and 25.0 $NaHCO_3$. The solution was gassed continuously with 95% O_2-5% CO_2 (pH 7.4 at 37°C). HEPES solution contained (in mmol/l) 143.0 NaCl, 4.8 KCl, 1.2 $MgSO_4$, 1.2 KH_2PO_4, 1.4 $CaCl_2$, 11.0 glucose, and 6.0 HEPES. The pH was adjusted to 7.4 at 37°C by the addition of HCl, and the solution was gassed continuously with 100% O_2. Both solutions were filtered (pore size 5 µm) before use and maintained at 37°C throughout the experiment. Coronary flow was monitored using an in-line flow detector (model T206 Animal Research Flowmeter with 2N probe, Transonic Systems, New York, NY) with a linear detection range of 0.1–150 ml/min. After 15 min of perfusion at constant pressure, the basal coronary flow rate was recorded, and perfusion was switched to a constant-flow system (supplied by a Gilson Minipuls 3 roller pump) at the basal flow rate. When required (see later), this enabled the infusion of drug solutions or vehicle via a sidearm at a known percentage of the total flow rate. The heart was maintained in a temperature-regulated chamber at 37°C throughout the experiment, and the right atrium was continuously superfused with oxygenated perfusion solution (37°C) at 6 ml/min.

Drug Administration and Study Protocol

HOE-694 has limited long-term solubility in physiological buffer solutions. Therefore HOE-694 and DMA were dissolved in deionized water at 1.25, 12.5, or 125 µmol/l. When required, these solutions were infused into the perfusion line supplying the coronary vasculature at 8% of the total flow rate; this resulted in final perfusate drug concentrations of 0.1, 1, or 10 µmol/l, respectively. Control hearts received vehicle (deionized water) at the same infusion rate during the corresponding period. The concentrations of DMA and HOE-694 were selected in accordance with published data (15, 32).

At the end of the initial 15-min period of constant-pressure perfusion, basal cardiac function (see *Measured Variables*) was assessed and perfusion was switched to a constant-flow system, as described above. In the various study subsections (n = 8/group), drug or vehicle was infused into the perfusion line supplying the coronary vasculature for 5 min immediately preceding ischemia (pre-I), for the first 5 min of reperfusion (R), or during both of these periods (pre-I + R). All hearts were subjected to 20 min of zero-flow global ischemia and 45 min of reperfusion with constant-flow perfusion maintained throughout the reperfusion period. Reperfusion frequently resulted in the induction of episodes of ventricular tachyarrhythmias; however, all hearts reverted to a stable sinus rhythm within 35 min of reperfusion. Postischemic cardiac function was assessed at the end of the 45-min period of reperfusion, by which time each heart had been in stable sinus rhythm for an interrupted period of ≥10 min. All experiments in each study subsection were carried out in a prospectively randomized manner with contemporary controls.

Measured Variables

Left ventricular developed pressure (LVDP) and LVEDP were obtained from a pressure transducer attached to the intraventricular balloon through a fluid-filled catheter. Coronary vascular resistance was derived from the coronary flow rate monitored via the in-line flow detector and the perfusion pressure (monitored through a sidearm of the aortic cannula). A unipolar electrogram (electrocardiogram) was obtained through a silver electrode inserted into the free wall of the left ventricle and a reference electrode connected to the aorta, from which heart rate was derived. The electrocardiogram, left ventricular pressure (LVDP and LVEDP), perfusion pressure, and coronary flow rate were recorded on an ink-jet recorder (model RS3400, Gould). Values for LVDP, LVEDP, coronary vascular resistance, and heart rate were noted 1 min before the start of vehicle or drug infusion (to obtain basal values), at 1-min intervals during vehicle or drug infusion, and at the end of 45 min of reperfusion (after ≥10 min of stable sinus rhythm; see *Drug Administration and Study Protocol*).

Expression of Results and Statistical Analysis

The recovery of LVDP after reperfusion was expressed as a percentage of the preischemic basal value (obtained before the start of drug or vehicle infusion) in the same heart. Statistical analyses were based on the guidelines described by Wallenstein et al. (31). Gaussian-distributed variables were expressed as means ± SE and were subjected to one-way analysis of variance. If a difference among mean values was established, comparison with controls was performed using Dunnett's test. $P < 0.05$ was considered significant.

RESULTS

Effects of DMA and HOE-694 on Preischemic Cardiac Function

The temporal changes in functional indexes that occurred during the preischemic infusion of vehicle, DMA (10 µmol/l), and HOE-694 (10 µmol/l) were assessed using data from all hearts that received such infusions (i.e., pre-I and pre-I + R protocols, n = 16/group).

Bicarbonate buffer. Basal values for LVDP, LVEDP, coronary vascular resistance, and heart rate in the various study groups are shown in Table 1. These values did not differ significantly between control, DMA, and HOE-694 groups within each study subsection. There were no significant changes in LVDP, LVEDP, coronary vascular resistance, and heart rate in control hearts during the preischemic infusion of vehicle. The infusion of DMA or HOE-694 also did not affect these indexes, such that there were no significant

Table 1. *Basal values for functional indexes*

	LVDP, mmHg	LVEDP, mmHg	Coronary Vascular Resistance, mmHg·ml⁻¹·min·g	Heart Rate, beats/min
Bicarbonate buffer				
Pre-I				
Control	125 ± 6	6.2 ± 0.7	8.4 ± 0.5	341 ± 16
DMA	122 ± 7	6.2 ± 0.7	8.3 ± 0.4	360 ± 13
HOE-694	117 ± 6	5.5 ± 0.5	8.5 ± 0.9	345 ± 20
R				
Control	114 ± 8	5.9 ± 0.4	5.5 ± 0.4	359 ± 14
DMA	118 ± 6	4.8 ± 0.3	4.8 ± 0.2	353 ± 18
HOE-694	101 ± 4	5.1 ± 0.6	5.8 ± 0.3	326 ± 16
Pre-I + R				
Control	132 ± 9	5.2 ± 1.0	4.5 ± 0.2	333 ± 17
DMA	124 ± 5	5.4 ± 0.8	4.8 ± 0.5	356 ± 16
HOE-694	134 ± 3	6.4 ± 0.6	4.4 ± 0.3	358 ± 12
HEPES buffer				
Pre-I				
Control	117 ± 4	5.7 ± 0.6	13.0 ± 1.2	276 ± 8
DMA	112 ± 4	4.3 ± 0.6	9.0 ± 0.7*	261 ± 5
HOE-694	121 ± 7	4.6 ± 0.7	10.0 ± 1.0	272 ± 6
R				
Control	106 ± 7	6.2 ± 0.6	11.9 ± 0.8	296 ± 8
DMA	93 ± 5	6.4 ± 1.1	12.8 ± 0.7	278 ± 9
HOE-694	89 ± 3	6.9 ± 0.4	13.0 ± 0.4	285 ± 7
Pre-I + R				
Control	95 ± 6	7.0 ± 0.9	14.6 ± 1.6	285 ± 5
DMA	97 ± 5	6.0 ± 0.8	13.1 ± 1.0	273 ± 11
HOE-694	94 ± 2	6.8 ± 0.8	13.2 ± 1.0	259 ± 4

Values are means ± SE. n = 8/group. DMA, 5-(*N*,*N*-dimethyl)amiloride; HOE-694, (3-methylsulfonyl-4-piperidinobenzoyl)guanidine methanesulfonate; Pre-I, drug/vehicle administration before ischemia; R, drug/vehicle administration during early reperfusion; Pre-I + R, drug/vehicle administration before ischemia plus during early reperfusion. Drug concentration was 10 µmol/l in all cases. All values were obtained before drug administration. *P < 0.05 vs. relevant control receiving vehicle.

differences between hearts receiving DMA or HOE-694 and controls at any time during this preischemic infusion period. The relevant data for coronary vascular resistance and heart rate are illustrated in Figs. 1*A* and 2*A*, respectively.

HEPES buffer. Basal values for functional indexes in hearts receiving perfusate containing HEPES buffer are also shown in Table 1. Once again, these values did not vary significantly between control, DMA, and HOE-694 groups within each study subsection for the majority of indexes. The only exception was the significantly lower basal coronary vascular resistance observed in the group that received DMA before ischemia. However, because the study was carried out in a prospectively randomized manner, this difference (which was observed before any drug infusion) probably occurred by chance. Once again, the preischemic infusion of vehicle did not result in a significant change in LVDP, LVEDP, coronary vascular resistance, and heart rate. During infusion of DMA or HOE-694, LVDP and LVEDP did not change significantly, and there were no significant differences from the control group at any time point during this period. However, in contrast to the observations made with bicarbonate buffer, the infusion of DMA or HOE-694 in the presence of HEPES buffer resulted in a progressive increase in coronary vascular resistance (Fig. 1*B*) and a corresponding decrease in heart rate (Fig. 2*B*). In the DMA and HOE-694 groups, coronary vascular resistance was significantly greater from 3 min (Fig. 1*B*) and heart rate was significantly lower from 1 min (Fig. 2*B*) after the onset of drug infusion than time-matched values from control hearts receiving vehicle.

Effects of DMA and HOE-694 on Postischemic Cardiac Function

Bicarbonate buffer. Postischemic recovery of LVDP in the various study groups is illustrated in Fig. 3*A*. When

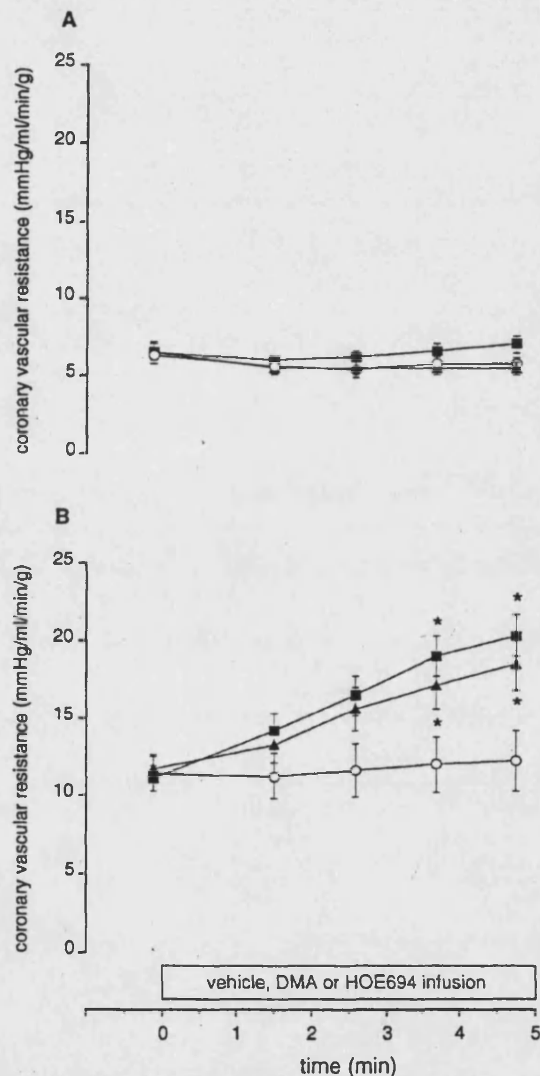

Fig. 1. Coronary vascular resistance during infusion of vehicle (○), 5-(*N*,*N*-dimethyl)amiloride (DMA, 10 µmol/l; ■), or (3-methylsulfonyl-4-piperidinobenzoyl)guanidine methanesulfonate (HOE-694, 10 µmol/l; ▲) before ischemia (n = 16/group). Experiments were carried out using bicarbonate (*A*) or HEPES (*B*) as buffer in perfusion solution. Values are means ± SE. *P < 0.05 vs. vehicle.

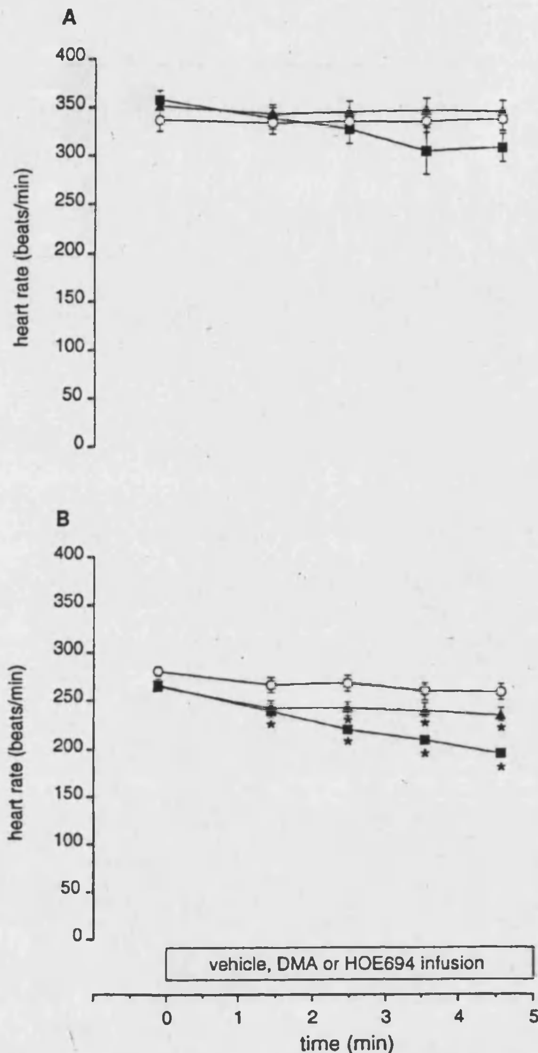

Fig. 2. Heart rate during infusion of vehicle (○), DMA (10 μmol/l; ■), or HOE-694 (10 μmol/l; ▲) before ischemia (n = 16/group). Experiments were carried out using bicarbonate (*A*) or HEPES (*B*) as buffer in perfusion solution. Values are means ± SE. $*P < 0.05$ vs. vehicle.

given before ischemia or before ischemia plus during early reperfusion, DMA (10 μmol/l) did not improve the recovery of LVDP. In contrast, HOE-694 (10 μmol/l) significantly improved the recovery of LVDP when infused before ischemia (from 39 ± 5% in controls to 66 ± 6%) and when given before ischemia plus during early reperfusion (from 33 ± 4% in controls to 65 ± 4%). When given during reperfusion alone, neither DMA nor HOE-694 improved the postischemic recovery for LVDP (Fig. 3*A*). The lack of effect on postischemic recovery was evident throughout the period of reperfusion (Fig. 4*A*).

Postischemic values of LVEDP, coronary vascular resistance, and heart rate in the various study groups are given in Table 2. When given before ischemia, DMA and HOE-694 resulted in a significant reduction in postischemic LVEDP, indicating an improved diastolic function. With HOE-694, this improvement of postischemic diastolic function was observed also when the drug was given before ischemia plus during reperfusion; however, this infusion protocol resulted in a loss of efficacy with DMA. Neither drug affected postischemic LVEDP significantly when given during reperfusion alone. Postischemic coronary vascular resistance and heart rate were not affected significantly by either drug, regardless of the infusion protocol. With all treatment protocols, 50–100% of hearts in each group exhibited ventricular tachyarrhythmias during reperfu-

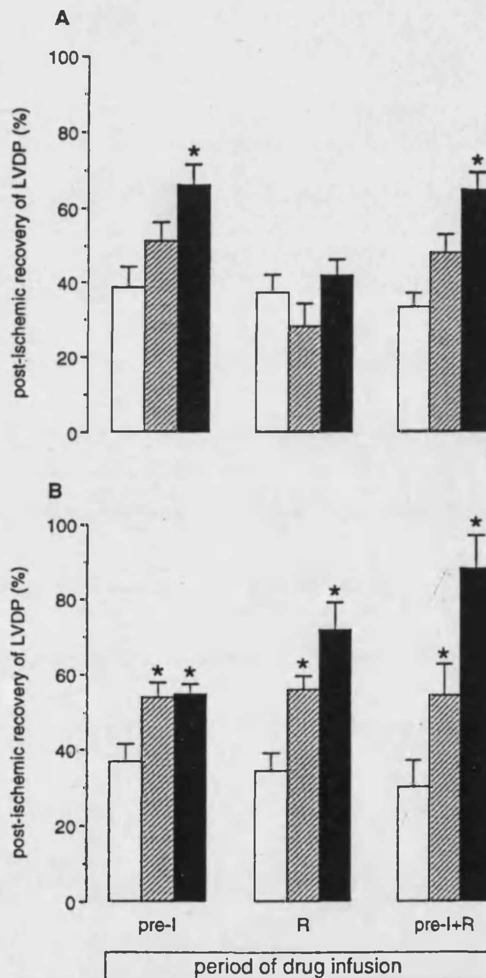

Fig. 3. Recovery of left ventricular developed pressure (LVDP) at end of 45 min of reperfusion expressed as percentage of preischemic basal value (n = 8/group). Hearts were perfused with vehicle (open bars), DMA (10 μmol/l, hatched bars), and HOE-694 (10 μmol/l, filled bars) before ischemia (pre-I), during reperfusion (R), and before ischemia plus during reperfusion (pre-I + R). Experiments were carried out using bicarbonate (*A*) or HEPES (*B*) as buffer in perfusion solution. Values are means ± SE. $*P < 0.05$ vs. vehicle.

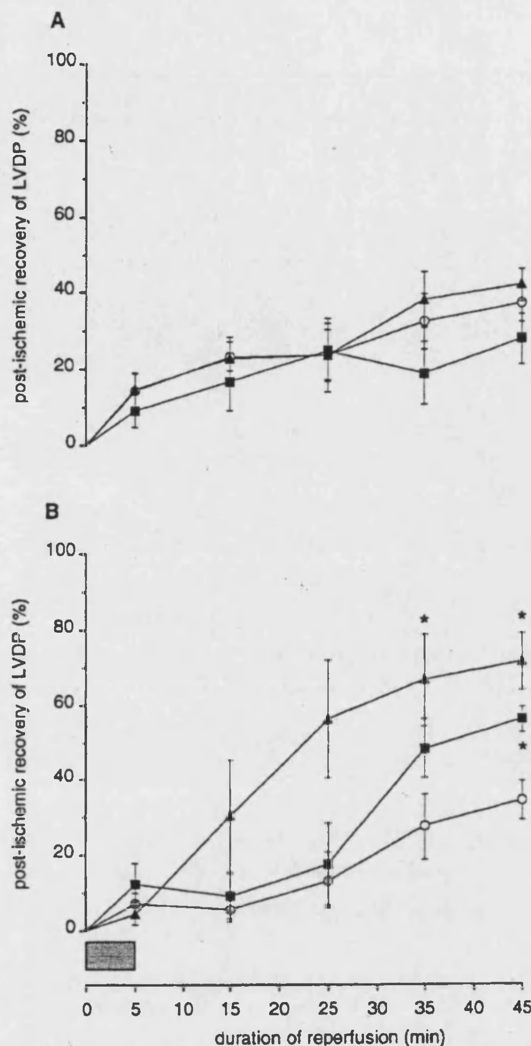

Fig. 4. Recovery of LVDP during 45 min of reperfusion expressed as percentage of preischemic basal value (n = 8/group). Hearts received vehicle (○), DMA (10 μmol/l; ■), or HOE-694 (10 μmol/l; ▲) during first 5 min of reperfusion, as indicated by shaded box above horizontal axis. Experiments were carried out using bicarbonate (A) or HEPES (B) as buffer in perfusion solution. Values are means ± SE, with recovery assumed as 0 in hearts that were in sustained tachyarrhythmia at any of data collection time points. *$P < 0.05$ vs. vehicle.

sion, indicating a lack of effect of DMA and HOE-694 on the incidence of such arrhythmias in this model.

HEPES buffer. In contrast to our observations with bicarbonate buffer, in the presence of HEPES buffer DMA (10 μmol/l) and HOE-694 (10 μmol/l) significantly improved the postischemic recovery of LVDP in all drug infusion protocols (Fig. 3B). When the drugs were given before ischemia, the postischemic recoveries of LVDP in control, DMA, and HOE-694 groups were 37 ± 5%, 54 ± 4% ($P < 0.05$), and 54 ± 3% ($P < 0.05$), respectively. When given before ischemia plus during early reperfusion, DMA improved the postischemic recovery of LVDP from 30 ± 7% in controls to 54 ± 8% ($P < 0.05$); with this drug infusion protocol, HOE-694 resulted in almost complete restoration of preischemic systolic function, with the postischemic recovery of LVDP reaching 88 ± 9% ($P < 0.05$). When the drugs were given during early reperfusion alone, the postischemic recoveries of LVDP were 34 ± 5% in controls, 56 ± 3% with DMA ($P < 0.05$), and 71 ± 8% with HOE-694 ($P < 0.05$). In addition to improving the ultimate recovery of LVDP, HOE-694 also accelerated the recovery of LVDP during the reperfusion period (Fig. 4B).

As shown in Table 2, DMA and HOE-694 did not significantly affect postischemic LVEDP, coronary vascular resistance, and heart rate in most cases. Indeed, the only significant differences from control were observed with DMA infusion before ischemia, which resulted in a lower postischemic coronary vascular resistance (which was probably a reflection of the lower basal coronary vascular resistance in this group) and a small reduction in postischemic heart rate. The incidence of tachyarrhythmias during reperfusion was not affected by either drug with any treatment protocol, with 50–100% of hearts in each group exhibiting such arrhythmias.

Dose-Response Relationships for DMA and HOE-694

To determine whether the reduced cardioprotective efficacy of HOE-694 and the loss of efficacy of DMA in

Table 2. *Postischemic values for functional indexes*

	LVEDP, mmHg	Coronary Vascular Resistance, mmHg·ml⁻¹·min·g	Heart Rate, beats/min
Bicarbonate buffer			
Pre-I			
Control	71 ± 5	9.4 ± 0.4	319 ± 30
DMA	58 ± 4*	9.5 ± 0.6	326 ± 16
HOE-694	49 ± 4*	9.3 ± 0.7	335 ± 28
R			
Control	69 ± 5	6.9 ± 0.3	329 ± 12
DMA	82 ± 8	6.6 ± 0.5	324 ± 23
HOE-694	67 ± 3	6.9 ± 0.3	274 ± 20
Pre-I + R			
Control	86 ± 7	6.9 ± 0.5	348 ± 18
DMA	82 ± 10	7.4 ± 0.6	320 ± 19
HOE-694	55 ± 4*	5.8 ± 0.2	333 ± 6
HEPES buffer			
Pre-I			
Control	60 ± 5	15.3 ± 2.7	313 ± 8
DMA	48 ± 3	8.9 ± 0.5*	263 ± 20*
HOE-694	50 ± 4	11.4 ± 0.8	293 ± 9
R			
Control	79 ± 4	14.6 ± 0.9	305 ± 16
DMA	71 ± 4	13.2 ± 1.3	285 ± 11
HOE-694	66 ± 5	13.0 ± 0.8	298 ± 17
Pre-I + R			
Control	62 ± 4	20.1 ± 5.1	310 ± 10
DMA	56 ± 7	14.8 ± 2.6	290 ± 19
HOE-694	47 ± 8	13.4 ± 1.6	256 ± 14

Values are means ± SE; n = 8/group. Drug concentration was 10 μmol/l in all cases. All values were obtained at end of 45-min period of reperfusion. See Table 1 footnote for definition of abbreviations. *$P <$ 0.05 vs. relevant control receiving vehicle.

the presence of bicarbonate buffer could have been due to the use of excessive drug concentrations (which might produce unfavorable secondary effects), a dose-response study was carried out utilizing two additional lower concentrations of each drug. Figure 5 illustrates the postischemic recovery of LVDP in hearts that received vehicle and those that received DMA or HOE-694 at three concentrations (0.1, 1, or 10 μmol/l) before ischemia and during early reperfusion. HOE-694 increased the postischemic recovery of LVDP in a dose-dependent manner from 33 ± 4% in control hearts to 40 ± 7%, 48 ± 5% ($P < 0.05$), and 65 ± 4% ($P < 0.05$) at 0.1, 1, and 10 μmol/l, respectively. DMA did not significantly affect the ischemic recovery of LVDP at any concentration.

DISCUSSION

Our results suggest that, in the isolated rat heart, the relative degree of protection afforded by Na^+/H^+ exchanger inhibition during ischemia vs. during reperfusion is influenced significantly by the perfusate buffer composition. Furthermore, DMA and HOE-694 appear to exhibit differential cardioprotective efficacy, despite their comparable potency as inhibitors of NHE-1 (4), which is the predominant Na^+/H^+ exchanger isoform expressed in the rat heart (21).

Differential Efficacy of DMA and HOE-694

In the presence of bicarbonate, only HOE-694 improved the postischemic recovery of systolic function, and only when present during ischemia. This may indicate that under these conditions HOE-694 exhibits distinct cardioprotective properties (in addition to Na^+/H^+ exchanger inhibition) that are not shared by DMA. Alternatively, this observation may reflect the greater selectivity of HOE-694 for the Na^+/H^+ exchanger, particularly because this agent has been shown not to have a cardiodepressant effect in the rat (32), unlike amiloride and several of its 5-amino-substituted derivatives (23, 32). In other words, under these experimental conditions, cardiodepressant effects arising from secondary pharmacological properties of DMA may partially counteract the beneficial effects of Na^+/H^+ exchanger inhibition by this agent. The absence of a significant cardiodepressant effect during the preischemic infusion of DMA in the present study may at first appear contrary to this hypothesis. However, it should be noted that the negative effects of DMA on the contractile function of rat myocardium are much more pronounced after prolonged exposure to the drug (23). Therefore, in the present study, cardiodepressant effects of DMA may only become manifest during reperfusion, after prolonged exposure to the drug during its entrapment within the ischemic myocardium. In this regard, even under aerobic conditions, exposure of rat right ventricular myocardium to 20 μM DMA for 30 min (equivalent to the total duration of drug infusion plus global ischemia in the present study) has been shown to result in ~70% suppression of developed tension, with only incomplete recovery after washout of the drug (23). In the presence of bicarbonate, neither drug exhibited a protective effect when given only during reperfusion. This implies that, under such conditions, Na^+/H^+ exchanger activity during reperfusion may not play a significant role in determining the extent of postischemic functional recovery. Indeed, it is noteworthy that the majority of studies in which the administration of Na^+/H^+ exchanger inhibitors only during reperfusion has been reported to dramatically improve postischemic contractile function have been carried out in the absence of bicarbonate (14–17). Consistent with our observation, other studies have shown that the infusion of Na^+/H^+ exchanger inhibitors only during reperfusion in the presence of bicarbonate is ineffective (10, 18, 20) or provides only minimal protection (8, 29).

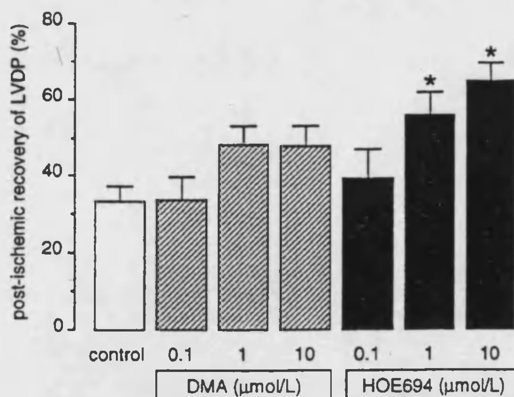

Fig. 5. Recovery of LVDP at end of 45 min of reperfusion expressed as percentage of preischemic basal value. Hearts (n = 8/group) received vehicle (open bar), DMA (0.1, 1, or 10 μmol/l; hatched bars), or HOE-694 (0.1, 1, or 10 μmol/l; filled bars) before ischemia plus during reperfusion. All experiments were carried out using bicarbonate as buffer in perfusion solution. Values are means ± SE. *$P < 0.05$ vs. vehicle.

In the presence of HEPES buffer, DMA and HOE-694 significantly improved the postischemic recovery of systolic function, not only when present during ischemia but also when given only during reperfusion. This observation is consistent with earlier studies carried out with Na^+/H^+ exchanger inhibitors in the absence of bicarbonate (14–17) and suggests that, under such conditions, exchanger activity during reperfusion may be of greater significance in determining the severity of postischemic contractile dysfunction. Similar to the observations made in the presence of bicarbonate, HOE-694 exhibited a greater cardioprotective efficacy than DMA, once again probably due to the greater selectivity of this agent for the Na^+/H^+ exchanger. Indeed, when HOE-694 was given before ischemia plus during reperfusion in the presence of HEPES buffer, an additive effect was observed such that by the end of the reperfusion period systolic function had recovered almost completely to its preischemic level. Nevertheless it should be stressed that under these conditions DMA also provided significant protection with all drug admin-

istration protocols. Thus, in bicarbonate-free conditions, the beneficial effects of Na^+/H^+ exchanger inhibition may outweigh any cardiodepressant effects arising from nonselective actions of DMA.

In this study, neither DMA nor HOE-694 affected the incidence of reperfusion-induced tachyarrhythmias, regardless of the perfusate buffer composition. This may at first appear contrary to our earlier observations (32) with HOE-694 and 5-(*N*-ethyl-*N*-isopropyl)amiloride (EIPA), another 5-amino-substituted derivative of amiloride. However, the present study used 20 min of global ischemia vs. 10 min of regional ischemia in our earlier study (32). Because reperfusion-induced arrhythmias are believed to originate within the ischemic/reperfused zone and are modulated significantly by the size of this zone (5), it is probable that the lack of effect of DMA and HOE-694 on reperfusion arrhythmias in the present study was a reflection of the greater arrhythmogenic insult presented by 20 min of global ischemia.

Impact of Perfusate Buffer Composition

The above observations suggest that the influence of Na^+/H^+ exchanger activity during reperfusion on the extent of postischemic functional recovery is significantly modulated by perfusate buffer composition. As a consequence, the cardioprotective efficacy of Na^+/H^+ exchanger inhibitors may be overestimated when used under bicarbonate-free conditions. In isolated myocytes (13) and whole hearts (7), recovery from intracellular acidosis in the presence of bicarbonate and under aerobic conditions has been shown to occur through not only the Na^+/H^+ exchanger but also the Na^+-HCO_3^- symporter, with the exchanger becoming the predominant pH recovery mechanism only in the absence of bicarbonate. In a similar manner, during reperfusion in the presence of bicarbonate, the Na^+/H^+ exchanger and the Na^+-HCO_3^- symporter appear to play comparable roles in intracellular pH recovery, and both may contribute significantly to a Na^+ influx (30). Despite this possibility, however, there is a paucity of studies investigating the effects of inhibition of the Na^+-HCO_3^- symporter on postischemic recovery of contractile function and intracellular Na^+ and Ca^{2+} homeostasis, probably due to a lack of selective inhibitors (30). If the Na^+-HCO_3^- symporter also plays a significant role in ischemia and reperfusion-induced loss of ionic homeostasis and contractile dysfunction, then inhibition of the Na^+/H^+ exchanger would be expected to result in only partial protection in the presence of bicarbonate. Conversely, when this mechanism is inhibited through the removal of bicarbonate from the perfusion medium, the Na^+/H^+ exchanger could assume a more significant role in mediating such dysfunction. Consequently, the cardioprotective efficacy of Na^+/H^+ exchanger inhibitors would be enhanced, particularly during reperfusion. This hypothesis is consistent with the observations made in the present study and needs to be tested further by studies designed specifically to determine the role of bicarbonate-dependent mechanisms in myocardial ischemia and reperfusion.

Effects of DMA and HOE-694 on Preischemic Cardiac Function

In the presence of bicarbonate, neither drug had a significant effect on contractile function, coronary vascular resistance, or heart rate. In contrast, in the presence of HEPES buffer, DMA and HOE-694 produced a reduction in heart rate and an increase in coronary vascular resistance, although contractile function was once again unaffected. Significant coronary vasoconstrictive and negative chronotropic effects in the presence of bicarbonate have been reported previously with prolonged infusion of EIPA in isolated hearts from the guinea pig (22) and the rat (32). However, in the absence of similar effects with HOE-694 under identical conditions, such actions of EIPA have been attributed to nonselective effects of the drug (32). In contrast, the similarity of the effects of DMA and HOE-694 on heart rate and coronary vascular resistance during their short-term infusion in the presence of HEPES buffer in the present study suggests that these effects may have been mediated through the common mechanism of action of these agents, that is, Na^+/H^+ exchanger inhibition. The cellular mechanisms downstream of exchanger inhibition cannot be ascertained from the present study, although they may involve alterations in intracellular pH in the relevant cell types (i.e., sinoatrial node and coronary vascular smooth muscle cells) under these conditions. Nevertheless, with regard to the therapeutic potential of Na^+/H^+ exchanger inhibitors, it is important to stress that such vasoconstrictive and negative chronotropic effects are not seen with Na^+/H^+ exchanger inhibitory concentrations of 5-(*N*-methyl-*N*-isobutyl)-amiloride (M. Karmazyn, personal communication) or with newer agents such as HOE-694 (32), under physiological conditions where bicarbonate-dependent pH regulatory mechanisms remain operative.

Although the present study was not designed specifically to compare cardiac function in the presence and absence of bicarbonate, in hearts perfused with solution containing HEPES basal values for LVDP and heart rate were lower and those for coronary vascular resistance were higher. It is unclear, however, whether these differences were due to the absence of bicarbonate or the presence of HEPES, particularly because there is some evidence (2) that HEPES may influence electrical and mechanical activity in ventricular muscle independently of its function as a buffer.

Therapeutic Potential of Na^+/H^+ Exchanger Inhibitors

Our results have shown that the cardioprotective efficacy of Na^+/H^+ exchanger inhibition during reperfusion may be overestimated in in vitro studies employing bicarbonate-free conditions. Nevertheless the present study and many previous studies have shown that Na^+/H^+ exchanger inhibitors can provide significant cardioprotection in isolated rat hearts, even in the presence of bicarbonate, particularly when given as pretreatment before the onset of ischemia. Such protec-

tion has been observed with amiloride (10, 29), with a variety of its 5-amino-substituted analogues (3, 17, 18, 29), and with novel inhibitors such as HOE-694 (28) and its structural congener HOE-642 (27). Indeed, Na^+/H^+ exchanger inhibitors have also been shown to be cardioprotective (with this effect manifest as improved postischemic contractile function or reduced cell necrosis) in isolated hearts from other species [e.g., guinea pig (18) and rabbit (20)] perfused with aqueous media, as well as in blood-perfused preparations, including rabbit myocardium in vitro (8, 9) and porcine hearts in vivo (25). This unusual degree of conformity among different investigators, drugs, species, and models suggests that Na^+/H^+ exchanger inhibitors may possess genuine cardioprotective potential in a variety of settings associated with ischemia and reperfusion.

Concluding Comments

The present study has demonstrated that, in isolated rat hearts perfused with an aqueous medium and subjected to global ischemia and reperfusion, the buffer composition of the medium has a significant impact on the cardioprotective efficacy of two structurally distinct inhibitors of the Na^+/H^+ exchanger. This phenomenon may be mediated by the inactivation of bicarbonate-dependent pH regulatory mechanisms (such as the Na^+-HCO_3^- symporter) in the presence of bicarbonate-free medium, such that the Na^+/H^+ exchanger becomes the predominant mechanism of H^+ extrusion linked to Na^+ influx. As a result, the cardioprotective efficacy of Na^+/H^+ exchanger inhibitors, particularly during reperfusion, may be overestimated under bicarbonate-free conditions, although these agents clearly retain significant efficacy, even under more physiological conditions.

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Na^+/H^+ Exchanger Activity Does Not Contribute to Protection by Ischemic Preconditioning in the Isolated Rat Heart

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Background Despite evidence that pharmacological inhibition of the Na^+/H^+ exchanger (NHE) is cardioprotective, activation of NHE has been proposed as a protective mechanism of ischemic preconditioning (PC).

Methods and Results In isolated rat ventricular myocytes (n=8 to 11 per group) loaded with the fluorescent pH indicator C-SNARF-1, we showed that HOE-642 (HOE) was a potent inhibitor of the sarcolemmal NHE (80% inhibition at 1 μmol/L); such inhibition was readily reversible by washout of the drug. We confirmed that 1 μmol/L HOE produces significant and reversible inhibition of NHE activity in isolated rat hearts as well (n=4), and in this model, we tested (n=8 per group) whether the presence of the drug during (1) the prolonged period of ischemia (40 or 60 minutes) or (2) the preceding brief periods of PC ischemia (3 minutes plus 5 minutes) modulates the protective efficacy of PC. In protocol 1, HOE was infused for 5 minutes immediately before the prolonged ischemic period. With 40 minutes of prolonged ischemia, the postischemic recovery of left ventricular developed pressure (LVDP) was 15±2% in controls and was improved to 45±7% with HOE ($P<.05$), 55±5% with PC ($P<.05$), and 68±2% with PC+HOE ($P<.05$ versus all groups). When the prolonged ischemic period was extended to 60 minutes, an additive effect of PC and HOE was readily apparent and LVDP recovery with PC+HOE (66±2%) was almost double that observed with HOE (37±4%) or PC (34±5%) alone ($P<.05$). In protocol 2, HOE was infused for 3 minutes immediately before each episode of PC ischemia and was subsequently washed out before a 40-minute prolonged ischemic period (HOE+PC). LVDP recovery was 34±4% in controls and was improved to 57±2% with PC ($P<.05$) and 55±3% with HOE+PC ($P<.05$). Improved recovery of LVDP was matched by reduced creatine kinase leakage in all cases.

Conclusions Because coadministration of HOE (at a concentration sufficient to inhibit NHE activity) did not reduce the efficacy of PC in either protocol, we conclude that NHE activity does not contribute to the cardioprotective actions of PC. On the contrary, NHE inhibition during the prolonged ischemic period may enhance the protection afforded by PC. (*Circulation*. 1997;96:3617-3625.)

Key Words • ischemia • sodium • myocytes

The remarkable cardioprotective efficacy of ischemic preconditioning has stimulated an intense investigative effort aimed at delineating its cellular mechanisms (for a recent review, see Downey and Cohen[1]). One noteworthy consequence of ischemic preconditioning, which has been reported by several independent investigators,[2-6] is a significant reduction in the severity of intracellular acidosis during the prolonged period of ischemia. This "antiacidotic" effect has received considerable attention as a potential mechanism mediating the cardioprotective actions of ischemic preconditioning.[7-10] However, despite the general agreement that ischemic preconditioning attenuates intracellular acidosis during subsequent ischemia, there is no consensus regarding the mechanism that underlies this effect. Recently, Ramasamy and colleagues[8] proposed that the sarcolemmal Na^+/H^+ exchanger (NHE), which is a primary route for H^+ efflux from cardiac myocytes,[11,12] may be stimulated after ischemic preconditioning and that this may contribute to both the attenuation of intracellular acidosis and the amelioration of ischemia/reperfusion-induced injury in preconditioned hearts.[8]

On the basis of available evidence, it is reasonable to suggest that ischemic preconditioning may result in a greater activity of the sarcolemmal NHE and that this may contribute to a reduced severity of intracellular acidosis during the prolonged ischemic period. However, the proposal[8] that such stimulation of NHE may be a cardioprotective mechanism appears contrary to the substantial body of evidence suggesting that in the setting of myocardial ischemia and reperfusion, pharmacological inhibition of NHE is protective, whereas pharmacological activation of NHE is detrimental (for recent reviews, see References 12 through 15). An alternative possibility is that activation of NHE is an epiphenomenon that accompanies ischemic preconditioning but is not causally involved in its cardioprotective actions. On the contrary, it is possible that any activation of NHE may limit the extent of the cardioprotection afforded by preconditioning.

In light of the above, the primary objective of the present study was to determine whether NHE activity during either (1) the prolonged ischemic period or (2) the brief preconditioning ischemic periods contributes to the cardioprotective effect of ischemic preconditioning.

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Selected Abbreviations and Acronyms

C-SNARF-1 = carboxy-seminaphthorhodafluor-1
LVDP = left ventricular developed pressure
LVEDP = left ventricular end-diastolic pressure
LVSP = left ventricular systolic pressure
NHE = Na^+/H^+ exchanger
PC = preconditioning
pH_i = intracellular pH

To attain this objective, we first determined the effects of HOE-642, a novel benzoyl guanidine–based NHE inhibitor,[16] on sarcolemmal NHE activity in rat ventricular myocytes. Subsequently, we subjected isolated rat hearts to an ischemic preconditioning protocol (which we have previously shown[6,17,18] to provide significant cardioprotection) in conjunction with the administration of HOE-642 at a concentration sufficient to inhibit sarcolemmal NHE activity.

Methods

Animals

Adult male Wistar rats (200 to 300 g body weight; B&K Universal Ltd, Hull, UK) were used in all studies. The animals were anesthetized with sodium pentobarbital (60 mg/kg IP) and systemically anticoagulated with heparin (300 IU IV). After a transverse thoracotomy, the heart was excised and immediately immersed in perfusion solution at 4°C for subsequent use in isolated myocyte or whole-heart studies. The investigation was conducted in accordance with the Home Office Guidance on the Operation of the Animals (Scientific Procedures) Act 1986, published by Her Majesty's Stationery Office, London, UK.

Effects of HOE-642 on NHE Activity in Isolated Myocytes

Myocyte Isolation

Ventricular myocytes were isolated by a collagenase-based enzymatic digestion technique, as we have described previously.[19] In brief, hearts were retrogradely perfused (37°C) in the Langendorff mode at a constant flow rate of 10 $mL \cdot min^{-1} \cdot g^{-1}$ for four sequential periods, as follows: (1) with Tyrode's solution (containing, in mmol/L, NaCl 137, KCl 5.4, $CaCl_2$ 1.8, $MgCl_2$ 0.5, HEPES 10, and glucose 10, adjusted to pH 7.4 at 34°C with NaOH) for 5 minutes, (2) with nominally Ca^{2+}-free Tyrode's solution (NaCl 135, KCl 5.4, NaH_2PO_4 0.33, $MgCl_2$ 1.0, HEPES 10, and glucose 10, adjusted to pH 7.2 at 34°C with NaOH) for 5.5 minutes, (3) with nominally Ca^{2+}-free Tyrode's solution containing collagenase (Worthington type 1, 100 U/mL) for 10 minutes, and (4) with storage buffer (KOH 78, KCl 30, KH_2PO_4 30, $MgSO_4$ 3, EGTA 0.5, HEPES 10, glutamic acid 50, taurine 20, and glucose 10, adjusted to pH 7.2 at 34°C with KOH) for 5 minutes. All solutions were gassed with 100% O_2. After the perfusion procedure, the ventricles were removed and chopped into several pieces in storage buffer. The tissue fragments were then gently agitated to facilitate cell dispersion, and the cell suspension was maintained in storage buffer at 25°C for at least 1 hour before use in the microepifluorescence studies.

Measurement of pH_i and NHE Activity

pH_i was measured in single ventricular myocytes with the pH-sensitive fluorescent dye C-SNARF-1, as we have described previously.[19] Cells loaded with C-SNARF-1 were allowed to settle on a glass coverslip at the bottom of a chamber mounted on the stage of an inverted microscope (Nikon Diaphot) and were superfused (3.5 mL/min, 34°C) with Tyrode's solution. Cells were excited with light at 540 nm, and the resulting fluorescence emission intensity from a selected area of a single myocyte was measured simultaneously at 580 nm (I_{580}) and 640 nm (I_{640}) with a dual-emission photometer system (model D104C, Photon Technology International). The emission intensity ratio (I_{580}/I_{640}) was calculated and converted to a pH_i scale by use of in situ calibration data obtained by exposing cells loaded with C-SNARF-1 to nigericin-containing calibration solutions.[19]

All experiments were carried out in the nominal absence of HCO_3^- (thereby precluding an involvement of HCO_3^--dependent pH_i-regulatory mechanisms), such that the rate of acid efflux (J_H) could be used as a direct index of sarcolemmal NHE activity.[19] J_H was estimated during recovery from acute intracellular acidosis from the equation $J_H=\beta_i \cdot dpH_i/dt$, where β_i is the intrinsic buffering power and dpH_i/dt is the rate of recovery of pH_i.

Experimental Protocol

The main objective of these studies was to determine the inhibitory efficacy of HOE-642 on sarcolemmal NHE activity. To this end, intracellular acidosis was induced (to activate sarcolemmal NHE) in the cells by the washout of NH_4Cl (20 mmol/L) after its transient (3 minutes) application. The initial (1 minute) washout of NH_4Cl was with Na^+-free Tyrode's solution (NaCl replaced by choline chloride) to ensure NHE inactivity during H^+ loading. Subsequently, NHE was reactivated by the reintroduction of Na^+-containing Tyrode's solution in the absence or presence of various concentrations of HOE-642 (0.0001 to 1 μmol/L; n=8 to 11 cells per group). J_H was calculated from the initial dpH_i/dt value (obtained by linear regression analysis of pH_i data collected during the first 1 minute after the reintroduction of Na^+) and the β_i value corresponding to the appropriate pH_i (estimated from the equation $\beta_i=-34.9 \cdot pH_i+273.5$).[19]

In additional experiments, the reversibility of sarcolemmal NHE inhibition by HOE-642 was studied. Myocytes (n=3) were subjected to two consecutive acid pulses by the NH_4Cl washout method, separated by 15 minutes of normal superfusion. During both pulses, NH_4Cl washout was with normal Tyrode's solution; however, during the second pulse the initial (3 minutes) washout solution additionally contained 1 μmol/L HOE-642.

Effects of HOE-642 on NHE Activity in Whole Hearts

Isolated Heart Perfusion

Hearts were retrogradely perfused in the Langendorff mode at a constant coronary flow rate of 12 mL/min via a roller pump (Gilson Minipuls 3). The nominally HCO_3^--free perfusion solution was of the following composition (in mmol/L): NaCl 143.5, KCl 4.7, $MgSO_4$ 1.2, KH_2PO_4 1.2, HEPES 20.0, $CaCl_2$ 1.4, and glucose 11.0 (adjusted to pH 7.4 at 37°C with NaOH, maintained at 37°C, and gassed continuously with 100% O_2). The solution contained, in addition, 20 mmol/L NH_4Cl when required. The pulmonary artery was incised to facilitate coronary effluent drainage. Left ventricular pressure was monitored by means of an intraventricular, isovolumic balloon[20] connected to a pressure transducer and was recorded with an ink-jet recorder. The right atrium was excised, and hearts were paced at 360 bpm throughout via an electrode attached to the right ventricle to avoid potential complications in data interpretation arising from pH-induced changes in sinus rate and/or atrioventricular conduction. HOE-642 (a gift from Hoechst-Marion-Roussel, Frankfurt, Germany) was dissolved in perfusion solution immediately before use to obtain a drug concentration of 14.3 μmol/L. When required, this solution was infused into the perfusion line at 7% of the total flow rate to give a final drug concentration of 1 μmol/L (chosen on the basis of the isolated myocyte studies; see below).

Experimental Protocol

The main objective here was to confirm that 1 μmol/L HOE-642 was sufficient to produce significant inhibition of sarcolemmal NHE activity in the whole heart. In the absence of a facility for the continuous measurement of pH_i in the whole heart (eg, nuclear magnetic resonance spectroscopy), we used LVDP as a surrogate for pH_i during the infusion and washout of NH_4Cl (20 mmol/L). The selection of this surrogate index was based on the work of Grace et al,[21] who used hearts perfused with HCO_3^--free solution under conditions of constant coronary flow and heart rate (as in the present study), which has shown that (1) changes in LVDP during NH_4Cl pulses mirror closely the changes that occur in pH_i and (2) NHE inhibition suppresses the recovery of LVDP after NH_4Cl washout, which reflects a delay in the recovery of pH_i from acidosis. After 15 minutes of perfusion with the standard perfusion solution, hearts (n=4) were perfused with solution containing NH_4Cl for 5 minutes and with standard solution for a further 20 minutes (first acid pulse). Subsequently, this cycle was repeated (second acid pulse), but this time with HOE-642 (1 μmol/L) also present during the infusion of NH_4Cl and the first 10 minutes of its washout.

During initial perfusion with standard solution, the intraventricular balloon was inflated to obtain a LVEDP of 4 mm Hg, and the balloon volume was kept constant thereafter. LVDP was calculated as the difference between LVEDP and LVSP and was noted at 1- to 2-minute intervals throughout each experiment.

Effects of HOE-642 on Cardioprotective Efficacy of Ischemic Preconditioning

Isolated Heart Perfusion

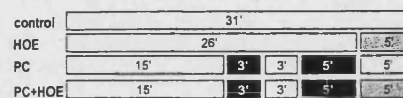
Hearts were perfused in the Langendorff mode as described above, but this time at a constant perfusion pressure of 75 mm Hg. Furthermore, the perfusion solution contained the physiological buffer HCO_3^- (25.0 mmol/L $NaHCO_3$) rather than HEPES and was gassed continuously with a mixture of 95% O_2/5% CO_2 (pH 7.4 at 37°C). During preischemic perfusion, hearts were paced at 360 bpm via an electrode attached to the right atrium; pacing was discontinued from 2 minutes into the prolonged ischemic period and recommenced on reperfusion. HOE-642 was dissolved in deionized water to make a 1 mmol/L stock solution, which was stored at 4°C for a maximum of 5 days. The stock solution was diluted in perfusion solution to obtain a final drug concentration of 1 μmol/L immediately before use.

Experimental Protocols

As summarized schematically in Fig 1, there were two main protocols in this part of the project (n=8 hearts per group), which were designed to determine the effects of NHE inhibition during either (1) the prolonged ischemic period (protocol 1) or (2) the preceding brief preconditioning ischemic periods (protocol 2). After the interventions illustrated in Fig 1, hearts were subjected to normothermic global zero-flow ischemia for 40 or 60 minutes in protocol 1 and for 40 minutes in protocol 2, followed in each case by 40 minutes of reperfusion. Associated with protocol 2, an additional experiment was performed to confirm the adequate washout of HOE-642 before the prolonged ischemic period, as described in "Results."

Left ventricular pressure was monitored via an intraventricular balloon, as described above, and coronary flow rate was measured by timed collection of the coronary effluent. Basal values of LVDP and coronary flow rate were measured at the end of the initial 15-minute period of aerobic perfusion. Left ventricular pressure was also monitored during the period of ischemic arrest and throughout reperfusion to allow assessment of the temporal profiles of the development of ischemic contracture and the postischemic recovery of contractile function. The final postischemic recoveries of LVDP and coronary

PROTOCOL I: NHE inhibition during prolonged ischemic period



PROTOCOL II: NHE inhibition during preconditioning ischemic periods

FIG 1. Schematic of main experimental protocols for PC studies. Only periods before induction of prolonged ischemia are shown; open bars indicate aerobic perfusion with standard perfusion solution, shaded bars aerobic perfusion with perfusion solution containing HOE-642 (1 μmol/L), and solid bars global zero-flow ischemia. All hearts (n=8/group) were subsequently subjected to 40 minutes (protocols 1 and 2) or 60 minutes (protocol 1 only) of global zero-flow ischemia, followed by 40 minutes of reperfusion. Functional indices were measured at end of reperfusion period and expressed as percentage of their respective values obtained at end of initial 15 minutes of aerobic perfusion.

flow were assessed by expressing the values obtained at the end of the reperfusion period as a percentage of their respective basal values. Total creatine kinase leakage (expressed as IU/g heart dry wt) was assessed by spectrophotometric analysis of enzyme activity in the coronary effluent collected during reperfusion with a commercially available kit (Sigma Diagnostics).

Statistical Analysis

Experiments within each protocol were carried out in a prospectively randomized manner. Gaussian-distributed variables were expressed as mean±SEM and were subjected to one-way ANOVA. If a difference among mean values was established, intergroup comparisons were performed with the Student-Newman-Keuls test. A value of $P<.05$ was considered significant.

Results

Effects of HOE-642 on NHE Activity in Isolated Myocytes

Basal and Minimal pH_i

Values for basal pH_i (measured immediately before exposure to NH_4Cl) and minimal pH_i (measured at the end of NH_4Cl washout with Na^+-free Tyrode's solution) did not differ significantly between the various study groups. The ranges observed were from 7.16±0.04 to 7.24±0.04 for basal pH_i and from 6.68±0.04 to 6.77±0.05 for minimal pH_i.

Sarcolemmal NHE Activity

Fig 2A shows representative recordings of pH_i during NH_4Cl pulses in a control cell and in a cell in which extracellular Na^+ was reintroduced in the presence of 1 μmol/L HOE-642. There was a rapid recovery of pH_i from acidosis under control conditions, whereas pH_i recovery was slowed significantly by HOE-642. The quantitative effects of HOE-642 on sarcolemmal NHE activity are illustrated in Fig 2B, which shows J_H as a function of drug concentration. J_H was 7.51±1.49 mmol/L per minute in control cells and was reduced by HOE-642 in a concentration-dependent manner, by 10%, 27%, 50% ($P<.05$), 68% ($P<.05$), and 77% ($P<.05$) at 0.0001, 0.001, 0.01, 0.1, and 1 μmol/L, respectively.

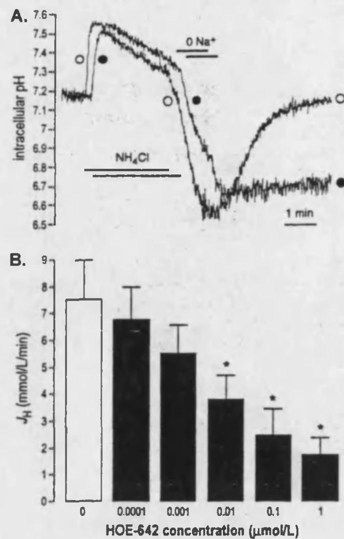


FIG 2. A, Intracellular pH recordings during acid pulses (induced by transient exposure to 20 mmol/L NH_4Cl in HCO_3^--free medium) in control myocyte (open symbols) and one that received 1 µmol/L HOE-642 during reintroduction of extracellular Na^+ (solid symbols). B, Initial H^+ efflux rate (J_H) in control myocytes (open bar) and those that were exposed to various concentrations of HOE-642 during reintroduction of extracellular Na^+ (solid bars) (n=8 to 11/group). *$P<.05$ vs control.

Fig 3 shows representative pH_i recordings from a myocyte subjected to two consecutive acid loads, the first in the absence of HOE-642 and the second in the transient presence of 1 µmol/L HOE-642. As expected from the above observations, pH_i recovery was markedly suppressed in the presence of HOE-642; however, pH_i recovery (at a rate similar to that under control conditions) was rapidly restored on removal of HOE-642 from the superfusion solution, indicating that the inhibition of exchanger activity was readily reversible.

Effects of HOE-642 on NHE Activity in Whole Hearts

Basal Cardiac Function

The basal value of LVDP, measured immediately before the first exposure to NH_4Cl, was 117±7 mm Hg.

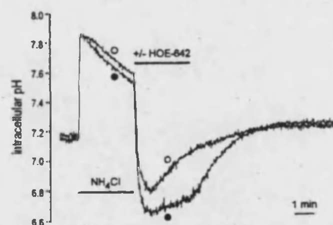


FIG 3. Intracellular pH recordings in an isolated rat ventricular myocyte during two consecutive acid pulses (induced by transient exposure to 20 mmol/L NH_4Cl in HCO_3^--free medium). During first pulse (open symbols), NH_4Cl washout was under control conditions. During second pulse (solid symbols), initial (3 minutes) washout of NH_4Cl was in presence of 1 µmol/L HOE-642, which was subsequently removed from superfusion solution. Recording shown is representative of values obtained from three such experiments.

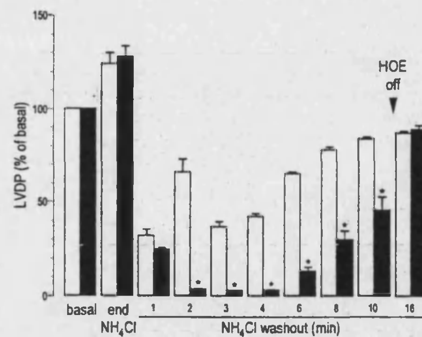


FIG 4. LVDP at various time points during two consecutive NH_4Cl pulses (comprising the 5-minute infusion and 20-minute washout of 20 mmol/L NH_4Cl) in isolated hearts perfused with nominally HCO_3^--free solution. LVDP is expressed as percentage of basal value obtained immediately before start of NH_4Cl infusion during each pulse. Open bars indicate first pulse; solid bars, second pulse. During second pulse, 1 µmol/L HOE-642 was present during NH_4Cl infusion and first 10 minutes of its washout. *$P<.05$ vs first pulse.

LVDP declined slightly after recovery from the first acid pulse, and the basal value measured immediately before the second exposure to NH_4Cl was 104±5 mm Hg.

Cardiac Function During Acid Pulses

Fig 4 illustrates LVDP, expressed as a percentage of the basal value, at various time points during the two consecutive acid pulses. As can be seen, during both pulses, the 5-minute infusion of NH_4Cl produced a positive inotropic effect (probably because of a rise in pH_i[21]), and the washout of NH_4Cl depressed LVDP within 1 minute (probably because of a rapid drop in pH_i[21]). In the first pulse, which occurred in the absence of HOE-642, there was a rapid biphasic recovery of LVDP. In the second pulse, which occurred in the presence of 1 µmol/L HOE-642, LVDP was further depressed by 2 minutes of washout, and recovery was markedly delayed, with a significant difference in LVDP values between the pulses during the first 2 to 10 minutes of NH_4Cl washout. This most likely reflected a delayed recovery of pH_i from acidosis due to drug-induced inhibition of NHE activity,[21] the major H^+ extrusion pathway under these experimental conditions. After the removal of HOE-642 from the perfusion solution, there was a rapid secondary recovery of LVDP, such that there was no significant difference between the pulses in LVDP values by 16 minutes of NH_4Cl washout.

Effects of HOE-642 on Cardioprotective Efficacy of Ischemic Preconditioning

Basal Cardiac Function

Basal values of LVDP and coronary flow did not differ significantly between groups within each study protocol and ranged from 136±4 to 148±5 mm Hg and from 11.4±0.5 to 12.2±0.5 mL/min, respectively.

Postischemic Cardiac Function

Protocol 1: Effects of NHE inhibition during prolonged ischemic period. In this protocol, the objective was to determine whether NHE inhibition during the prolonged ischemic period influences the cardioprotection

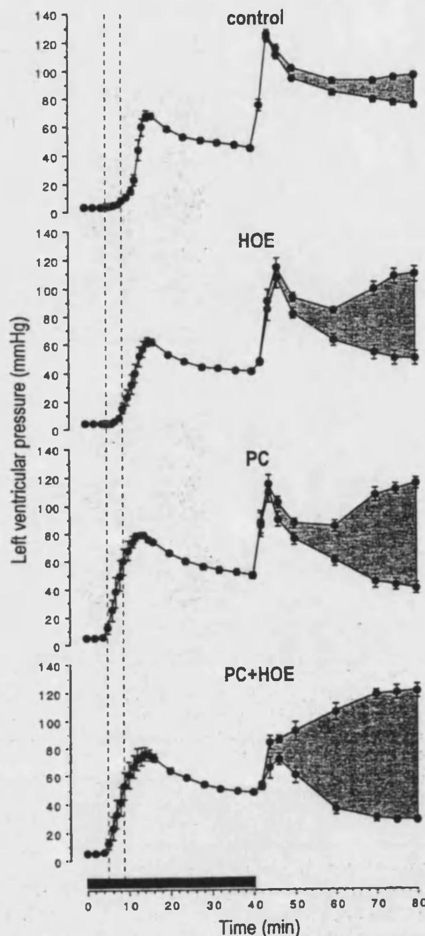


FIG 5. Left ventricular pressure profiles during the 40-minute period of prolonged ischemia and subsequent reperfusion in various study groups in protocol 1 (n=8/group). HOE indicates hearts that received HOE-642 (1 μmol/L) immediately before prolonged ischemic period; PC, preconditioned hearts; and PC+HOE, preconditioned hearts that also received HOE-642 (1 μmol/L) immediately before prolonged ischemic period (see Fig 1 for details). Horizontal black bar above time axis illustrates period of ischemia, and vertical dashed lines indicate times to onset of ischemic contracture in control and PC groups. At each time point during reperfusion, higher symbol indicates LVSP and lower symbol LVEDP; thus, difference (shaded area) represents LVDP.

afforded by ischemic preconditioning. Fig 5 shows the left ventricular pressure profiles during 40 minutes of ischemia and subsequent reperfusion in the four study groups. As can be seen, the time to onset of ischemic contracture (defined as the time at which left ventricular pressure rose 4 mm Hg above baseline) was significantly shorter in PC (5.0±0.2 minutes) relative to control (9.0±0.6 minutes). HOE-642 did not alter the time to onset of ischemic contracture when given alone (HOE; 9.0±0.6 minutes) and did not inhibit the acceleration of the onset of ischemic contracture by PC when given in combination (PC+HOE; 5.0±0.2 minutes). It is also apparent from this figure that in all three treatment groups, the postischemic recovery of contractile function was markedly improved relative to control, with end-reperfusion LVSP values of 96±2, 111±4 (P<.05), 115±4 (P<.05), and 121±3 (P<.05) mm Hg and LVEDP values of 75±2, 53±6 (P<.05), 40±4 (P<.05), and 28±2 (P<.05) mm Hg in control, HOE, PC, and PC+HOE, respectively. At this time, LVDP recovery was 15±2% in controls. This was significantly increased, to 45±7% by HOE-642 alone (HOE) and to 55±5% by ischemic preconditioning alone (PC). With the combination of both interventions (PC+HOE), LVDP recovery was further improved to 68±2%, a value that was significantly greater than those obtained in HOE and in PC. Creatine kinase leakage during reperfusion measured 494±49 IU/g in controls and was reduced significantly to 350±35, 291±36, and 272±33 IU/g in HOE, PC, and PC+HOE, respectively (with no significant difference between the values obtained in the three treatment groups). Postischemic recovery of coronary flow was 56±3% in the untreated control group; this was significantly increased in all treatment groups, to 78±3%, 72±3%, and 80±4% in HOE, PC, and PC+HOE, respectively.

As shown above, although the recovery of contractile function after 40 minutes of prolonged ischemia was significantly enhanced in PC+HOE relative to PC or HOE alone, the improvement in final LVDP recovery was small and was not matched by a significant reduction in creatine kinase leakage during reperfusion. Therefore, we performed an additional study with the objective of testing whether any additive protection afforded by the combination of ischemic preconditioning and HOE-642 would be more readily revealed under more severe conditions. To this end, the duration of prolonged ischemia was extended from 40 minutes to 60 minutes. Fig 6 shows the postischemic recovery of LVDP (Fig 6A) and creatine kinase leakage during reperfusion (Fig 6B) in the control, HOE, PC, and PC+HOE groups. Under these conditions also, LVDP recovery was significantly improved and creatine kinase leakage significantly reduced in all three treatment groups relative to control. However, with this extended duration of prolonged ischemia, LVDP recovery in PC+HOE (66±2%) was almost double that in HOE (37±4%) or PC (34±5%); furthermore, this time the improved contractile recovery was accompanied by a significant reduction in creatine kinase leakage, supporting an additive effect.

Protocol 2: Effects of NHE inhibition during preconditioning ischemic periods. In this protocol, the objective was to determine whether NHE inhibition during the short periods of preconditioning ischemia influences the cardioprotection afforded by ischemic preconditioning. Relative to control, the postischemic recovery of LVDP was once again significantly increased in PC (57±2% versus 34±4%), and this effect was accompanied by a significant reduction in creatine kinase leakage during reperfusion (410±25 versus 523±31 IU/g) (Fig 7). However, in contrast to our observations with the infusion of HOE-642 immediately before the prolonged ischemic period, when the drug was infused before each of the preconditioning ischemic periods and subsequently washed out (HOE+PC), there was no significant difference in LVDP recovery or creatine kinase leakage relative to PC (Fig 7). A similar pattern was seen with

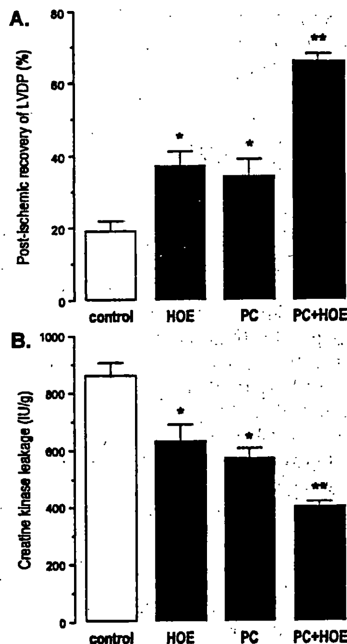


FIG 6. A, Postischemic recovery of LVDP and B, creatine kinase leakage during reperfusion in various study groups in protocol 1 (n=8/group), which were subjected to 60 minutes of prolonged ischemia. HOE indicates hearts that received HOE-642 (1 μmol/L) immediately before prolonged ischemic period; PC, preconditioned hearts; and PC+HOE, preconditioned hearts that additionally received HOE-642 (1 μmol/L) immediately before prolonged ischemic period (see Fig 1 for details). *$P<.05$ vs control; **$P<.05$ vs control, HOE, and PC.

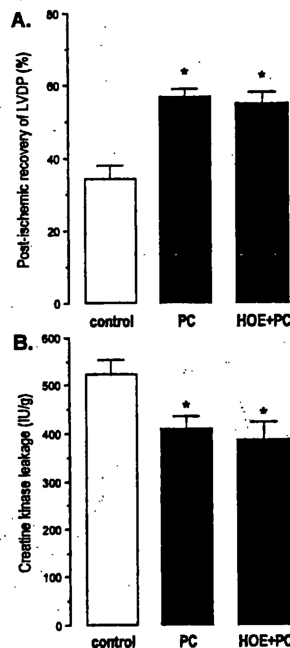


FIG 7. A, Postischemic recovery of LVDP and B, creatine kinase leakage during reperfusion in the various study groups in protocol 2 (n=8/group), which were subjected to 40 minutes of prolonged ischemia. PC indicates preconditioned hearts; HOE+PC, preconditioned hearts that also received HOE-642 (1 μmol/L) for 3 minutes immediately before each of two preconditioning ischemic periods (drug was washed out before prolonged ischemic period; see Fig 1 for details). *$P<.05$ vs control.

respect to the postischemic recovery of coronary flow: 57±4% in control, 70±3% in PC ($P<.05$), and 72±2% in HOE+PC ($P<.05$). The time to onset of ischemic contracture was once again significantly shortened in PC (from 10.9±0.4 minutes in control to 6.4±0.4 minutes), and this effect was unaffected by the coadministration of HOE-642 (7.3±0.6 minutes in HOE+PC, $P<.05$ versus control).

A potential complication in the interpretation of the above study is the possibility that the washout of HOE-642 in the HOE+PC group might have been inadequate. Thus, even if NHE inhibition during the brief preconditioning ischemic periods did abolish the cardioprotective actions of preconditioning, such an effect might have been obscured by any cardioprotection arising from residual drug presence during the prolonged ischemic period. To test whether the washout period used was sufficient, an additional study was performed in which 1 μmol/L HOE-642 was infused for 6 minutes (equivalent to the total duration of drug infusion in the above protocol) and hearts were subjected to ischemia either (1) immediately after drug infusion or (2) after 15 minutes of washout (equivalent to the duration of drug washout in the above protocol). The control group once again received no intervention. Postischemic recovery of LVDP was significantly improved, from 32±2% in control to 46±4% by the infusion of HOE-642 immediately before ischemia. In contrast, there was no significant change in LVDP recovery (31±4%) when the drug was washed out for 15 minutes before the induction of ischemia, indicating that a 15-minute washout period was sufficient to reduce the tissue drug content to a level that does not affect postischemic cardiac function.

Discussion

The present study has demonstrated that, in isolated rat hearts, administration of HOE-642 (at a concentration sufficient to inhibit sarcolemmal NHE activity in isolated myocytes and delay recovery of contractile function from acidosis-induced depression in whole hearts) before either the prolonged ischemic period or the preceding brief preconditioning ischemic periods fails to impair the cardioprotective efficacy of ischemic preconditioning. This finding questions the suggested role[8] of NHE activity as a determinant of the cardioprotective effect of ischemic preconditioning.

Is NHE Activity Necessary for Cardioprotection by Preconditioning?

Ramasamy et al[8] recently proposed a role for stimulation of NHE activity in the protective action of ischemic preconditioning in the isolated rat heart. This proposal was based on the observation that the intracoronary infusion of ethylisopropylamiloride (EIPA), an inhibitor of NHE, immediately before the prolonged ischemic period could attenuate the improved LVDP recovery and reduced creatine kinase leakage afforded by ischemic preconditioning. This finding con-

trasts with a large body of evidence (for recent reviews, see References 12 through 15), obtained with a variety of pharmacological NHE inhibitors (including EIPA) and species, that inhibition of NHE is cardioprotective. Indeed, in accordance with our earlier work,[22] the present study has confirmed that infusion of the NHE inhibitor HOE-642 immediately before the prolonged ischemic period affords significant cardioprotection. Furthermore, the present study has shown that the cardioprotective effects of ischemic preconditioning and NHE inhibition, as assessed by increased LVDP recovery and reduced creatine kinase leakage (as in the study by Ramasamy et al[8]), are additive rather than counteractive.

The observations of the present study do not support a role for NHE activity in determining the cardioprotective consequences of ischemic preconditioning. On the contrary, in light of the present study, it may be speculated that any increase in NHE activity in preconditioned hearts (the evidence for which is critically assessed below) may represent an undesirable side effect of ischemic preconditioning, which detracts from its cardioprotective efficacy. Thus, the true protective potential of ischemic preconditioning may be revealed only by concomitant inhibition of NHE activity during the prolonged ischemic period. The observation in the present study that the combination of ischemic preconditioning and infusion of HOE-642 immediately before the prolonged ischemic period afforded significantly greater protection than either intervention alone is consistent with this hypothesis.

Cardiac Actions of NHE Inhibitors

The factors that may potentially account for the divergent findings of the present study compared with that by Ramasamy and colleagues[8] must be considered. Both studies used identical species, models, and functional end points, although the mode of perfusion (constant pressure versus constant flow) differed and may have contributed to the divergence in findings. However, the most significant factor is likely to have been the difference between the studies in the characteristics and concentration of the pharmacological NHE inhibitor used. Although 5-amino-substituted derivatives of amiloride (such as EIPA) are potent inhibitors of NHE, they are relatively nonspecific[23] and have been shown to produce electrophysiological abnormalities[24,25] and cardiodepressant effects,[25,26] particularly at concentrations that exceed 1 μmol/L.[25] For this reason, we chose not to use an amiloride derivative and selected HOE-642, which is a novel, benzoylguanidine-based NHE inhibitor that exhibits marked selectivity for the cardiac isoform of the exchanger.[16] Indeed, in our myocyte studies, we confirmed that HOE-642 is a potent inhibitor of the sarcolemmal NHE in rat ventricular myocytes, with the 1 μmol/L concentration (as used in our preconditioning studies) resulting in ≈80% inhibition of exchanger activity at a pH_i of ≈6.75. This finding is consistent with recent work with HOE-694 (a structural congener of HOE-642) in guinea pig ventricular myocytes.[27] It is important to note that these benzoylguanidine derivatives do not affect the activity of other pH_i-regulating carriers[27] or Na^+ transport mechanisms.[16] Furthermore, unlike EIPA, they do not appear to exhibit cardiodepressant effects at NHE-inhibitory concentrations,[16,26] a property that enhances their value as pharmacological tools in the delineation of the physiological/pathophysiological role(s) of NHE. In light of the above arguments, it may be speculated that the results of the study by Ramasamy and colleagues[8] were complicated by the use of a relatively high concentration of a less selective NHE inhibitor, whose nonspecific actions might have contributed to the apparent abolition of the protective effect of ischemic preconditioning. In this regard, it is important to note that Bugge and Ytrehus[28] showed that coadministration of EIPA at a lower concentration (1 μmol/L versus 3 μmol/L in the study by Ramasamy et al[8]) provides additional protection to preconditioned rat hearts, which is in keeping with our findings with HOE-642.

Role of NHE Activity During Preconditioning Ischemic Periods

In both previous studies in which EIPA was used in combination with ischemic preconditioning,[8,28] the NHE inhibitor was present during the prolonged ischemic period. In the present study, we additionally addressed, for the first time, the question of whether NHE activity during the preconditioning ischemic periods might be involved in the signaling mechanism(s) mediating the protective response. Our observation that the infusion of HOE-642 before each of the preconditioning ischemic periods (followed by its washout) does not diminish the cardioprotective action of preconditioning suggests that NHE activity during these periods also is not involved in the underlying protective mechanisms. It may be argued that residual drug presence during the prolonged ischemic period might have complicated the interpretation of these studies. However, our demonstration of the ready reversibility by drug washout of (1) HOE-642–induced depression of pH_i recovery in acid-loaded myocytes (Fig 3), (2) HOE-642–induced depression of LVDP recovery in acid-loaded hearts (Fig 4), and (3) the cardioprotective effect of HOE-642 in hearts subjected to ischemia/reperfusion would argue against significant residual drug presence.

NHE Activity and Ischemic Contracture

In the present study, the infusion of HOE-642 immediately before the prolonged ischemic period did not alter the time to onset of ischemic contracture. However, consistent with recent observations from our laboratory,[6,18] ischemic preconditioning significantly accelerated the onset of ischemic contracture. The combination of the two interventions resulted in an accelerated contracture profile similar to that observed with ischemic preconditioning alone. On the basis of these observations, it can be concluded that, in the isolated rat heart, NHE activity is not a determinant of the rate of development of ischemic contracture (although different observations have been made in the rabbit heart[29]). Previous studies by Hearse et al[30] in the isolated rat heart have shown that the onset of ischemic contracture is closely linked to the rate at which the tissue ATP content declines, a relationship that appears to hold true in preconditioned hearts as well.[6,18] Thus, the inability of HOE-642 to modify the profile of ischemic contracture may be due to the inability of NHE inhibition to significantly alter the rate of ATP depletion during global zero-flow ischemia,

as revealed by studies that used NMR spectroscopy for continuous analysis of tissue ATP content.[29,31,32]

Is NHE Activity Increased by Preconditioning?

Within the context of the present study and the arguments presented above, a key issue to consider is whether ischemic preconditioning actually increases NHE activity. Before the evidence for this can be critically assessed, it should be stressed that the primary activator of NHE is intracellular acidosis.[33] Activation by other stimuli, such as α_1-adrenoceptor agonists[34,35] and thrombin,[19] arises from a change in the pH_i sensitivity of the exchanger, so that at a given pH_i the exchanger has greater activity after stimulation. Therefore, comparisons of NHE activity between two or more groups are informative only if activity is determined at a similar pH_i in all cases.

The primary evidence for an increased NHE activity after ischemic preconditioning is the observation by Ramasamy et al[8] that preconditioned rat hearts exhibit an enhanced ability to recover from acute intracellular acidosis induced in the absence of ischemia. However, because that study was carried out in hearts perfused with HCO_3^--containing medium, it is impossible to ascribe the accelerated recovery from acidosis to an increase in NHE activity.[36] Furthermore, it should be noted that the method used to induce acute intracellular acidosis (transient exposure to NH_4Cl) resulted in greater acidosis in preconditioned (pH_i=6.54±0.02) than in control (pH_i=6.72±0.02) hearts.[8] Because the rate of acid-equivalent extrusion via not only NHE but also Na^+/HCO_3^- symport is inversely related to pH_i,[36] it is likely that the faster initial recovery from acidosis in preconditioned hearts may have arisen as a consequence of the lower starting pH_i in this group. Indeed, de Albuquerque and colleagues[37] recently showed that, in the presence of a similar acid load, the rate of pH_i recovery is similar in control and preconditioned rat hearts.

Ramasamy et al[8] provided additional evidence that ischemic preconditioning increases intracellular Na^+ accumulation during the prolonged ischemic period and that this effect is attenuated by EIPA. When taken together with the earlier reports of reduced acidosis,[2-6] an enhanced Na^+ accumulation that is sensitive to inhibition by EIPA is supportive of an increased NHE activity in preconditioned hearts. However, the reported[8] enhancement of Na^+ accumulation in preconditioned hearts contrasts with earlier observations by Steenbergen et al[5] in a similar model. Thus, it would appear that the question of whether ischemic preconditioning results in increased NHE activity cannot be resolved on the basis of the evidence currently available.

Potential Limitations of the Study

In the present study, sarcolemmal NHE activity in control versus preconditioned hearts, with and without coadministration of HOE-642, was not determined. Nevertheless, on the basis of our work with acid-loaded isolated ventricular myocytes (Fig 2) and whole hearts (Fig 4), it is highly likely that the 1 μmol/L concentration of HOE-642 used in our preconditioning studies was sufficient to inhibit sarcolemmal NHE activity. This is supported by the ability of this concentration of the drug to afford significant protection in hearts subjected to ischemia/reperfusion.

The present interpretation of the data from our preconditioning studies is contingent on NHE inhibition being the primary pharmacological action of HOE-642 and the sole mechanism of its cardioprotective effect at the 1 μmol/L concentration used. If the cardioprotective effect arose from a hitherto unidentified secondary action of the drug (that is distinct from NHE inhibition), then any diminution of the cardioprotective efficacy of ischemic preconditioning by HOE-642–induced NHE inhibition might be masked by such a secondary action. Although this possibility cannot be discounted, because HOE-642 is a new drug whose actions may not yet be comprehensively characterized, it should also be noted that there is currently no evidence to support it.

Finally, caution should be exercised in extrapolating the findings of the present study to other species or models, particularly when a different index of injury (eg, infarct size, arrhythmias) might be used to quantify the cardioprotective efficacy of HOE-642 or ischemic preconditioning.

Concluding Comments

The present study has shown that the application of a potent NHE inhibitor in combination with ischemic preconditioning does not attenuate the cardioprotective efficacy of ischemic preconditioning; on the contrary, the NHE inhibitor provides additional protection when present during the prolonged ischemic period. Assuming that NHE inhibition is the principal action of the drug at the concentration used, these observations indicate that NHE activity during either the prolonged ischemic period or the preceding brief preconditioning ischemic periods does not contribute to the cardioprotection afforded by ischemic preconditioning. Furthermore, they dispute the proposal[8] that increased NHE activity may represent a protective mechanism of ischemic preconditioning.

Acknowledgments

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Editorial

Protection of the Myocardium During Ischemia and Reperfusion

Na^+/H^+ Exchange Inhibition Versus Ischemic Preconditioning

Metin Avkiran, PhD

The hypothesis that sarcolemmal Na^+/H^+ exchanger activity may contribute to myocardial injury during ischemia and reperfusion was first published in 1985,[1] preceding by 1 year the first description of the ischemic preconditioning phenomenon.[2] Initial pharmacological evidence in support of the Na^+/H^+ exchanger hypothesis was subsequently provided by Karmazyn,[3] who showed that amiloride (an inhibitor of the exchanger) enhanced the postischemic recovery of contractile function and reduced creatine kinase leakage in rat hearts subjected to global ischemia and reperfusion. Since then, a number of Na^+/H^+ exchange inhibitors, including highly specific novel inhibitors such as HOE-694, HOE-642 (cariporide), and EMD-85131, have been shown to afford cardioprotective benefit in a variety of animal models of ischemia and reperfusion.[4] Nevertheless, as an innovative approach to the protection of ischemic myocardium, Na^+/H^+ exchange inhibition has failed to capture the imagination of cardiologists (experimental and clinical alike) to quite the same extent as ischemic preconditioning. Indeed, a survey of articles published in *Circulation* and *Circulation Research* over the past decade reveals only 14 articles whose title or abstract contains the keywords "Na^+/H^+ exchange(r) and ischemia," whereas 115 articles are identified when the combination "preconditioning and ischemia" is used. Is this a fair reflection of the relative cardioprotective efficacy, and perhaps the therapeutic potential, of these interventions?

See p 2519

In this issue of *Circulation*, Gumina and colleagues[5] report on a comparison of the efficacy of Na^+/H^+ exchange inhibition (achieved with BIIB-513, the latest addition to the family of novel Na^+/H^+ exchange inhibitors) versus ischemic preconditioning in limiting infarct size in dog hearts subjected to regional ischemia and reperfusion in vivo. This is the first such comparison in a large animal, and the findings carry additional weight because they originate from a laboratory that has made a major contribution to the characterization of the powerful preconditioning phenomenon. The most striking result of the study is that extending the duration of index ischemia from 60 to 90 minutes abolishes the protective effect of ischemic preconditioning but does not affect the protection afforded by Na^+/H^+ exchange inhibition. This indicates that at least in dog myocardium, Na^+/H^+ exchange inhibition may afford greater protection against ischemia and reperfusion-induced injury than does ischemic preconditioning. Furthermore, other data reported in the article[5] suggest that the combination of partial Na^+/H^+ exchange inhibition and ischemic preconditioning may be more effective in limiting infarct size than either intervention alone. In considering the mechanistic and therapeutic implications of these findings, it is appropriate to address several pertinent questions.

Does Ischemic Preconditioning Alter Na^+/H^+ Exchanger Activity?

Some of the issues raised in the study by Gumina et al[5] warrant a brief overview of the potential effects of ischemic preconditioning on Na^+/H^+ exchanger activity. Intracellular acidosis occurs even during the brief periods of ischemia that are used to trigger preconditioning and is a potent stimulus for sarcolemmal Na^+/H^+ exchanger activity. In addition to such allosteric activation of the exchanger by intracellular acidosis, ischemic preconditioning may also initiate post-translational regulatory mechanisms (eg, phosphorylation of the exchanger and/or its regulatory proteins) that increase Na^+/H^+ exchanger activity by altering its sensitivity to intracellular H^+. In this regard, it is notable that stimuli that can mimic ischemic preconditioning, such as α_1-adrenergic receptor stimulation,[6] also increase sarcolemmal Na^+/H^+ exchanger activity, through an enhancement of its sensitivity to intracellular H^+.[7] Nevertheless, it has been shown recently that, after the induction of an intracellular acid load of comparable severity, the rate of recovery of intracellular pH is identical in preconditioned and nonpreconditioned myocardium under aerobic conditions.[8] From this, it appears that any stimulation of sarcolemmal Na^+/H^+ exchanger activity in preconditioned myocardium may not persist beyond the triggering ischemic episodes.

During index ischemia, a commonly observed consequence of ischemic preconditioning is a reduction in the severity of intracellular acidosis. Although other potential mechanisms for this "antiacidotic" effect exist (eg, reduced metabolic H^+ production[9]), the pertinent question within the context of this editorial is whether this effect arises from increased cellular H^+ extrusion via the sarcolemmal Na^+/H^+ exchanger. Al-

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though the evidence outlined above[8] would argue against such a possibility, 2 recent studies[9,10] have addressed this question by determining the effects of Na^+/H^+ exchange inhibitors on intracellular pH. Unfortunately, the results have been contradictory, with Na^+/H^+ exchange inhibition shown both not to alter[9] and to significantly attenuate[10] the antiacidotic effect of ischemic preconditioning. Furthermore, it has been suggested that this effect may arise from increased H^+ extrusion through an alternative pathway.[11] Therefore, from the available data, it is not possible to deduce that ischemic preconditioning increases Na^+/H^+ exchanger activity during index ischemia. Conversely, unless ischemic preconditioning increases the exchanger's sensitivity to intracellular H^+, attenuated intracellular acidosis during index ischemia in preconditioned hearts would be expected to reduce Na^+/H^+ exchanger activity.

Na^+/H^+ Exchanger Inhibition and Ischemic Preconditioning: Counteractive or Additive?

Regardless of whether ischemic preconditioning alters sarcolemmal Na^+/H^+ exchanger activity, an important question is whether an active exchanger is necessary to achieve cardioprotective benefit from this intervention. A number of studies have addressed this issue, again by using pharmacological inhibitors to suppress Na^+/H^+ exchanger activity at various times during the experimental protocol: during the cycles of triggering ischemia,[12,13] during index ischemia,[9,12,14–16] or during both periods.[17] Although contradictory findings have also been reported,[13,14] the majority of these studies[9,12,15–17] have shown that the cardioprotective benefit of ischemic preconditioning is not attenuated by Na^+/H^+ exchange inhibition, indicating that an active exchanger is not necessary to achieve such benefit. To the contrary, in some studies,[12,17] the combination of Na^+/H^+ exchange inhibition with ischemic preconditioning has been shown to provide an additive benefit, with the limitation of infarct size[17] or the improvement in the recovery of contractile function[12] appearing to be significantly greater with the combined intervention relative to either intervention alone. Provided that the Na^+/H^+ exchange inhibitor doses and ischemic preconditioning protocols used in these studies were those that each afforded the maximum attainable protection, then the additive effects observed[12,17] may indicate independent mechanisms of action.

It is notable that both of the previous studies that have shown additive benefit with the combination of Na^+/H^+ exchange inhibition and ischemic preconditioning were carried out in rat hearts[12,17] and that no similar additive effect has been observed in the rabbit.[15,16] At first consideration, this might appear to indicate species-specific responses, with Na^+/H^+ exchange inhibition and ischemic preconditioning possessing distinct mechanisms of action in the rat but sharing a common mechanism of action in the rabbit. This scenario is unlikely, however, because in the rabbit heart, interventions that abolish the cardioprotective benefit of ischemic preconditioning (such as protein kinase C inhibition and ATP-sensitive K^+ channel blockade) do not seem to affect the cardioprotective efficacy of Na^+/H^+ exchange inhibition.[15,18] Furthermore, the study by Gumina et al[5] in this issue provides evidence that, in the dog heart also, the combination of Na^+/H^+ exchange inhibition and ischemic preconditioning affords greater cardioprotective benefit than either intervention alone, with an index ischemia of 90 minutes' duration. A unique feature of this study[5] is that a marked reduction in infarct size was afforded by the combination of Na^+/H^+ exchange inhibition by low-dose BIIB-513 and ischemic preconditioning, even though either intervention alone did not produce a statistically significant effect. The authors describe this effect of the combined intervention as "greater than additive,"[5] which is akin to the textbook definition of synergism. However, the data may not reflect a true synergistic interaction between Na^+/H^+ exchange inhibition and ischemic preconditioning, because each intervention alone tended to reduce infarct size by ≈25% (Figure 3 in Reference 5) and the effect of the combined intervention was not substantially greater than the sum of the individual effects.[5] Instead, the apparent greater-than-additive effect could have arisen by chance, in view of the intragroup variability in infarct size, which most likely reflected a variable collateral flow. Another potential confounding factor is that low-dose BIIB-513 is unlikely to have produced a complete suppression of Na^+/H^+ exchanger activity (as evidenced by the enhanced protection afforded by a 4-fold greater dose), which makes it difficult to interpret mechanistically the effects of combining this intervention with ischemic preconditioning. Regardless of these issues, however, the important new data provided by Gumina et al,[5] considered together with other pertinent evidence in the literature, strongly suggest that Na^+/H^+ exchange inhibition and ischemic preconditioning can each afford significant cardioprotection in ischemia and reperfusion (most likely through independent mechanisms) and that the former intervention does not counteract (but may add to) the protection afforded by the latter.

Na^+/H^+ Exchange Inhibition Versus Ischemic Preconditioning: Is One Superior to the Other?

A recent Special Report in *Circulation*[19] stated that "... other than early reperfusion, preconditioning is the strongest form of in vivo protection against myocardial ischemic injury." While there can be no argument that reperfusion is an absolute prerequisite for the salvage of ischemic myocardium and that ischemic preconditioning is a powerful cardioprotective intervention, the new evidence provided by Gumina et al[5] and other published data do not wholly support this statement. The Table summarizes the protocols and main findings of 5 studies, 2 of them in vivo, which (to the best of the author's knowledge) constitute all of the studies in the literature that have directly compared the cardioprotective efficacy of Na^+/H^+ exchange inhibition versus ischemic preconditioning. In these studies, ischemic preconditioning was shown to afford marked protection against ischemia-and-reperfusion–induced injury, which was manifest as an attenuation of contractile dysfunction and creatine kinase leakage[12] or a limitation of infarct size.[5,15–17] The earlier studies[12,15–17] all showed that Na^+/H^+ exchange inhibition is equally as effective as ischemic preconditioning in protecting

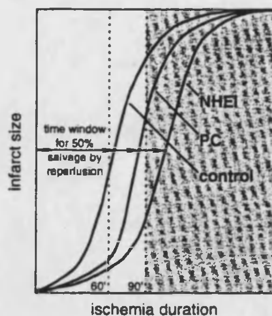
Relative Cardioprotective Efficacy of Na^+/H^+ Exchange Inhibition Versus Ischemic Preconditioning

Reference	Model	Ischemia, min		Reperfusion, min		NHEI	Relative Efficacy
		Trigger	Index	Trigger	Index		
Bugge et al[17]	Rat, in vitro	5/5/5	30	5/5/5	120	EIPA	NHEI=PC
		5/5/5	45	5/5/5	120	EIPA	NHEI=PC
Shipolini et al[12]	Rat, in vitro	3/5	40	3/5	40	HOE-642	NHEI=PC
		3/5	60	3/5	40	HOE-642	NHEI=PC
Sato et al[15]	Rabbit, in vitro	5	30	10	120	EIPA	NHEI=PC
		5	45	10	120	EIPA	NHEI=PC
Munch-Ellingsen et al[16]	Rabbit, in vivo	5	45	10	150	EIPA	NHEI=PC
Gumina et al[5]	Dog, in vivo	5	60	10	180	BIIB-513	NHEI=PC
		5	90	10	180	BIIB-513	NHEI>PC
		5/5/5/5	90	5/5/5/5	180	BIIB-513	NHEI>PC

NHEI indicates Na^+/H^+ exchange inhibitor; EIPA, 5-(*N*-ethyl-*N*-isopropyl)-amiloride; and PC, ischemic preconditioning. HOE-642 is also known as cariporide.

the myocardium during ischemia and reperfusion. The current findings of Gumina and colleagues[5] similarly demonstrate comparable efficacies with both interventions in dog hearts subjected to 60 minutes of index ischemia, but they suggest that Na^+/H^+ exchange inhibition may afford superior protection when this is extended to 90 minutes.

In discussing the relative efficacy of Na^+/H^+ exchange inhibition and ischemic preconditioning, Gumina et al[5] coined the phrase "ceiling of protection" to describe the minimum duration of index ischemia against which a particular intervention cannot afford significant protection. Their data suggest that in the dog in vivo and with infarct size as the index of injury, this ceiling is between 60 and 90 minutes for ischemic preconditioning but >90 minutes for Na^+/H^+ exchange inhibition. Although it may not be entirely helpful to extend the architectural analogy, it is probable that the higher ceiling for Na^+/H^+ exchange inhibition simply reflects a further extension of the time window during which myocardial salvage can be achieved by reperfusion. The Figure illustrates this concept by showing hypothetical "injury curves" that describe the relationship between ischemia duration and infarct size in 3 groups of hearts: untreated controls, hearts subjected to ischemic preconditioning, and hearts pretreated with the maximally effective dose of a Na^+/H^+ exchange inhibitor. It should be stressed that although the early sections of these curves have been drawn with the guidance of data from Gumina et al,[5] the sections beyond 90 minutes (shaded area) are speculative. Nevertheless, the figure illustrates that the greater limitation of infarct size afforded by Na^+/H^+ exchange inhibition versus ischemic preconditioning after extended ischemia may arise from a greater delay in the progression of ischemic injury, depicted as a further shift to the right of the injury curve. By increasing the amount of viable tissue remaining at the time of reperfusion, this would then allow significant myocardial salvage to be achieved even after extended ischemia.



Hypothetical curves describing relationship between ischemia duration and infarct size in hearts without treatment (control) and after ischemic preconditioning (PC) or treatment with a Na^+/H^+ exchange inhibitor (NHEI). See text for details.

The scheme proposed above does not take into account any potential contribution of reperfusion injury to the infarct size measured after ischemia and reperfusion. Recent work by Matsumura and colleagues[20] suggests that in dog hearts subjected to 90 minutes of ischemia followed by reperfusion (as in the study by Gumina et al[5]), a substantial proportion of the infarcted myocardium is viable at the end of the ischemic period but loses viability after 180 minutes of reperfusion. In this context, it is important to highlight earlier evidence from Gumina and colleagues,[21] also in the dog, that has shown that administration of a Na^+/H^+ exchange inhibitor shortly before reperfusion can produce a significant limitation of infarct size. Interestingly, preliminary evidence for this mode of action has been obtained in humans as well, in a recent study in which patients with anterior myocardial infarction received the Na^+/H^+ exchange inhibitor cariporide shortly before undergoing primary percutaneous transluminal coronary angioplasty.[22] Therefore, it is reasonable to suggest that Na^+/H^+ exchange inhibition may provide superior protection by limiting the loss of myocardial viability not only during ischemia but also during reperfusion.[5]

The preclinical evidence[4] that Na^+/H^+ exchange inhibition represents an effective approach to the limitation of myocardial injury during ischemia and reperfusion has been strengthened substantially by the work reported by Gumina and colleagues,[5] whose data suggest that, at least in the dog, the

protection afforded by this intervention may be superior to that provided by ischemic preconditioning. Because specific and apparently well-tolerated inhibitors of the Na^+/H^+ exchanger are now available, a further advantage of Na^+/H^+ exchange inhibition over ischemic preconditioning may arise from the greater practicability of assessing its therapeutic potential. Indeed, a multicenter clinical trial, designed with the objective of assessing the potential benefits of cariporide in patients with acute coronary syndromes,[23] was recently completed. Although the preliminary results of this trial (as presented at the American College of Cardiology Scientific Session in March 1999) have not shown a significant overall benefit, subgroup differences suggest that cariporide treatment may have provided benefit when ischemia was terminated by reperfusion. This finding, if confirmed by detailed analysis, would be wholly consistent with the established actions of Na^+/H^+ exchange inhibitors in animal models.[4] The challenge now is to design and perform further trials that reflect the knowledge that has been accumulated from such recent clinical experience and through extensive preclinical investigation; ultimately, only these can provide the acid test for Na^+/H^+ exchange inhibition as a novel therapeutic approach in ischemic heart disease.

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KEY WORDS: Editorials ■ myocardial infarction ■ ischemia ■ reperfusion

Na^+/H^+ Exchanger Inhibitor HOE-642 Improves Cardioplegic Myocardial Preservation Under Both Normothermic and Hypothermic Conditions

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Background The sarcolemmal Na^+/H^+ exchanger has been implicated in the pathogenesis of myocardial injury during ischemia/reperfusion. We determined the cardioprotective efficacy of the Na^+/H^+ exchanger inhibitor HOE-642 (HOE) as an alternative, adjunct, or additive to cardioplegia (CP).

Methods and Results In isolated working rat hearts (n=6 per group) subjected to 25 minutes of ischemia at 37°C, the postischemic recovery of aortic flow (AF) was 5±3% in controls; this was improved to 18±4% by the preischemic infusion of 1 μmol/L HOE ($P<.05$ versus control) and to 53±7% by CP ($P<.05$ versus control and HOE). In hearts subjected to CP and 35 minutes of ischemia at 37°C, AF recovered to 9±3% with CP alone; this was improved to 18±3% by the adjunctive administration of HOE during early reperfusion (CP+repHOE, $P<.05$ versus CP) and to 27±4% by the use of HOE as an additive to CP (CP+HOE, $P<.05$ versus CP and CP+repHOE). With 120 minutes of ischemia at 28°C, AF recoveries were 16±3% in CP, 32±3% in CP+repHOE ($P<.05$ versus CP) and to 50±4% in CP+HOE ($P<.05$ versus CP and CP+repHOE). With 300 minutes of ischemia at 7.5°C, the corresponding values were 30±4%, 45±5% ($P<.05$ versus CP), and 63±5% ($P<.05$ versus CP and CP+repHOE). Improved recovery of pump function was often accompanied by a reduction in creatine kinase leakage during reperfusion.

Conclusions (i) HOE alone affords significant protection at normothermia but is not a superior alternative to CP, and (ii) the use of HOE as an adjunct or additive to CP provides significant benefit at normothermia, moderate hypothermia, and severe hypothermia. (***Circulation*. 1997;96[suppl II]:II-266-II-273.**)

Key Words • cardioplegia • hypothermia • ischemia • Na^+/H^+ exchanger • HOE-642

The introduction of CP has dramatically improved myocardial protection during cardiac surgery.[1] Nevertheless, in some cases, ischemia and reperfusion-induced cardiac dysfunction remains an important factor in the development of perioperative complications; consequently, efforts continue to further improve the efficacy of cardioprotective strategies.

In recent years, considerable experimental evidence has accumulated to suggest that activation of the sarcolemmal Na^+/H^+ exchanger may be an important factor in the development of myocardial injury during ischemia and reperfusion, probably by contributing to the disturbance of Na^+ and Ca^{2+} homeostasis (for reviews, see Karmazyn and Moffat[2] and Avkiran[3]). Much of this evidence is based on the cardioprotective properties of amiloride-based Na^+/H^+ exchanger inhibitors; however, the utility of these agents as pharmacological tools or potential therapeutic agents is compromised by their nonspecificity.[4,5] In 1993, HOE-694, a novel compound with a structure distinct from amiloride, was reported to be a potent Na^+/H^+ exchanger inhibitor.[6] Additionally, HOE-694 was shown to possess cardioprotective properties during ischemia and reperfusion,[6] an observation which has been supported by subsequent studies.[7-11] More recently, it has been demonstrated that HOE, a structural congener of HOE-694, may have even greater potency than HOE-694 as a Na^+/H^+ exchanger inhibitor and a cardioprotective agent.[12] HOE also exhibits greater selectivity than HOE-694 for the NHE-1 isoform of the exchanger[12] (believed to be the predominant isoform expressed in the heart[13]), and its therapeutic efficacy is currently being evaluated in humans. In view of the marked cardioprotective efficacy of HOE and the evidence that Na^+/H^+ exchanger activity may contribute to the loss of ionic homeostasis during ischemia even under hypothermic conditions,[14] this agent may prove to be of clinical value as an alternative, adjunct, or additive to CP in cardiac surgery. However, experimental evidence for this is currently lacking.

In the light of the above, the objectives of the present study were to use an isolated working rat heart model to assess: (i) the potential of HOE as an alternative to CP and (ii) the efficacy of HOE as an adjunct or additive to CP. When used as an adjunct to CP, HOE was administered during early reperfusion; when used as an additive, it was included as a component of the cardioplegic solution.

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Methods

All experiments were performed in accordance with the Home Office Guidance on the Operation of the Animals

Selected Abbreviations and Acronyms

CP = cardioplegia
HOE = 4-isopropyl-3-methylsulfonylbenzoyl-guanidine
HOE-694 = 3-methylsulfonyl-4-piperidinobenzoyl-guanidine

(Scientific Procedures) Act 1986, published by Her Majesty's Stationery Office, London, UK.

Experimental Preparation

Adult male Wistar rats (B & K, Hull, UK) weighing 250 to 320 g were anesthetized with sodium pentobarbitone (60 mg/kg intraperitoneally). Thirty seconds after administration of heparin (300 IU intravenously), the chest was opened, and the heart was excised and immersed in cold (4°C) perfusion medium, thus achieving ventricular standstill within 10 to 15 s. The aorta was immediately cannulated, and retrograde perfusion in the Langendorff mode was initiated at a constant pressure of 75 mm Hg. During Langendorff perfusion, the pulmonary artery was incised to facilitate coronary drainage, and the left atrium was cannulated to complete a working left heart circuit with a fixed preload of 15 mm Hg and afterload of 75 mm Hg. During perfusion in the working mode, aortic flow was measured by a flowmeter (Metergate, Hemel Hempstead, UK) positioned in-line with the aortic cannula, and coronary flow was calculated by timed collection of the coronary effluent. Aortic pressure was monitored via a pressure transducer connected to a side arm of the aortic cannula and recorded on a Gould RS3400 inkjet chart recorder (Cleveland, Ohio). Heart rate was derived from the pressure trace. Hearts that did not achieve an aortic flow of ≥40 mL/min and a spontaneous heart rate of ≥300 beats/min during an initial 20-minute period of aerobic perfusion in the working mode were excluded from the study (excluded hearts numbered <10% of the total used). The cardiac output (aortic flow plus coronary effluent) was filtered (5 μm pore size) and returned to the perfusion circuit via a roller pump (Watson Marlow, Falmouth, UK).

Solutions

All solutions were filtered (5 μm pore size) before use. The standard perfusion solution contained (in mmol/L): NaCl 118.5, KCl 4.7, $MgSO_4$ 1.2, KH_2PO_4 1.2, $NaHCO_3$ 25.0, $CaCl_2$ 1.4, and glucose 11.0. The perfusion solution was maintained at 37°C and gassed continuously with a mixture of 95% O_2 + 5% CO_2 (pH 7.4). St Thomas' Hospital cardioplegic solution (composition in mmol/L: NaCl 110, $MgCl_2$ 16, KCl 16, $CaCl_2$ 1.2, $NaHCO_3$ 10, pH adjusted to 7.8) was infused retrogradely via the aortic cannula at a constant perfusion pressure of 45 mm Hg at 37, 28 or 7.5°C. HOE (Hoechst-Marion-Roussel, Frankfurt, Germany) was dissolved in de-ionized water to make a stock solution of 1 mmol/L, which was stored at 4°C for up to 5 days. This was diluted in the perfusion solution or cardioplegic solution immediately before use to obtain a final concentration of 1 μmol/L. This concentration was chosen on the basis of our preliminary work, which has shown 1 μmol/L HOE to inhibit sarcolemmal Na^+/H^+ exchanger activity in rat ventricular myocytes by >75% and to provide optimal protection[15] (of a magnitude comparable to that afforded by ischemic preconditioning[16]) in isolated rat hearts subjected to normothermic global ischemia.

Study Protocols

In all protocols, basal values for aortic flow, coronary flow, cardiac output (the sum of aortic flow and coronary flow), and heart rate were measured before ischemia (at the end of the initial 20-minute period of perfusion in the working mode). In all cases, values obtained at the end of reperfusion (15 minutes in the Langendorff mode and 30 minutes in the working mode) were expressed as a percentage of their respective preischemic basal values to assess functional recovery. In some protocols, coronary effluent was collected during the Langendorff reperfusion period for assessment of creatine kinase leakage as an additional index of myocardial injury. Furthermore, because it has been suggested that Na^+/H^+ exchanger inhibition may preserve tissue high-energy phosphates by reducing Na^+ influx, and thereby Na^+/K^+ ATPase activity,[17] hearts were freeze-clamped at the end of the total reperfusion period for analysis of high-energy phosphate content.

Potential of HOE as an Alternative to CP

The objective of this study was to determine the cardioprotective efficacy of preischemic infusion of HOE relative to that of CP under normothermic conditions. To this end, after the assessment of basal function, three groups of hearts (n=6 per group) were perfused for an additional 5 minutes with standard perfusion solution (control), perfusion solution containing 1 μmol/L HOE, or St Thomas' Hospital cardioplegic solution, all at 37°C. Subsequently, hearts were subjected to global zero-flow ischemia at 37°C for 25 minutes.

Efficacy of HOE as an Adjunct or Additive to CP

Our objective was to test whether the use of HOE as an adjunct (ie, given during early reperfusion) or an additive (ie, included as a component of the cardioplegic solution) can enhance the cardioprotection afforded by CP. Studies were carried out at three temperatures; at each temperature, the ischemic duration used was one that resulted in a postischemic functional recovery of 10 to 30% in the CP alone group, thus allowing sufficient scope for any improvement arising from the adjunctive or additive use of HOE to be readily demonstrated.

Normothermia (37°C). All groups (n=6 hearts per group) underwent CP (37°C) for 5 minutes immediately before the induction of normothermic ischemia for 35 minutes. One group (CP) received no other intervention. In a second group (CP+repHOE), hearts received 1 μmol/L HOE as an adjunct during the first 5 minutes of Langendorff reperfusion, whereas in a third group (CP+HOE), 1 μmol/L HOE was used as an additive to the cardioplegic solution.

Moderate hypothermia (28°C). All groups (n=6 hearts per group) were again arrested by a 5-minute infusion of cardioplegic solution, this time at 28°C. Furthermore, the myocardium was maintained at this temperature during a subsequent 120-minute period of ischemia, with a 5-minute reinfusion of the cardioplegic solution after the first 60 minutes of ischemia. As above, one group (CP) received no additional intervention. In a second group (CP+repHOE), hearts received 1 μmol/L HOE as an adjunct during the first 5 minutes of Langendorff reperfusion, whereas in a third group (CP+HOE), 1 μmol/L HOE was used as an additive to the cardioplegic solution.

Severe hypothermia (7.5°C). As in the above two protocols, all groups (n=6 hearts per group) were subjected to CP for 5 minutes immediately before the induction of ischemia. However, this time the cardioplegic solution was infused at 7.5°C and hearts were subjected to 300 minutes of ischemia at this temperature. Once again, one group (CP) received no additional intervention, another (CP+repHOE) received 1 μmol/L HOE as an adjunct during the first 5 minutes of Langendorff reperfusion, and a third (CP+HOE) received 1 μmol/L HOE as an additive to the cardioplegic solution.

Biochemical Analyses

Creatine Kinase Leakage

Creatine kinase content of the coronary effluent collected during Langendorff reperfusion was assayed spectrophotometrically using a kit from Sigma Diagnostics (Poole, UK). Creatine kinase leakage was expressed in International Units (IU)/heart.

Basal (Preischemic) Values for Functional Indices

Study Group	Aortic Flow (mL/min)	Cardiac Output (mL/min)	Coronary Flow (mL/min)	Heart Rate (beats/min)
Potential of HOE as an alternative to cardioplegia				
Control	55±2	71±3	16±1	327±19
HOE	56±2	73±3	17±1	325±16
CP	56±3	73±3	17±1	340±6
Efficacy of HOE as an adjunct or additive to cardioplegia				
Normothermia (37°C)				
CP	59±2	77±2	18±1	340±17
CP+repHOE	58±2	73±3	15±1	331±15
CP+HOE	58±2	75±3	17±1	345±14
Moderate hypothermia (28°C)				
CP	63±2	82±2	19±1	330±15
CP+repHOE	67±2	85±3	19±1	354±8
CP+HOE	59±3	81±4	21±2	336±13
Severe hypothermia (7.5°C)				
CP	59±3	77±3	18±1	311±10
CP+repHOE	56±2	73±2	17±1	316±12
CP+HOE	58±4	76±4	18±1	310±10

n=6 hearts per group.
HOE indicates 1 μmol/L HOE-642 4-isopropyl-3-methylsulfonylbenzoyl-guanidine; CP, cardioplegia alone; CP+repHOE, cardioplegia plus HOE administered as an adjunct during the first 5 minutes of reperfusion; CP+HOE, cardioplegia with HOE included as an additive to the cardioplegic solution.

Myocardial High-Energy Phosphate Content

Frozen ventricular samples were stored in liquid nitrogen before lyophilization. Subsequently, the tissue contents of ATP, ADP, and creatine phosphate were analyzed by using high-pressure liquid chromatography, as previously described.[18] Data were expressed in micromoles per gram dry weight of ventricle.

Statistical Analyses

Experiments within each protocol were carried out in a randomized manner. Gaussian-distributed variables were expressed as mean±SEM and subjected to ANOVA. If a difference between groups was established, intergroup comparisons were performed using the Student-Newman-Keuls test. A value of $P<.05$ was considered significant.

Results

There was no significant difference among the study groups within each protocol in the basal values for functional indices (Table 1).

Potential of HOE as an Alternative to CP

The postischemic recovery of aortic flow was only 5±3% in the control group (with no recovery in three of the six hearts); this was improved to 18±4% with HOE ($P<.05$ versus control) and to 53±7% with CP ($P<.05$ versus control and HOE), with all hearts exhibiting some recovery of pump function in both of the latter groups. The postischemic recovery of cardiac output followed a pattern similar to that of aortic flow, with values of 16±3%, 35±4%, ($P<.05$ versus control), and 62±5% ($P<.05$ versus control and HOE) in the control, HOE, and CP groups, respectively. Recovery of coronary flow was 55±7% in the control group and was significantly improved to 85±7% and 86±6% in the HOE and CP groups, respectively. Heart rate recovered fully in all groups, excluding the three hearts in the control group, which exhibited no recovery of pump function.

Efficacy of HOE as an Adjunct or Additive to CP

Normothermia (37°C)

As illustrated in Fig 1A, the postischemic recovery of aortic flow was 9±3% in the CP group. This was significantly improved to 18±3% by the adjunctive use of HOE during early reperfusion (CP+repHOE). However, the best recovery of 27±4% ($P<.05$ versus CP and CP+repHOE) was observed when HOE was used as an additive to CP (CP+HOE). The postischemic recovery of cardiac output followed a similar pattern, with values of 21±3, 31±3 ($P<.05$ versus CP), and 41±4% ($P<.05$ versus CP and CP+repHOE) in the CP, CP+repHOE, and CP+HOE groups, respectively. The recovery of coronary flow was similar (74±3, 76±3, and 83±3%), and heart rate recovered to basal values in all groups. The intergroup differences in the recovery of pump function were reflected by differences in creatine kinase leakage (Fig 1B), which measured 12.6±1.6 IU in the control group, 9.7±1.3 IU in the CP+repHOE group, and 4.7±0.7 IU in the CP+HOE group ($P<.05$ versus CP and CP+repHOE).

Moderate Hypothermia (28°C)

Under conditions of moderate hypothermia with multidose cardioplegic infusion, the adjunctive use of HOE again improved the myocardial preservation afforded by CP; however, once again, the greatest benefit was obtained when the Na^+/H^+ exchanger inhibitor was used as an additive to the cardioplegic solution. Thus, the postischemic recovery of aortic flow was improved from 16±3% with CP to 32±3% with CP+repHOE ($P<.05$ versus CP) and to 50±4% with CP+HOE ($P<.05$ versus CP and CP+repHOE) (Fig 2A). The corresponding values for cardiac output were 30±3%, 40±3% ($P<.05$ versus CP), and 60±3% ($P<.05$ versus CP and CP+repHOE). Once again, coronary flow recovered to a similar extent (77±3%, 71±5%, and 85±4%), and heart rate recovered fully in all groups. Creatine kinase

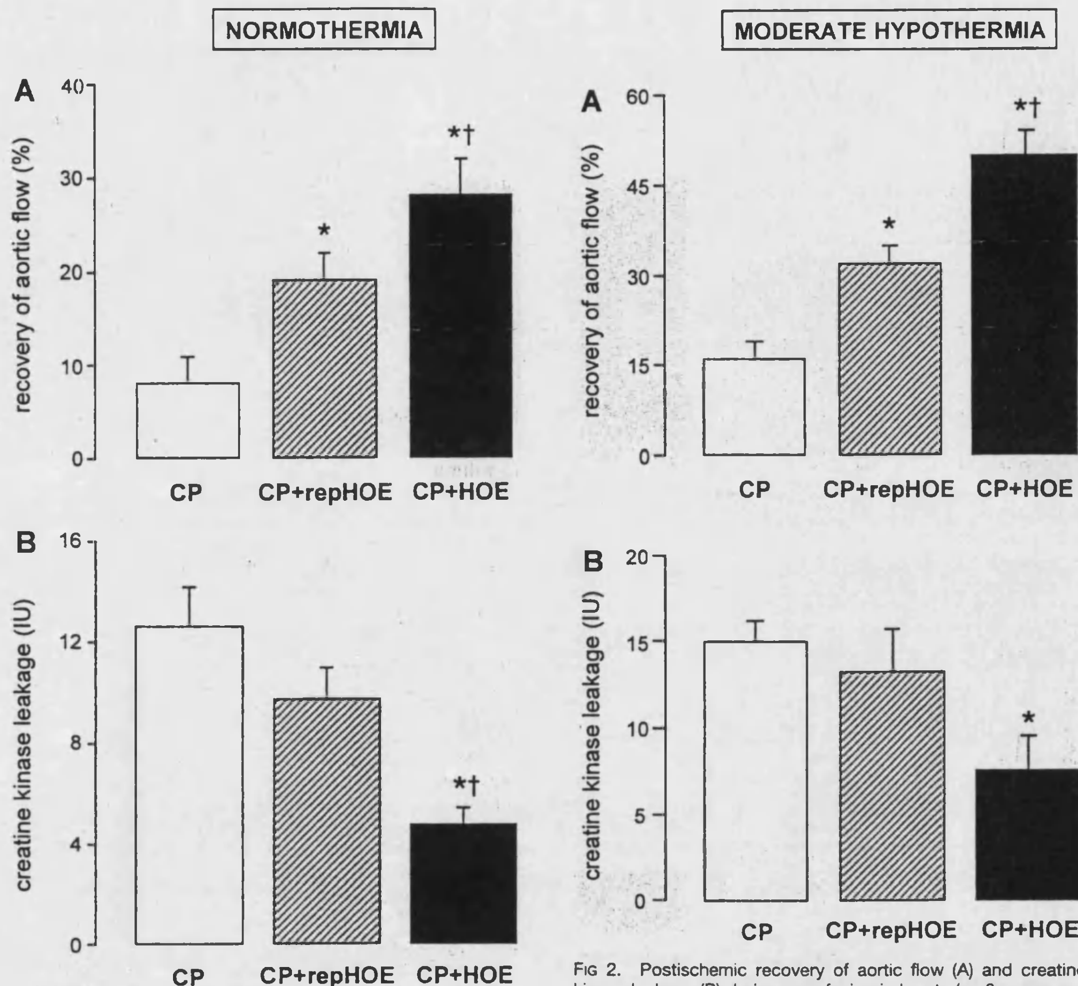

FIG 1. Postischemic recovery of aortic flow (A) and creatine kinase leakage (B) during reperfusion in hearts (n=6 per group) arrested by a 5-minute infusion of St Thomas' Hospital cardioplegic solution (37°C) and subjected to 35 minutes of global ischemia at 37°C. CP indicates cardioplegia alone; CP+repHOE, cardioplegia plus HOE (1 μmol/L) administered as an adjunct during the first 5 minutes of reperfusion; CP+HOE, cardioplegia with HOE (1 μmol/L) included as an additive to the cardioplegic solution. **P*<.05 versus CP, †*P*<.05 versus CP+repHOE.

FIG 2. Postischemic recovery of aortic flow (A) and creatine kinase leakage (B) during reperfusion in hearts (n=6 per group) arrested by a 5-minute infusion of St Thomas' Hospital cardioplegic solution (28°C) and subjected to 120 minutes of global ischemia at 28°C, with a 5-minute reinfusion of the cardioplegic solution after 60 minutes. CP indicates cardioplegia alone; CP+repHOE, cardioplegia plus HOE (1 μmol/L) administered as an adjunct during the first 5 minutes of reperfusion; CP+HOE, cardioplegia with HOE (1 μmol/L) included as an additive to the cardioplegic solution. **P*<.05 versus CP, †*P*<.05 versus CP+repHOE.

leakage was unaltered by the adjunctive use of HOE (CP+repHOE) but was significantly reduced when the drug was used as an additive (CP+HOE) (Fig 2B). Despite the intergroup differences in the postischemic recovery of pump function and creatine kinase leakage, there were no significant differences in the tissue contents of ATP (10.3±0.6, 10.3±0.4, and 10.5±0.2 μmol/g), ADP (2.2±0.1, 2.2±0.1, and 2.3±0.1 μmol/g), and creatine phosphate (12.2±1.2, 12.2±1.0, and 15.8±1.1 μmol/g) in the CP, CP+repHOE, and CP+HOE groups, respectively, at the end of reperfusion.

Severe Hypothermia (7.5°C)

In this protocol involving cardioplegic arrest and an extended period (300 minutes) of global ischemia at 7.5°C, the use of HOE as an adjunct or additive to CP provided additional benefit, with the latter affording the greatest protection. As illustrated in Fig 3A, the postischemic recovery of aortic flow was 30±4% in the CP group, and this was significantly improved to 45±5% in the CP+repHOE group and to 63±5% in the CP+HOE group. The postischemic recovery of aortic flow with CP+HOE was significantly greater than that with CP+repHOE. The recovery of cardiac output was 44±4%, 56±5% (*P*<.05 versus CP), and 72±5% (*P*<.05

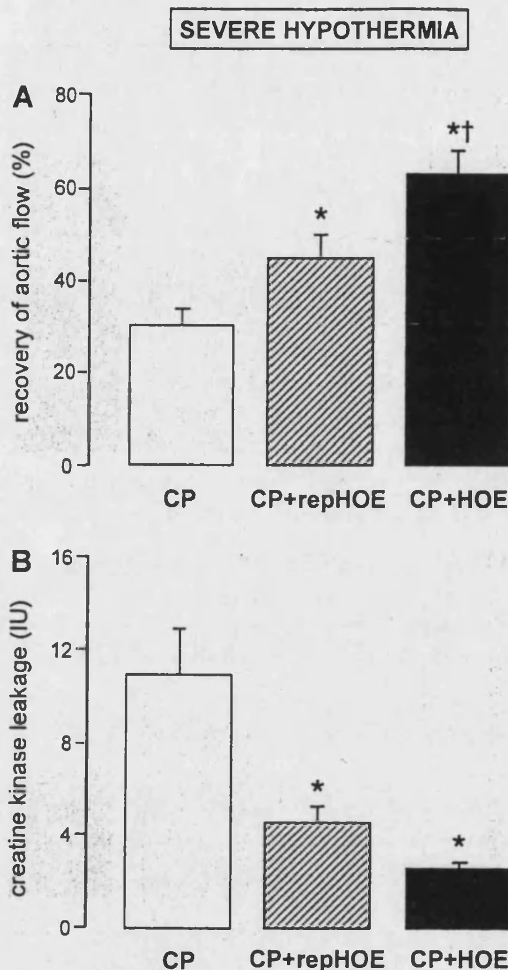

FIG 3. Postischemic recovery of aortic flow (A) and creatine kinase leakage (B) during reperfusion in hearts (n=6 per group) arrested by a 5-minute infusion of St Thomas' Hospital cardioplegic solution (7.5°C) and subjected to 300 minutes of global ischemia at 7.5°C. CP indicates cardioplegia alone; CP+repHOE, cardioplegia plus HOE (1 μmol/L) administered as an adjunct during the first 5 minutes of reperfusion; CP+HOE, cardioplegia with HOE (1 μmol/L) included as an additive to the cardioplegic solution. *P<.05 versus CP, †P<.05 versus CP+repHOE.

versus CP and CP+repHOE) in the CP, CP+repHOE, and CP+HOE groups, respectively. The corresponding values for coronary flow were 84±5%, 90±5%, and 96±4% (P=NS), and heart rate recovered to basal values in all groups. Under these conditions, creatine kinase leakage was significantly reduced from 11.0±2.0 IU with CP to 4.6±0.7 IU by the use of HOE as an adjunct (CP+repHOE) and to 2.6±0.3 IU by the use of the drug as an additive (CP+HOE) (Fig 3B). Once again, the significant intergroup differences in the postischemic recovery of pump function and creatine kinase leakage were not reflected by the tissue contents of ATP (15.7±0.4, 16.2±0.3, and 17.1±0.5 μmol/g), ADP (2.8±0.1, 3.2±0.1, and 3.1±0.1 μmol/g), and creatine phosphate (17.3±1.3, 19.0±1.9, and 20.3±0.5 μmol/g) in the CP, CP+repHOE, and CP+HOE groups, respectively, at the end of reperfusion.

Discussion

The present study is the first to characterize the cardioprotective efficacy of HOE as an alternative, adjunct, or additive to CP. Our findings show that the preischemic infusion of HOE affords significant cardioprotection under normothermic conditions; however, this is not as great as that provided by CP. Nevertheless, as an adjunct or an additive, HOE significantly enhances the cardioprotective efficacy of CP under both normothermic and hypothermic conditions.

Pharmacological Inhibitors of the Na/H Exchanger

Amiloride and its structural derivatives are known to inhibit the Na/H exchanger with variable potency[4] and have been frequently used as pharmacological tools to investigate the role of the sarcolemmal Na^+/H^+ exchanger in the genesis of myocardial injury during ischemia and reperfusion (for a recent review, see Avkiran[3]). However, the utility of these agents is limited by their nonspecificity[4] and, of particular relevance
to studies of cardiac pathophysiology, their ability to depress contractile function[5,7] and to induce electrophysiological effects[5,19–21] in ventricular myocardium. The recently developed benzoylguanidine-based agents HOE-694 and HOE offer significant advantages over amiloride and its derivatives, both as pharmacological tools and as potential therapeutic agents, because of: (i) a high inhibitory potency on the sarcolemmal Na^+/H^+ exchanger,[15,22] and (ii) a lack of effect on cardiac contractile function,[7,12] electrogenic Na^+ transport pathways (such as the Na^+/Ca^{2+} exchanger and the noninactivating Na^+ current),[12] and pH_i-regulatory mechanisms other than Na^+/H^+ exchanger.[22] As stated in a recent editorial,[23] HOE in particular represents a highly attractive potential therapeutic agent because of its high selectivity for the predominant Na^+/H^+ exchanger isoform expressed in the heart. Indeed, this agent has now been selected for clinical evaluation as the prototype of a novel cardioprotective approach in the therapy of acute coronary syndromes. For these reasons, in the present study, we chose to use HOE in preference to other Na^+/H^+ exchanger inhibitors.

Potential of HOE as an Alternative to CP

Consistent with our previous studies using HOE-694 in a similar model with less severe normothermic ischemia,[11] the present study has shown that HOE alone can provide significant cardioprotection when given as a pretreatment. Nevertheless, the protection was not as great as that afforded by CP, which suggests that HOE is unlikely to be a viable alternative to CP.

It is well established that the primary protective mechanism of CP is membrane depolarization and the rapid induction of diastolic arrest, resulting in the preservation of myocardial high-energy phosphates.[1] As part of a spectrum of beneficial actions in maintaining cellular integrity during ischemia and reperfu-

sion,[1] such preservation of tissue high-energy phosphates is also likely to aid the maintenance and/or restoration of cellular ionic homeostasis by preserving the activity of energy-consuming ion transporters (such as the sarcolemmal Na^+/K^+ ATPase and the sarcolemmal/sarcoplasmic reticular Ca^{2+} ATPases). Additionally, CP attenuates intracellular acidosis during early ischemia,[24] which would be expected to limit sarcolemmal Na^+/H^+ exchanger activity because the exchanger is stimulated primarily by a reduction in pH_i.[25] In contrast, the primary (and possibly the sole) cardioprotective mechanism of HOE, when given alone, is the direct inhibition of sarcolemmal Na^+/H^+ exchanger activity. Thus, it is probable that the greater cardioprotective efficacy of CP relative to HOE arises from the broader range of salutary actions of the former intervention on the detrimental processes that accompany ischemia and reperfusion.[1]

Efficacy of HOE as an Adjunct or Additive to CP at Normothermia

In the present study, the use of HOE either as an adjunct or as an additive was found to significantly enhance the cardioprotective efficacy of CP under normothermic conditions. However, the maximal benefit was obtained when HOE was infused into the coronary vasculature before the onset of ischemia, as an additive to the cardioplegic solution, rather than as an adjunct during early reperfusion. This is consistent with our earlier study with HOE-694 in the absence of CP,[11] which revealed superior protection with preischemic versus postischemic treatment in hearts perfused with bicarbonate-buffered medium. These findings suggest that in rat hearts subjected to cardioplegic arrest, Na^+/H^+ exchanger activity during both ischemia and subsequent reperfusion contributes to myocardial injury. Thus, HOE can provide significant benefit when used either as an additive or as an adjunct, and the superior protection afforded by the former approach probably arises from reduced Na^+/H^+ exchanger activity during ischemia, as well as during reperfusion. In this regard, it is worth noting that our results with HOE are consistent with those of a recent study by Koike et al,[26] in which the cardioprotective efficacy of dimethylamiloride (a nonspecific Na^+/H^+ exchanger inhibitor) as an additive to CP was investigated, and suggest that Na^+/H^+ exchanger inhibition may be a common mechanism of the benefit afforded by both drugs.

In considering the mechanism(s) that might underlie the superior myocardial protection afforded by the additive (cf. adjunctive) use of a Na^+/H^+ exchanger inhibitor, an important issue to address is whether the sarcolemmal Na^+/H^+ exchanger retains significant activity during ischemia in hearts subjected to cardioplegic arrest. In this regard, studies with nuclear magnetic resonance spectroscopy and amiloride-based Na^+/H^+ exchanger inhibitors[27,28] suggest that in the absence of cardioplegic arrest, sarcolemmal Na^+/H^+ exchanger activity contributes significantly to intracellular Na^+ accumulation during ischemia. Thus, the extracellular acidosis that develops during ischemia (and may in fact exceed intracellular acidosis[29]) does not seem to abolish sarcolemmal Na^+/H^+ exchanger activity, despite the known inhibitory effects of a low pH_o on the exchanger.[30,31] In this regard, Vaughan-Jones and Wu[31] have shown that H^+ efflux (and concomitant Na^+ influx) via the sarcolemmal Na^+/H^+ exchanger can be maintained even when the trans-sarcolemmal H^+ gradient is inwardly directed, provided pH_i is sufficiently low. Within the context of the present study, it is important to note that cardioplegic arrest attenuates but does not abolish the induction of intracellular acidosis during normothermic ischemia.[24] Thus, it is reasonable to propose that hearts subjected to cardioplegic arrest may retain significant Na^+/H^+ exchanger activity during ischemia and that inhibition of this activity may contribute to the superior protection afforded by the additive use of agents such as HOE and dimethylamiloride.

Efficacy of HOE Under Conditions of Hypothermia

In many surgical settings, cardioplegic arrest is combined with hypothermia to achieve optimal myocardial preservation.[1] It is therefore important to determine whether the benefit afforded by the adjunctive or additive use of HOE is sustained under hypothermic conditions. The importance of this is further highlighted by the ability of hypothermia per se to significantly alter cellular ionic homeostasis. With particular reference to Na^+/H^+ exchanger, there is evidence that hypothermia increases the activity of this exchanger, in both erythrocytes[32] and cultured chick embryonic myocytes.[33] Furthermore, hypothermia may inhibit the activity of the primary Na^+ extrusion pathway, the sarcolemmal Na^+/K^+ ATPase.[33] Thus, it is probable that although hypothermia undoubtedly provides substantial benefit when combined with chemical CP,[1] it may also induce a potentially detrimental response by promoting intracellular Na^+ accumulation. Of particular relevance to the present study, the studies of Askenasy and colleagues[34] in isolated rat hearts have shown that intracellular Na^+ accumulation during hypothermic ischemia increases with decreasing temperature and that an amiloride-sensitive mechanism contributes significantly to such accumulation under conditions of severe hypothermia (4°C).[14]

In the present study, we have studied the effects of using HOE as an adjunct or additive to CP under two hypothermic conditions: at 28°C with multidose cardioplegic infusion (as often employed during cardiac surgery) and at 7.5°C (as may be used for donor heart preservation for transplantation). It is possible that in the former setting, the moderate degree of hypothermia may limit the temperature-mediated effects on ion transporters such as the Na^+/H^+ exchanger and Na^+/K^+ ATPase; however, sarcolemmal Na^+/H^+ exchanger activity may still play an important role because this is likely to be stimulated by the washout of the extracellular medium (and, hence, the relative alkalinization of pH_o) during the second cardioplegic infusion. Indeed, superior benefit was afforded by the use of HOE as an additive at both 28 and 7.5°C. Under both hypothermic conditions, the myocardial high-energy phosphate content was well preserved by CP and was not altered significantly by the adjunctive or additive use of HOE. This supports the hypothesis that the benefit obtained from the use of HOE in combination with CP is likely to have arisen from an

improved maintenance of myocardial ionic homeostasis rather than from metabolic effects. Our findings are consistent with previous studies in hearts subjected to cardioplegic arrest under normothermic[26] and hypothermic (4°C)[35] conditions, in which the use of other Na^+/H^+ exchanger inhibitors in combination with CP has been shown to improve the postischemic recovery of contractile function without an effect on myocardial high-energy phosphate content.

Potential Limitations of Study

In the present study, changes in pH_i or the intracellular Na^+ concentration were not determined during cardioplegic arrest in the presence or absence of HOE. Thus, the mechanism(s) of the additive protective effect of HOE cannot be determined definitively. It is interesting to note that pretreatment with a Na^+/H^+ exchanger inhibitor (given either as an additive to CP[26] or alone[14]) has been shown to increase intracellular acidosis under normothermic[26] and hypothermic[14] conditions, but only after periods of ischemia longer than those used here under comparable conditions. It is possible that with such prolonged durations of ischemia, Na^+/H^+ exchanger inhibition may have an attenuated beneficial impact on functional recovery, perhaps by promoting apoptosis due to an exacerbation of intracellular acidosis.[36]

Clinical Implications

The present study has shown that in an experimental model of cardiopulmonary bypass, the use of the specific and potent Na^+/H^+ exchanger inhibitor HOE as an adjunct or additive to CP provides a significant improvement in myocardial preservation under conditions of normothermia, moderate hypothermia, and severe hypothermia. At all temperatures studied, the best preservation was obtained when HOE was used as an additive to CP. Provided the safety and cardioprotective efficacy of HOE in humans is proven by the ongoing clinical trials, then this agent may be of significant benefit when used in combination with CP for myocardial preservation in cardiac surgery and transplantation.

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Rational Basis for Use of Sodium-Hydrogen Exchange Inhibitors in Myocardial Ischemia

Metin Avkiran, PhD

The cardiac sarcolemmal Na^+/H^+ exchanger extrudes intracellular H^+ in exchange for Na^+, in an electroneutral process. Of the 6 mammalian exchanger isoforms identified to date, the Na^+/H^+ exchanger (NHE)-1 is believed to be the molecular homolog of the sarcolemmal Na^+/H^+ exchanger. The exchanger is activated primarily by a reduction in intracellular pH (intracellular acidosis), although such activation is subject to modulation by a variety of endogenous mediators (e.g., catecholamines, thrombin, endothelin) through receptor-mediated mechanisms. A large body of preclinical evidence now suggests that inhibition of the sarcolemmal Na^+/H^+ exchanger attenuates many of the unfavorable consequences of acute myocardial ischemia and reperfusion. Much of this evidence has been obtained with recently developed potent, selective inhibitors of the exchanger, such as HOE-642 (cariporide) and its structurally related congener HOE-694, in studies using both in vitro and in vivo models of ischemia and reperfusion in a variety of species. The data from these studies indicate that Na^+/H^+ exchange inhibition leads to a decreased susceptibility to severe ventricular arrhythmia, attenuates contractile dysfunction, and limits tissue necrosis (i.e., decreases infarct size) during myocardial ischemia and reperfusion. Such protection is likely to arise, at least in part, from attenuation of "Ca^{2+} overload," which has been linked causally with all of these pathologic phenomena. The consistent and marked cardioprotective benefit that has been observed with cariporide and related compounds in preclinical studies suggests that Na^+/H^+ exchange inhibition may represent a novel and effective approach to the treatment of acute myocardial ischemia in humans. **©1999 by Excerpta Medica, Inc.**

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Na^+/H^+ exchangers (NHEs) are membrane proteins that transport H^+ in exchange for Na^+ in an electroneutral manner, utilizing for their driving force the transmembrane Na^+ gradient.[1-3] To date, 6 mammalian isoforms of NHE have been identified; these have been termed NHE-1–6.[4-7] NHE-1–4 are located in the plasma membrane; little is known about the localization and function of NHE-5, and NHE-6 appears to be the molecular homolog of the mitochondrial exchanger. With respect to tissue distribution, NHE-1 and NHE-6 are ubiquitous (the latter expressed abundantly in tissues with high mitochondrial content), whereas NHE-2–5 show restricted expression.[4-7] To date, only NHE-1 and NHE-6 transcripts have been detected in the heart,[4,7] and it is believed that the sarcolemmal NHE of cardiac myocytes is composed predominantly, if not exclusively, of the NHE-1 isoform.[8]

Recently, the sarcolemmal NHE has received attention as a potential mediator of various physiologic and pathophysiologic phenomena in myocardium, such as increased myofilament sensitivity to Ca^{2+} in response to stimulation of a variety of G protein-coupled receptors[9-11] and the induction of hypertrophy by both mechanical[12] and neurohormonal[13] stimuli. However, perhaps the strongest evidence for an important role for the sarcolemmal NHE in cardiac pathophysiology is that which implicates the exchanger in the unfavorable sequelae of ischemia and reperfusion, such as arrhythmia, contractile dysfunction, and infarction. Such evidence was obtained initially with nonspecific NHE inhibitors (NHEIs), such as amiloride and its 5-amino-substituted derivatives (for reviews, see Avkiran[14] and Fröhlich and Karmazyn[15]), and has been substantiated by more recent studies with novel benzoylguanidine derivatives, such as HOE-694,[16] HOE-642 (cariporide),[17] and EMD-85131.[18] These agents have proved to be of considerable investigative value since they are potent inhibitors of NHE,[16-18] do not appear to inhibit other ion transport[16,17] or pH regulatory[19] systems, and exhibit marked selectivity for the NHE-1 isoform of the exchanger.[17,18,20] Published studies that have investigated the effects of these novel NHEIs on arrhythmia, contractile dysfunction, and infarct size in animal models of myocardial ischemia and reperfusion are briefly reviewed below.

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CARDIOPROTECTIVE EFFECTS OF NHEI IN ISCHEMIA AND REPERFUSION

Ventricular arrhythmia: Several studies have shown NHEIs to exhibit marked efficacy against severe ventricular arrhythmia, in particular ventricular fibrillation (VF), during both ischemia and reperfusion. Scholz et al[16] were the first to report that HOE-694, included in the perfusion solution throughout, inhibits reperfusion-induced VF in isolated rat hearts subjected to 15 minutes of regional ischemia. In the same study,[16] evidence was also presented that preischemic administration of HOE-694 abolishes the incidence of

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VF during 15 minutes of regional ischemia in the anesthetized rat in vivo. Yasutake et al[21] subsequently showed that selective infusion of HOE-694 into the ischemic/reperfused zone of isolated rat hearts subjected to 10 minutes of regional ischemia suppresses the incidence of reperfusion-induced VF in a dose-dependent manner. Interestingly, similar protection was observed, regardless of whether HOE-694 was administered before ischemia and during reperfusion or during reperfusion alone, suggesting that NHE activity during reperfusion was the most important determinant of susceptibility to reperfusion-induced VF in this model.[21] The potent inhibitory effect of HOE-694 on reperfusion-induced VF has also been demonstrated in the pig in vivo.[22] More recent studies have shown that cariporide shares the antiarrhythmic efficacy of HOE-694 in the setting of myocardial ischemia and reperfusion, indicating that this is not a drug-specific, but a class effect. Thus, cariporide, given before coronary artery occlusion, has been shown to inhibit ischemia and reperfusion-induced VF in isolated rat hearts,[17] and in rat[17,23] and dog[23] hearts in vivo. Therefore, the evidence that NHEIs inhibit potentially lethal ventricular arrhythmia appears well established; it is important to note, however, that this effect is not mediated via a classic antiarrhythmic drug action (i.e., inhibition of electrogenic ion current[s]) and most likely occurs as a consequence of decreased severity of myocardial injury during ischemia and reperfusion.

Contractile dysfunction: A critical consequence of ischemia is a deficit in myocardial contractile capacity (and hence in ventricular function) that is maintained after the restoration of coronary flow. In most species, the contractile dysfunction observed during reperfusion after relatively short periods (up to 15–20 minutes) of ischemia is reversible, and normal function can be restored after prolonged reperfusion (this phenomenon has been termed "myocardial stunning"). After longer periods of ischemia, the contractile dysfunction observed during reperfusion is not reversible since it arises, at least in part, from myocardial necrosis. The evidence in the literature suggests that NHEIs can protect against both forms of contractile dysfunction.

In isolated working rat hearts subjected to 20 minutes of global ischemia, du Toit and Opie[24] showed that HOE-694 attenuated myocardial stunning to a similar extent when given before ischemia or during early reperfusion. In contrast, Shimada et al[25] reported that, in rat hearts subjected to 20 minutes of global ischemia in the presence of bicarbonate-containing perfusate (as in the study by du Toit and Opie[24]), HOE-694 provided no significant improvement in postischemic contractile recovery when given only during reperfusion. Nevertheless, the drug afforded substantial benefit when it was administered before the onset of ischemia.[25] That pretreatment with HOE-694 improves the postischemic recovery of contractile function has been demonstrated also in a porcine model of myocardial stunning, in which HOE-694 was given as a preischemic intravenous bolus followed by continuous infusion.[22] Thus, although there is agreement among studies from different laboratories that NHEIs can significantly attenuate contractile dysfunction after relatively short periods of ischemia, some controversy exists as to whether the primary benefit arises from NHE inhibition during ischemia itself or during subsequent reperfusion.

The efficacy of NHEIs against postischemic contractile dysfunction has also been studied with longer durations of ischemia in a variety of models. Hendrikx et al[26] found that, in isolated blood-perfused rabbit hearts subjected to 45 minutes of global ischemia, preischemic administration of HOE-694 significantly improved the recovery of contractile function after reperfusion. In contrast, administration of HOE-694 during reperfusion alone provided only marginal benefit.[26] Electron-microscopic assessment of tissue sections in this study revealed marked preservation of ultrastructural integrity in myocytes from hearts pretreated with the NHEI, with complete absence of classic manifestations of irreversible injury.[26] In pig hearts in vivo subjected to 45 minutes of regional ischemia, preischemic administration of HOE-694 significantly improved regional systolic shortening after 24 hours of reperfusion, whereas no such benefit was seen when the drug was given shortly before reperfusion.[27] Similarly, in isolated working rat hearts subjected to global low-flow ischemia for 60 minutes, the presence of HOE-694 in the perfusion medium from 15 minutes before the onset of ischemia significantly improved recovery of aortic flow during reperfusion. In contrast, late administration of the drug (from 15 or 30 minutes after the onset of ischemia) was of no significant benefit.[28] Thus, as with shorter periods of ischemia, the consistent finding is that preischemic treatment with NHEI significantly improves recovery of contractile function during reperfusion. In contrast, any benefit afforded by these drugs when given only during reperfusion is insignificant or, at best, inconsistent.

In a recent study, Shipolini et al[29] have compared the ability of cariporide to improve postischemic contractile function with that of ischemic preconditioning, which is widely accepted as a potent cardioprotective intervention. It was found that, in isolated rat hearts subjected to 60 minutes of global ischemia, preischemic administration of cariporide and ischemic preconditioning afforded similar improvements in the recovery of left ventricular-developed pressure during reperfusion.[29] Interestingly, with each cardioprotective intervention, improved recovery of contractile function during reperfusion was associated with decreased creatine kinase leakage, suggesting that at least part of the functional benefit arose from a reduction in myocardial necrosis.[29]

Myocardial necrosis: The first direct evidence that novel NHEIs inhibit the development of myocardial necrosis during ischemia and reperfusion came to light in 1995, in the form of 2 reports on the effects of HOE-694 on infarct size in pig hearts subjected to regional ischemia in vivo.[27,30] Rohmann et al[30] reported that HOE-694 given before ischemia signifi-

cantly decreased infarct size (assessed at the end of reperfusion by tetrazolium staining and expressed relative to the volume of ischemic ventricular myocardium at risk of necrosis), from 78 ± 4% in controls to 4 ± 1%. Interestingly, HOE-694 given shortly before reperfusion also decreased infarct size significantly (to 38 ± 6%), which was presented as evidence for the existence of "reperfusion injury."[30] Consistent findings with respect to the effects of pretreatment with HOE-694 were reported by Klein et al,[27] who also showed significant reduction in infarct size, from 65 ± 7% in controls to 13 ± 3%. In this study, however, HOE-694 produced no significant reduction in infarct size when given shortly before reperfusion.[27]

Since 1995, 4 additional studies have been published in which the relative efficacy in decreasing infarct size of preischemic versus prereperfusion treatment with an NHEI (cariporide or EMD-85131) has been reported. Garcia-Dorado et al[31] and Miura et al[32] have obtained data in the pig and rabbit, respectively, that are consistent with the observations of Klein et al,[27] while Linz et al[33] in the rabbit and Gumina et al[18] in the dog have reported findings that support the observations of Rohmann et al.[30] The main findings of these important and potentially clinically relevant studies are summarized in Figure 1. Despite the differences in the NHEI and/or the dose used, the species under study, and the durations of ischemia and reperfusion, the consistent finding, which once again emerges, is that a large and significant cardioprotective benefit is obtained when the NHEI is administered before onset of ischemia. In contrast, when the NHEI is administered shortly before reperfusion, only an intermediate (and, in half of the studies, insignificant) effect is observed. It appears, therefore, that although NHE activity during both ischemia and reperfusion may contribute to the overall extent of myocardial injury, exchanger activity during ischemia is the more important factor, such that inhibition of the exchanger during this period slows progression of injury and allows greater myocardial salvage by reperfusion.

The Klein group has recently published 2 further studies[34,35] that may help better quantify the benefit afforded by pretreatment with an NHEI and ascertain the period during which the exchanger needs to be inhibited to achieve reduction in infarct size. The first of these studies[34] showed that infarct size obtained in pigs pretreated with cariporide and subjected to 70 minutes of regional myocardial ischemia was comparable to that seen in untreated controls subjected to 45 minutes of ischemia. The investigators interpreted this to suggest that, in this model, NHE inhibition increases tolerance to ischemia/reperfusion-induced injury by approximately 25 minutes. An alternative interpretation of these data may be that, in this model, the window for myocardial salvage (see later) is extended by at least 25 minutes, since precise quantification of the "bought time" would require determination of the duration of ischemia that results in maximum infarct size with and without pretreatment with the NHEI. In a subsequent study, in pigs subjected to 60 minutes of regional ischemia with residual flow, Klein et al[35] showed that a significant reduction in infarct size could be obtained with cariporide administered 15 minutes after the onset of ischemia. However, such benefit was lost when cariporide was administered 45 minutes after the onset of ischemia, suggesting that, even in the presence of residual flow, inhibition of NHE during early ischemia is essential to achieve reduction in infarct size.

MECHANISM(S) OF CARDIOPROTECTIVE ACTIONS OF NHEI

The most likely mechanism through which NHEIs exert their cardioprotective action is attenuation of the loss of Na^+ and Ca^{2+} homeostasis, and subsequent "Ca^{2+} overload," during ischemia and reperfusion,[15] particularly since Ca^{2+} overload has been causally implicated in the genesis of VF, contractile dysfunction, and tissue necrosis in this setting. The original "Lazdunski hypothesis"[36] proposed that increased sarcolemmal NHE activity, arising from the rapid normalization of extracellular pH and the generation of an outwardly directed H^+ gradient upon reperfusion, may play a role in reperfusion injury. Although this hypothesis is supported by studies that have shown cardioprotective benefit with NHEIs when given shortly before or during reperfusion, it is not consistent with the majority of studies that have shown superior benefit when the NHEI is given before or early during ischemia (see above). In this regard, the evidence that NHEIs attenuate intracellular Na^+[37–39] and Ca^{2+}[38] accumulation during ischemia in parallel with their cardioprotective effects also suggests that NHE activity is maintained during ischemia and contributes substantially to the loss of ionic homeostasis and the overall extent of injury.

Figure 2 summarizes the likely sequence of events that involves the sarcolemmal NHE during ischemia and reperfusion, as suggested by currently available data. Although the exchanger is quiescent under basal conditions, it becomes activated during ischemia in response to intracellular acidosis,[36,40] which is known to develop rapidly,[41] and possibly to other NHE-stimulatory factors (see below). The resulting influx of Na^+, occurring in the presence of ischemia-induced inhibition of Na^+/K^+ adenosine triphosphatase (the primary Na^+ extrusion pathway from the cardiac myocyte), causes the intracellular accumulation of Na^+. Such an increase in intracellular Na^+ concentration in turn alters the reversal potential of the Na^+/Ca^{2+} exchanger in a manner that inhibits Ca^{2+} efflux and/or enhances Ca^{2+} influx through this bidirectional mechanism—the end result being a pathologic increase in intracellular Ca^{2+} (i.e., Ca^{2+} overload). NHEIs are likely to afford their cardioprotective effect during ischemia, at least in part by inhibiting this sequence at an early stage, through the limitation of Na^+ influx. An important caveat to this probable scheme is that the studies that showed NHEIs to decrease intracellular Na^+ accumulation during ischemia used amiloride and its analogs,[37–39] and the pos-

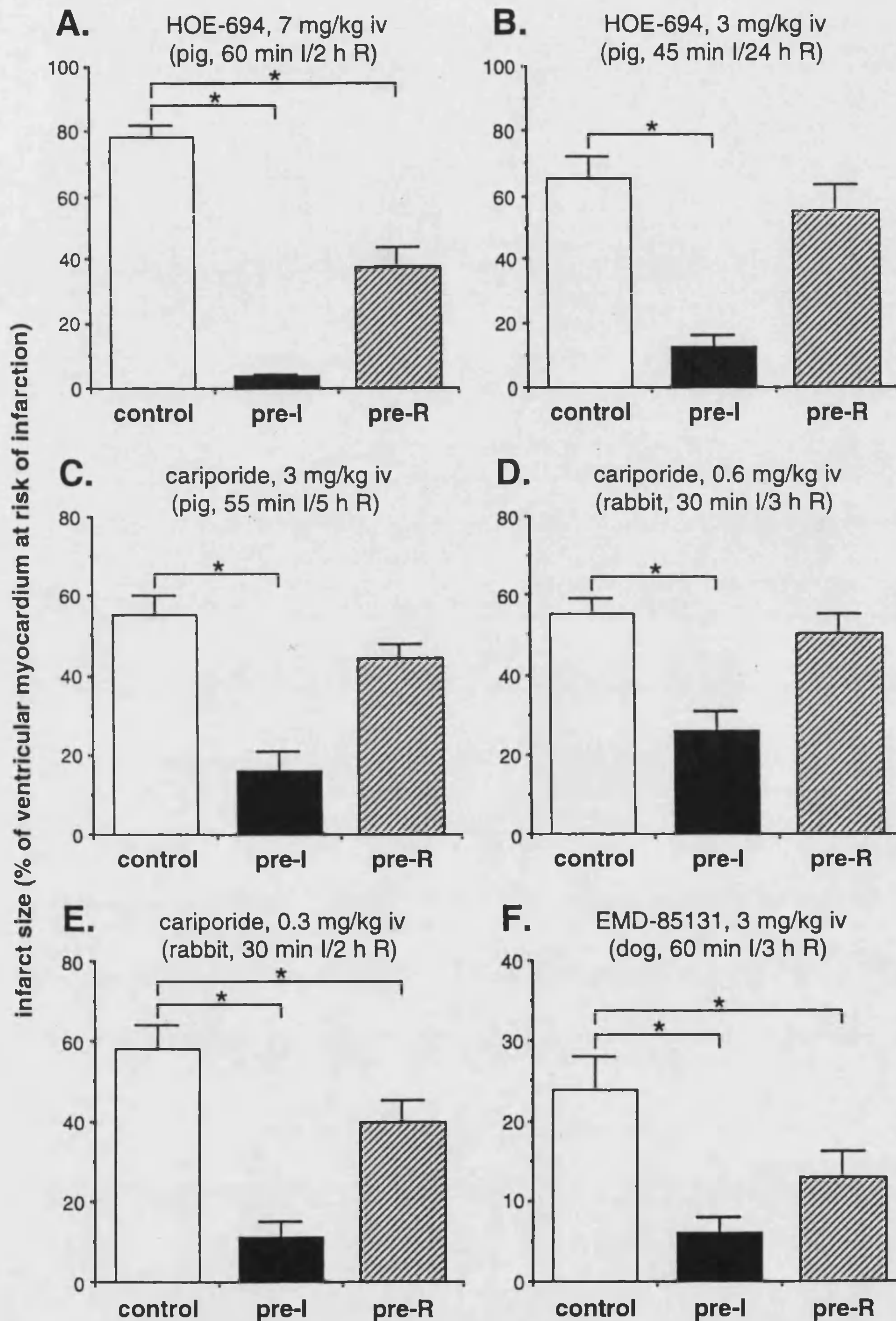

FIGURE 1. Infarct size, assessed at the end of the reperfusion period by tetrazolium staining and expressed as a percentage of the volume of ischemic ventricular myocardium at risk of infarction, in animals receiving an intravenous (iv) injection or short infusion of an Na^+/H^+ exchange inhibitor (HOE-694, cariporide, or EMD-85131), either 10–15 minutes before occlusion of an epicardial coronary artery to induce regional ischemia (pre-I) or 5–15 minutes before release of the occluder to institute reperfusion (pre-R). Data are from (*A*) Rohmann et al,[30] (*B*) Klein et al,[27] (*C*) Garcia-Dorado et al,[31] (*D*) Miura et al,[32] (*E*) Linz et al,[33] and (*F*) Gumina et al.[18] Note that, under control conditions, dog hearts (*F*) exhibit a smaller infarct size compared with pig (*A–C*) and rabbit (*D,E*) hearts subjected to an equivalent or shorter duration of ischemia, due to a greater collateral flow in the first species. I = ischemia; R = reperfusion. *$p < 0.05$.

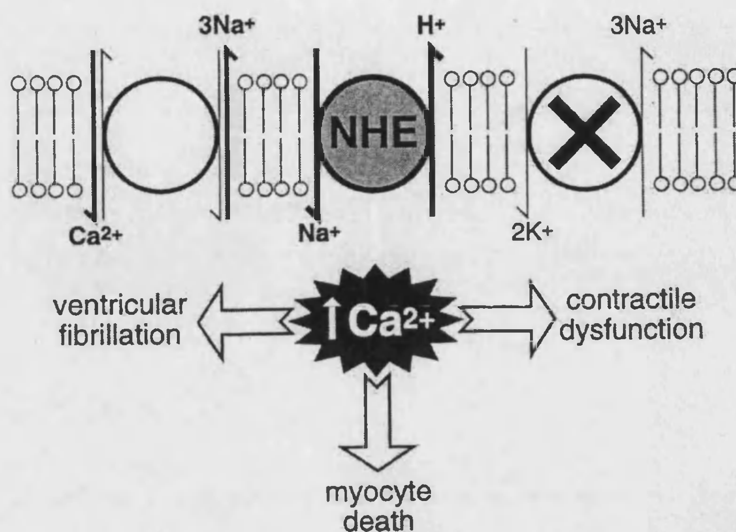

FIGURE 2. The likely sequence of events that involves the sarcolemmal Na^+/H^+ exchanger (NHE) during ischemia and reperfusion. Although the exchanger is quiescent under basal conditions, it becomes activated during ischemia in response to intracellular acidosis and other NHE-stimulatory factors. The resulting influx of Na^+, occurring in the presence of ischemia-induced inhibition of the Na^+/K^+ adenosine triphosphatase (the primary Na^+ extrusion pathway), causes the intracellular accumulation of Na^+. Such an increase in the intracellular Na^+ concentration in turn alters the reversal potential of the Na^+/Ca^{2+} exchanger in a manner that inhibits Ca^{2+} efflux and/or enhances Ca^{2+} influx through this bidirectional mechanism. The end result is a pathologic increase in intracellular Ca^{2+} (Ca^{2+} overload), which has been causally implicated in the genesis of ventricular fibrillation, contractile dysfunction, and myocardial necrosis. NHE inhibitors are likely to afford their cardioprotective effects, at least in part, by inhibiting this sequence at an early stage, through the limitation of Na^+ influx.

sibility cannot be discounted that the observed effects might have arisen through inhibition of Na^+ influx pathway(s) other than the sarcolemmal NHE, such as the noninactivating Na^+ channel.[42] There appears to be a need, therefore, to test whether novel NHEIs that do not inhibit this channel (e.g., cariporide[17]) also attenuate Na^+ (and Ca^{2+}) accumulation during ischemia.

Although the sarcolemmal NHE is activated by intracellular acidosis, it is subject to inhibition by extracellular acidosis,[40,43] which also develops rapidly during ischemia.[44] Thus, exchanger activity would be expected to decline with prolonged ischemia (which may help explain the requirement to inhibit NHE early during ischemia to achieve cardioprotective benefit[28,35]). The rapid normalization of extracellular pH during early reperfusion would be expected to disinhibit the sarcolemmal NHE, reactivate the sequence depicted in Figure 1, and lead to further injury, in accordance with the Lazdunski hypothesis.[36] Thus, inhibition of the sarcolemmal NHE during ischemia and reperfusion could be of benefit through an identical mechanism, namely, the attenuation of Na^+ influx. Other potential mechanisms through which NHE inhibition could be of benefit during ischemia and reperfusion include: (1) slowing of the recovery of intracellular pH from acidosis during reperfusion,[26,39] which may limit the development of hypercontracture[26,45,46]; (2) inhibition of myocardial edema[47]; and (3) attenuation of myocyte apoptosis.[48]

SARCOLEMMAL NHE ACTIVITY IN ISCHEMIA

How does the sarcolemmal NHE retain significant activity, at least during early ischemia, despite the rapid development of extracellular acidosis? First, although the sarcolemmal NHE is undoubtedly inhibited by extracellular acidosis,[40,43] such inhibition is not absolute. Contrary to a common misconception, the primary regulator of NHE activity is not the transmembrane H^+ gradient, but the intracellular pH, through the interaction of intracellular H^+ with the so-called "H^+ sensor" site of the exchanger protein.[2] Indeed, the sarcolemmal NHE can remain active and extrude H^+ against an inwardly directed H^+ gradient, provided the intracellular pH is sufficiently low.[43] Second, certain processes associated with ischemia, such as the accumulation of lipid metabolites and the imposition of oxidant stress, may upregulate NHE activity, since exogenous lysophosphatidylcholine[49] and hydrogen peroxide[50,51] have both recently been shown to stimulate the sarcolemmal exchanger in cultured neonatal[50] and freshly isolated adult[49,51] rat ventricular myocytes. Finally, various endogenous receptor-mediated stimuli of relevance to myocardial ischemia have been shown to stimulate sarcolemmal NHE activity, apparently by increasing the sensitivity of the exchanger to intracellular H^+. Such stimuli include thrombin,[52] endothelin,[9,53,54] and α_1-adrenergic agonists.[55–57] Recent data suggest that α_1-adrenergic stimulation of sarcolemmal NHE activity is likely to

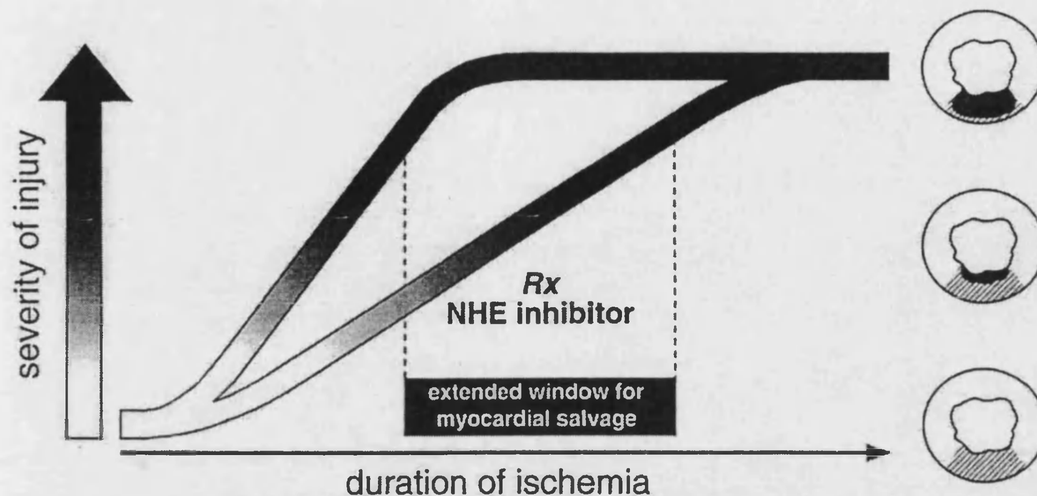

FIGURE 3. The temporal and spatial progression of myocardial injury during ischemia and the potential therapeutic benefit of Na^+/H^+ exchange (NHE) inhibitors. Through an extension of the time window for myocardial salvage by reperfusion, NHE inhibitors may decrease infarct size (and thereby improve ventricular function and decrease mortality) even after relatively long periods of ischemia (see text for further detail). Additional benefit may be provided by NHE inhibitors through the suppression of ventricular fibrillation (and thereby of sudden cardiac death) and amplification of the benefits of reperfusion by inhibition of NHE-mediated reperfusion injury.

be mediated selectively via the α_{1A}-adrenoceptor (AR) subtype,[58] which may have clinical significance since the α_{1A}-AR is the dominant α_1-AR subtype expressed in human myocardium.[59] Notably, α_1-adrenergic stimulation retains its ability to increase sarcolemmal NHE activity in the presence of extracellular acidosis,[55] which has important implications for NHE regulation under ischemic conditions.

In considering adrenergic regulation of the sarcolemmal NHE, it is important to also note that, in contrast to the effect of α_1-AR stimulation, β_1-AR stimulation inhibits sarcolemmal NHE activity.[53,57,60] Therefore, the effects of the endogenous catecholamines norepinephrine and epinephrine on sarcolemmal NHE activity may depend on the relative density or availability of α_1- versus β_1-ARs, which can be modulated by diseases such as heart failure (which is associated with β_1-AR downregulation) and myocardial ischemia (which enhances α_1-AR signaling), as well as by pharmacologic therapy (particularly with adrenergic antagonists).

THERAPEUTIC POTENTIAL OF NHEI IN ACUTE MYOCARDIAL ISCHEMIA

Myocardial ischemia initiates a continuum of progressively more severe cellular changes that culminate in cell death and tissue necrosis.[61] This progression from reversible to irreversible injury (depicted schematically in Figure 3) may be viewed at the whole organ level as the development of an endocardial infarct and the progression of a wavefront of cell death to form a transmural infarct.[62] The absolute prerequisite for myocardial salvage in this setting is early reperfusion, during the period of ischemia in which most or part of the ischemic myocardium has not yet progressed to irreversible injury. This concept of a temporal progression of ischemic injury has been established not only in animal models[62] but also in humans.[63] Thus, a recent analysis of 4 prospective randomized trials of thrombolytic therapy has revealed that each 30-minute increase in symptom (i.e., ischemia) duration before reperfusion is associated with an increase in infarct size of 1% of the myocardium.[63] Accordingly, although thrombolysis achieved significant myocardial salvage (the extent of which decreased with increasing symptom duration) when instituted within 4 hours after symptom onset, comparable infarct sizes were seen in patients treated 4–6 hours after symptom onset and in those who did not receive thrombolytic therapy.[63]

The preclinical data with NHEIs, in particular the substantial evidence that these agents decrease infarct size significantly when given before the onset of ischemia, suggest that NHE inhibition slows progression of myocardial injury during ischemia. Thus, as illustrated in Figure 3, a key benefit of treatment with an NHEI may be an extension of the time window for myocardial salvage by reperfusion, resulting in decreased infarct size (and hence improved ventricular function and decreased mortality) even after relatively long periods (>4 hours) of ischemia. In addition to this potential extension of the window for myocardial salvage, NHEIs are expected to be of therapeutic value by suppressing occurrence of VF (and thereby decreasing sudden cardiac death) and amplifying the undoubted benefits of reperfusion through inhibition of any NHE-mediated reperfusion injury.

CONCLUSIONS

As presented above, there is now a wealth of evidence from preclinical studies that NHEIs provide significant cardioprotection in the setting of ischemia and reperfusion, particularly when administered before the onset of ischemia (a requirement that has been incorporated into the design of the GUARD During Ischemia Against Necrosis [GUARDIAN] study, in which patients with acute coronary syndromes receive the NHEI cariporide during the period of risk for myocardial infarction[64]). The uncommon degree of conformity between different investigators, drugs, species, and models regarding the cardioprotective efficacy of NHEIs suggests a genuine therapeutic potential, which may be translated into development of a novel and effective approach to the treatment of acute myocardial ischemia in humans.

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DISCUSSION

Pierre Théroux, MD (Montreal, Quebec, Canada): Before moving to the more clinical part of the symposium, we will take some questions on concepts and on the basics of the exchange system.

Audience member: Amiloride is a diuretic that blocks the Na^+/H^+ exchange system in the kidneys. How is cariporide different from this diuretic?

Metin Avkiran, PhD (London, United Kingdom): There is plenty of experimental evidence that amiloride and amiloride-based compounds afford cardioprotection as do the newer, benzoylguanidine-based compounds like cariporide. However, the concentrations of amiloride that are required to inhibit NHE-1 are in excess of 1 mmol/L, compared with 1 μmol/L for cariporide. Such high plasma concentrations of amiloride are not achieved through use of this drug as a diuretic. I don't know if there is any clinical evidence that patients who are taking amiloride as a diuretic tolerate acute myocardial ischemia better.

Dr. Théroux: Is hyperkalemia observed with cariporide?

Dr. Avkiran: No, I don't think there is any evidence for that.

Dr. Théroux: You said that Na^+/H^+ exchange systems can be expressed differently in various organs. Can you elaborate on this?

Dr. Avkiran: There are at least 6 different isoforms of the Na^+/H^+ exchanger. By that I mean 6 have been identified and cloned. NHE-1 is ubiquitous but is the only one expressed in the cardiac sarcolemma. NHE-2–4 are localized to epithelial cells of the gastrointestinal tract and kidneys. NHE-5 is not very well known; it is only a partial clone at the moment. NHE-6 was discovered only a couple of months ago; it is believed to be the mitochondrial Na^+/H^+ exchanger. The data we have available in the literature suggest strongly that cariporide and related compounds are selective for the NHE-1 isoform.

Audience member: I expect the Na^+/H^+ exchanger functions only during anoxia or hypoxia. So if you constantly inhibit it, would it have any effect on the nonischemic heart?

Dr. Avkiran: That is a very good point, one that I tried to illustrate in the first part of my talk. It is important to restress that the exchanger is inactive under normal physiologic conditions. It only becomes active under pathologic conditions, such as ischemia. Therefore, its inhibition under normal circumstances is not expected to have much of an effect. In many respects, cariporide can be seen as an ischemia-selective agent. The exchanger becomes active only in conditions such as ischemia, which produce intracellular acidosis; its inhibition would only have a functional impact under those conditions.

Dr. Théroux: So we should not expect depression of function in nonischemic myocardial cells during regional ischemia?

Dr. Avkiran: That is correct. This has been looked at by a number of investigators, including in my own laboratory. The inhibitors of the Na^+/H^+ exchanger don't depress myocardial function under normal aerobic conditions.

Audience member: Does sodium influx influence the action potential of the cell, making it unstable?

Dr. Avkiran: There is no evidence of direct electrophysiologic effects of cariporide. Indeed, you wouldn't expect to see such an effect because the exchange system is electroneutral. For each positively charged sodium ion that enters the cell, a positively charged hydrogen ion leaves. From the studies in the literature, there are no direct effects on the action potential. The antiarrhythmic effects of drugs like cariporide are likely to arise from inhibition of sodium and calcium overload during ischemia and reperfusion, and attenuation of its arrhythmogenic consequences, such as disruption of cell-to-cell conduction and generation of delayed afterpotentials.

Audience member: I was quite intrigued by the potentiation between preconditioning and cariporide. Could you add to this observation?

Dr. Avkiran: I don't really want to upset any preconditioning people in the audience. All I can say is that Na^+/H^+ exchanger inhibition appears to be as

protective as ischemic preconditioning, at least in our hands; there also is evidence for this from other laboratories, measuring different endpoints such as infarct size. Interestingly, 2 weeks ago, at the North American Section meeting of the International Society for Heart Research, Dr. Garrett Gross and colleagues from Milwaukee presented some evidence obtained in a canine model of ischemia and reperfusion that the protective effect of ischemic preconditioning wanes with very prolonged ischemia, whereas the protection afforded by Na^+/H^+ exchanger inhibition is still present. These results need to be confirmed.

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FOCUSSED ISSUE: Na^+/H^+-exchange

Metin Avkiran

Protection of the ischaemic myocardium by Na^+/H^+ exchange inhibitors: potential mechanisms of action

Introduction

As reviewed recently (1, 16) and discussed elsewhere in this issue of Basic Research in Cardiology, there is substantial experimental evidence that pharmacological inhibitors of the Na^+/H^+ exchanger (NHE) attenuate the detrimental consequences of myocardial ischaemia and reperfusion, such as arrhythmias, contractile dysfunction, and tissue necrosis. The objective of this review is to consider the cellular mechanisms through which NHE inhibitors may preserve myocardial integrity and function during ischaemia and reperfusion.

Modulation of trans-sarcolemmal cation (Na^+, Ca^{2+}, H^+) transport

The mechanism which has been most commonly cited as the principal mechanism underlying the cardioprotective actions of NHE inhibitors during ischaemia and reperfusion is the attenuation of the loss of Na^+ and Ca^{2+} homeostasis and subsequent "Ca^{2+} overload". The original hypothesis by Lazdunski et al. (21) proposed that increased sarcolemmal NHE activity, arising from the rapid normalisation of extracellular pH and the generation of an outwardly directed H^+ gradient upon reperfusion, may play a role in reperfusion injury. Although this hypothesis is supported by the studies which have shown cardioprotective benefit with NHE inhibitors when given from shortly before or early during reperfusion (discussed elsewhere in this issue), it is not consistent with the majority of studies (starting with the original observations of Karmazyn (15)) which have shown superior benefit when the NHE inhibitor is given prior to the onset of or early during ischaemia (5, 12, 17, 18, 25, 35, 37). In this context, it has been suggested that the relative inefficacy of NHE inhibitors when given from shortly before or early during reperfusion in intact hearts may arise from inadequate drug delivery to the site of action (24). However, the recent work of Klein et al. (18) has shown that late administration of an NHE inhibitor by intracoronary infusion does not limit infarct size in pigs *in vivo*, even when an effective drug concentration is achieved in coronary venous blood prior to the onset of reperfusion. Furthermore, the evidence obtained with NHE inhibitors of the amiloride class, which have been shown to attenuate intracellular Na^+ (26, 28, 41) and Ca^{2+} (26) accumulation during ischaemia in parallel with their cardioprotective effects, suggests that NHE activity is maintained and contributes substantially to the loss of ionic homeostasis during ischaemia, and that this is a key determinant of the overall extent of injury during ischaemia and reperfusion. Nevertheless, the conclusions of these studies have been open to conjecture, on the basis that amiloride and its derivatives can additionally inhibit ion transporting proteins other than the sarcolemmal NHE that may also contribute to the loss of Na^+ and Ca^{2+} homeostasis during ischaemia (10). Therefore, it is important to emphasise recent studies which have shown that the highly specific NHE inhibitor cariporide (previously known as HOE-642 (34)) also attenu-

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ates the intracellular accumulation of Na^+ (11) and Ca^{2+} (40) during ischaemia and improves the post-ischaemic recovery of contractile function (11, 40). These findings provide further support for the concept that NHE activity contributes significantly to the loss of ionic homeostasis during ischaemia and, through this mechanism, to myocardial injury and post-ischaemic contractile dysfunction.

Figure 1 summarises the likely sequence of events that involve the sarcolemmal NHE during ischaemia. Although the exchanger is relatively quiescent under basal conditions, it becomes activated during ischaemia in response to intracellular acidosis and possibly to other NHE-stimulatory factors (see below). The resulting influx of Na^+, occurring in the presence of ischaemia-induced inhibition of the Na^+/K^+ ATPase (the primary Na^+ extrusion pathway from the cardiac myocyte), causes the intracellular accumulation of Na^+. Such a rise in the intracellular Na^+ concentration alters the reversal potential of the Na^+/Ca^{2+} exchanger in a manner that inhibits Ca^{2+} efflux and/or enhances Ca^{2+} influx through this bi-directional mechanism, thus producing intracellular Ca^{2+} overload. NHE inhibitors are likely to afford a cardioprotective effect during ischaemia by inhibiting this sequence at an early stage, through the limitation of Na^+ influx.

Although the sarcolemmal NHE is activated by intracellular acidosis, it is subject to inhibition by extracellular acidosis, which also develops during ischaemia. Thus, during a prolonged period of ischaemia, NHE activity would be expected to be high soon after the onset of ischaemia but to decline subsequently, which may help explain the requirement to inhibit NHE early during ischaemia in order to achieve maximum cardioprotective benefit (17, 18). The rapid normalization of extracellular pH during early reperfusion would be expected to disinhibit the sarcolemmal NHE, reactivate the sequence depicted in Fig. 1, and lead to further injury (in accordance with the Lazdunski hypothesis (21)). Thus, inhibition of the sarcolemmal NHE during ischaemia and reperfusion could be of benefit through an identical mechanism, that is the attenuation of Na^+ influx. Indeed, data from van Emous et al. (42) indicates the presence of NHE-mediated Na^+ influx during early reperfusion. However, in accordance with recent evidence (14), it is likely that Na^+ accumulation during ischaemia is the more critical factor and that the principal mechanism through which NHE inhibitors protect the ischaemic and reperfused myocardium is the attenuation of Na^+ influx during the ischaemic period.

Regardless of the relative importance of NHE-mediated Na^+ influx during ischaemia versus during reperfusion, there is substantial evidence that increased sarcolemmal NHE activity contributes significantly to H^+ efflux during the latter period. Thus, NHE inhibitors of the amiloride class (28) as well as the newer agents such as HOE-694 (12) and cariporide (11, 40) have been shown to slow the recovery of intracellular pH from ischaemia-induced acidosis during early reperfusion. Furthermore, such slowed recovery of intracellular pH during early reperfusion has been proposed as a cardioprotective mechanism of NHE inhibition, potentially through the desensitisation of myofilaments and consequent inhibition of the development of hypercontracture (12, 20, 31). It appears, therefore, that the cardioprotective consequences of NHE inhibition arise from a combination of

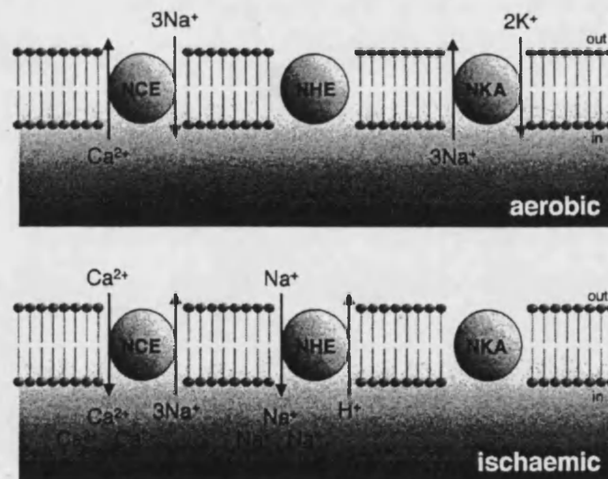

Fig. 1 Putative mechanism through which sarcolemmal Na^+/H^+ exchanger (NHE) activity contributes to the intracellular accumulation of Na^+ and Ca^{2+} during myocardial ischaemia. *NCE* Na^+/Ca^{2+} exchanger, *NKA* Na^+/K^+ ATPase. See text for details.

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1) the reduced intracellular accumulation of Na^+ and Ca^{2+}, particularly during ischaemia, and 2) the maintenance of a lower intracellular pH during early reperfusion. It should be re-emphasised, however, that the former mechanism is likely to predominate.

Role of intracellular Na^+ and Ca^{2+} accumulation – discordant findings

Contrary to much of the evidence discussed above, some recent studies have provided data that contradict a key role for reduced intracellular Na^+ and Ca^{2+} accumulation during ischaemia in the cardioprotective mechanisms of NHE inhibitors. For example, studies in rat isolated ventricular myocytes have shown that NHE inhibition by cariporide does not affect the intracellular accumulation of Na^+ and Ca^{2+} during anoxia, but inhibits the development of hypercontracture upon reoxygenation (31, 33). It is likely that the metabolic challenge associated with anoxia in quiescent myocytes *in vitro* differs significantly from that imposed by ischaemia in beating intact hearts *in vitro* or *in vivo*. Furthermore, the myocyte model is devoid of the neurohormonal stimuli that may regulate sarcolemmal NHE activity during ischaemia in the intact heart (see below). Therefore, it is not possible to conclude, on the basis of the myocyte studies (31, 33), that the protection afforded by NHE inhibitors in the intact heart occurs independently of an attenuation of Na^+ and Ca^{2+} accumulation during ischaemia. Nevertheless, the observation that NHE inhibitors can attenuate reoxygenation induced hypercontracture in isolated myocytes, without affecting Na^+ and Ca^{2+} accumulation during the preceding period of anoxia, raises the intriguing possibility that these agents may possess additional, currently unidentified, cardioprotective properties.

Contrary to many earlier studies (11, 14, 23, 26, 28, 42), a recent report has suggested that the intracellular Na^+ concentration does not rise significantly during ischaemia of the intact rat heart in vitro, even in the absence of an NHE inhibitor (27). This apparent contradiction is likely to have arisen from methodological differences. For example, the fluorimetric method used by Park et al. (27) detects Na^+ signals primarily from an epicardial layer of cells, whereas NMR spectroscopy, as used in the other studies (11, 14, 23, 26, 28, 42), provides a global estimate of the intracellular Na^+ concentration in ischaemic myocardium. Importantly, the measurement of the intracellular Na^+ concentration by an independent method (Na^+-selective intracellular electrodes) has also shown significant Na^+ accumulation during ischaemia, in arterially perfused rabbit papillary muscles subjected to ischaemia in an anoxic medium (46). Furthermore, the intracellular accumulation of Na^+ during ischaemia was accelerated when the muscles were exposed to a thrombin receptor-activating peptide (46), which was subsequently shown to stimulate sarcolemmal NHE activity (47). Finally, while Park et al. (27) found no increase in the intracellular Na^+ concentration during ischaemia in hearts which were paced at 120 beats/min (after destruction of the sinoatrial and atrioventricular nodes), they did observe a significant increase when the hearts were paced at 300 beats/min, which is closer to the spontaneous sinus rate of the rat heart *in vitro*. In this regard, in the NMR spectroscopy studies which have reported significant increases in the intracellular Na^+ concentration in isolated rat hearts during ischaemia, the hearts were either allowed to beat spontaneously (23, 26) or paced at 240–300 beats/min (14, 28, 42).

Regulation of sarcolemmal NHE activity during ischaemia

If it is accepted that intracellular Na^+ accumulation occurs during ischaemia and that Na^+ influx through the sarcolemmal NHE contributes significantly to this accumulation, an important question to address is how the exchanger retains its activity, at least during early ischaemia, despite the rapid development of extracellular acidosis. Firstly, although the sarcolemmal NHE is undoubtedly inhibited by extracellular acidosis, such inhibition is not absolute. Contrary to a common misconception, the primary regulator of NHE activity is not the trans-sarcolemmal H^+ gradient, but the intracellular pH, through the interaction of intracellular H^+ with the so-called "H^+ sensor" site of the exchanger protein (44). Indeed, the sarcolemmal NHE can remain active and extrude H^+ against an inwardly directed H^+ gradient, provided the intracellular pH is sufficiently low (43). Secondly, processes associated with ischaemia, such as the accumulation of lipid metabolites and the imposition of oxidant stress, may upregulate NHE activity, since exogenous lysophosphatidylcholine (13) and hydrogen peroxide (32, 38) have both been shown recently to stimulate the sarcolemmal NHE in rat ventricular myocytes. Finally, various endogenous receptor-mediated stimuli of relevance to myocardial ischaemia have been shown to stimulate sarcolemmal NHE activity, apparently by increasing the sensitivity of the exchanger to intracellular H^+. Such stimuli include thrombin (47), endothelin (19), angiotensin II (9) and α_1-adrenergic agonists (45). Recent data suggest that α_1-adrenergic stimulation of sarcolemmal NHE activity is likely to be mediated selectively via the α_{1A}-adrenoceptor subtype (39, 48); this may have clinical significance since the α_{1A}-adrenoceptor is the dominant α_1-adrenoceptor subtype expressed in human myocardium (29). It is notable also that α_1-adrenergic stimulation retains its ability to increase sarcolemmal NHE activity in the presence of extracellular acido-

sis (30), which has important implications for NHE regulation under ischaemic conditions.

A role for NHE inhibition in non-myocardial cells?

It is apparent from the evidence reviewed above that much of the work aimed at identification of the cardioprotective mechanisms of NHE inhibitors during ischaemia and reperfusion has, perhaps not surprisingly, focused on the cardiac sarcolemmal NHE. It should be borne in mind, however, that the novel NHE inhibitors which have been developed as putative cardioprotective agents (such as cariporide (34), EMD-85131 (8), BIIB-513 (7), SL-59.1227 (22) and CP-597396 (4)) all exhibit marked selectivity for the NHE-1 isoform, which is expressed not only in the cardiac sarcolemma but also in the plasma membrane of almost every cell type in the body (3). In the context of myocardial ischaemia and reperfusion in the *in vivo* setting, it is possible that inhibition of the plasma membrane NHE of other cellular components of the cardiovascular system (such as neutrophils, endothelial cells, platelets) may also modulate the extent of myocardial injury and dysfunction that arises. In support of this, there is preliminary evidence to suggest that inhibition of the neutrophil NHE may attenuate neutrophil-induced contractile dysfunction during reperfusion (36) and that NHE inhibitors may inhibit neutrophil adherence to activated vascular endothelium (2). Furthermore, in a recent comprehensive study, Gumina et al. (6) have shown that NHE inhibition attenuates neutrophil accumulation within ischaemic/reperfused myocardium, in an *in vivo* canine model of regional ischaemia and reperfusion. Their data additionally indicate that the *in vitro* activation of canine isolated neutrophils by platelet activating factor or a phorbol ester is inhibited by pretreatment of the cells with the NHE inhibitor BIIB-513 (6). On the basis of these findings, further work appears warranted to determine the contribution of reduced NHE activity in non-myocardial cells to the cardioprotective actions of NHE-1-selective inhibitors.

Conclusions

The balance of published evidence indicates that the potent cardioprotective actions of NHE inhibitors in experimental models of ischaemia and reperfusion arise primarily from the attenuation of the intracellular accumulation of Na^+ (and consequently Ca^{2+}) in ischaemic myocardium, with any attenuation of a further disturbance in Na^+ homeostasis during early reperfusion likely to play a less critical role. A slowed recovery from intracellular acidosis during early reperfusion may also contribute to the protection afforded by NHE inhibitors, by inhibiting the development of myocardial hypercontracture. Finally, there is suggestive evidence that, particularly in the *in vivo* setting, inhibition of NHE activity in cell types other than cardiac myocytes may also help ameliorate myocardial injury and dysfunction, although further investigation is required to substantiate the contribution of this mechanism.

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Thrombin Activates the Sarcolemmal Na^+-H^+ Exchanger

Evidence for a Receptor-Mediated Mechanism Involving Protein Kinase C

Masahiro Yasutake, Robert S. Haworth, Anna King, Metin Avkiran

Abstract Thrombin can activate the plasma membrane Na^+-H^+ exchanger in a variety of noncardiac cells. We have studied (1) the effect of thrombin on the activity of the sarcolemmal Na^+-H^+ exchanger in freshly isolated quiescent ventricular myocytes from the adult rat heart and (2) the signaling mechanism(s) underlying any effect. Reverse-transcription polymerase chain reaction analysis revealed thrombin receptor mRNA expression in a myocyte-enriched cell preparation. As an index of Na^+-H^+ exchanger activity, acid efflux rates (J_Hs) were determined in single myocytes (n=4 to 11 per group) loaded with the pH-sensitive fluoroprobe carboxy-seminaphthorhodafluor-1 after two consecutive intracellular acid pulses (induced by transient exposure to 20 mmol/L NH_4Cl) in bicarbonate-free medium. At a pH_i of 6.9, J_H did not change significantly during the second pulse relative to the first in control cells. However, when the second pulse occurred in the presence of 0.2, 1, or 5 U/mL thrombin, J_H increased by 30%, 62% ($P<.05$), and 87% ($P<.05$), respectively. A hexameric thrombin receptor–activating peptide (SFLLRN) mimicked the effect of thrombin and increased J_H by 73% ($P<.05$) at 25 μmol/L. In contrast, an inactive control peptide (FLLRN) was without effect at 25 μmol/L. In cells pretreated with 100 nmol/L GF109203X or 5 μmol/L chelerythrine (protein kinase C inhibitors), neither 5 U/mL thrombin nor 25 μmol/L SFLLRN produced a significant increase in J_H. In the presence of 10 μmol/L HOE-694 (a Na^+-H^+ exchanger inhibitor), pH_i did not recover after an acid load, even during exposure to 5 U/mL thrombin or 25 μmol/L SFLLRN, confirming that the Na^+-H^+ exchanger was the primary acid efflux mechanism under the conditions used. Neither 5 U/mL thrombin nor 25 μmol/L SFLLRN affected resting pH_i and Ca^{2+} or background acid loading. We conclude that (1) adult rat ventricular myocytes express a functional thrombin receptor, whose stimulation results in increased activity of the sarcolemmal Na^+-H^+ exchanger, and (2) this effect appears to occur through a protein kinase C–mediated mechanism. (*Circ Res.* 1996;79:705-715.)

Key Words • thrombin receptor • protein kinase C • Na^+-H^+ exchanger • heart • myocyte

The sarcolemmal Na^+-H^+ exchanger is a major acid extrusion system in cardiac myocytes and plays an important role in restoration of pH_i following an acid load.[1] Recent evidence suggests that the exchanger may also play an important role in determining the severity of the unfavorable sequelae of myocardial ischemia and reperfusion, such as arrhythmias, contractile dysfunction, and infarction.[1,2] In cardiac myocytes, as in other cell types, the activity of the Na^+-H^+ exchanger is regulated not only by pH_i but also by a number of extracellular stimuli (such as exposure to adrenergic agonists, endothelin, angiotensin II, and adenosine triphosphate) through receptor-mediated mechanisms (for recent review, see Pucéat and Vassort[3]).

Thrombin is a multifunctional protease, which, in addition to its established role in blood coagulation and thrombus formation, induces a variety of cellular responses through the recently cloned thrombin receptor (for review, see Coughlin[4]). The receptor can be activated not only by thrombin but also by synthetic peptides (thrombin receptor–activating peptides), which mimic the "tethered ligand" domain of the receptor that is revealed after cleavage by thrombin.[4] A number of cell types within the cardiovascular system, including platelets,[5] endothelial cells,[5] and vascular smooth muscle cells,[6] have been shown to express mRNA coding for the cloned thrombin receptor. Indeed, exposure to thrombin can produce a range of physiological effects in these cell types; these include activation of the plasma membrane Na^+-H^+ exchanger, which occurs (at least in part) through a PKC-mediated pathway.[7-9] With respect to cardiac myocytes, Steinberg et al[10] have shown that thrombin can alter phosphoinositide metabolism and cytosolic Ca^{2+} in cultured neonatal rat ventricular myocytes. In a similar model, Glembotski et al[11] have demonstrated recently that the cloned thrombin receptor is expressed and may mediate a hypertrophic response after exposure to thrombin. However, there is a paucity of data regarding the effects of thrombin in adult cardiac myocytes and the role of the cloned thrombin receptor in this cell type, particularly with regard to regulation of sarcolemmal Na^+-H^+ exchanger activity.

The primary objectives of the present study were to use freshly isolated adult rat ventricular myocytes to (1) obtain molecular evidence for expression of the cloned thrombin receptor in this cell type, (2) determine the effects of thrombin and a thrombin receptor–activating peptide on sarcolemmal Na^+-H^+ exchanger activity, and (3) delineate the roles of intracellular Ca^{2+} and PKC in mediating any regulation of exchanger activity via the

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Selected Abbreviations and Acronyms

β_i = intracellular intrinsic buffering power
C-SNARF-1 = carboxy-seminaphthorhodafluor-1
HOE-694 = 3-methylsulfonyl-4-piperidinobenzoyl guanidine
I_{405}, I_{485}, I_{580}, I_{640} = fluorescence emission intensity at 405, 485, 580, and 640 nm
J_H = rate of acid efflux
PCR = polymerase chain reaction
PKC = protein kinase C

thrombin receptor. Our results provide molecular and physiological evidence that adult rat ventricular myocytes express a functional thrombin receptor, whose stimulation results in increased activity of the sarcolemmal Na^+-H^+ exchanger. This effect occurs in the absence of a detectable increase in $[Ca^{2+}]_i$ and appears to involve a PKC-mediated mechanism.

Materials and Methods

The present investigation was performed in accordance with the Home Office Guidance on the Operation of the Animals (Scientific Procedures) Act of 1986, published by Her Majesty's Stationery Office, London, UK.

Isolation of Ventricular Myocytes

Ventricular myocytes were isolated from the hearts of adult male Wistar rats (200 to 250 g body weight) using a collagenase-based enzymatic digestion technique, which has been described previously.[12] In brief, rats were anesthetized by inhalation of diethyl ether, and hearts were excised and perfused (37°C) in the Langendorff mode for four sequential periods, as follows: (1) with Tyrode's solution (mmol/L: NaCl 137, KCl 5.4, $CaCl_2$ 1.8, $MgCl_2$ 0.5, HEPES 10, and glucose 10, adjusted to pH 7.4 at 34°C with NaOH) for 5 minutes, (2) with nominally Ca^{2+}-free Tyrode's solution (mmol/L: NaCl 135, KCl 5.4, NaH_2PO_4 0.33, $MgCl_2$ 1.0, HEPES 10, and glucose 10, adjusted to pH 7.2 at 34°C with NaOH) for 5.5 minutes, (3) with nominally Ca^{2+}-free Tyrode's solution containing collagenase (Worthington type 1, 200 U/mL) for 10 minutes, and (4) with storage buffer (mmol/L: KOH 78, KCl 30, KH_2PO_4 30, $MgSO_4$ 3, EGTA 0.5, HEPES 10, glutamic acid 50, taurine 20, and glucose 10, adjusted to pH 7.2 at 34°C with KOH) for 5 minutes. All solutions were gassed with 100% O_2. After the perfusion procedure, the ventricles were removed and chopped into several pieces in storage buffer. The tissue fragments were then gently agitated to facilitate cell dispersion, which commonly resulted in a myocyte yield of >80% rod-shaped cells. The cell suspension was maintained in storage buffer at 25°C for at least 1 hour before use in the microepifluorescence studies. When required for RNA extraction, myocyte-enriched preparations were obtained by centrifugation (25g for 5 minutes) of the cell suspension, followed by gravity sedimentation for 15 minutes.

Extraction of RNA From Myocyte-Enriched Preparations

Myocyte-enriched preparations (≈2×10^6 cells per heart) were pelleted by centrifugation at 160g for 5 minutes. The supernatant was discarded, and total RNA was prepared from the cell pellet using Trisolv reagent (Biotecx Laboratories Inc) according to the manufacturer's instructions. mRNA was subsequently prepared from total RNA using the Invitrogen FastTrack mRNA isolation kit (R & D Systems Europe Ltd) as recommended by the manufacturer.

Reverse-Transcription PCR

mRNA (1 μg) from myocyte-enriched preparations was reverse-transcribed in a volume of 20 μL, using the Invitrogen cDNA Cycle kit and oligo dT primer (R & D Systems Europe Ltd). Samples were extracted with phenol/chloroform, and the cDNA was precipitated with ethanol at −70°C for 20 minutes. The cDNA was dissolved in 20 μL RNase-free water before PCR. Aliquots (5 μL) were subjected to 35 cycles of PCR (1 minute at 94°C, 1 minute at 55°C, and 1 minute at 72°C) using primers (100 pmol) specific for the rat thrombin receptor (5′-TGTGAACTGATTATGTCAATT-3′ and 5′-TAACCAGGAAAAGGATATG-3′), as described by Glembotski et al.[11] As a negative control, reverse transcriptase was omitted from the cDNA synthesis step. As a positive control, plasmid (1 pg) containing subcloned rat thrombin receptor cDNA (see Glembotski et al[11]) was also subjected to PCR amplification. PCR products were incubated for 60 minutes in the presence or absence of the restriction enzyme *Eco*RV before separation on 5% acrylamide gels. Based on the known sequence of the rat thrombin receptor cDNA,[11] the intact PCR product obtained using these primers should be 946 bp, while cleavage at the *Eco*RV recognition site should result in two products of 585 and 361 bp. To further confirm the identity of the intact PCR product obtained from myocyte cDNA, this was additionally subjected to sequence analysis in both directions, using the primers described above and an ABI 373 automated sequencing system (Molecular Medicine Unit, King's College, London, UK).

Determination of Sarcolemmal Na^+-H^+ Exchanger Activity

Measurement of pH_i

pH_i was monitored in single ventricular myocytes using the pH-sensitive fluorescent dye C-SNARF-1, as described previously.[13,14] Briefly, aliquots of cells were loaded with C-SNARF-1 within 6 hours of their isolation by incubating them in a 4 μmol/L solution of the acetoxymethyl ester (Calbiochem) for 10 minutes at room temperature. Cells loaded with C-SNARF-1 were then allowed to settle on a glass coverslip at the bottom of a 100-μL chamber, mounted on the stage of an inverted microscope (Nikon Diaphot), and viewed using a ×40 oil immersion objective with a numerical aperture of 1.3. After adherence to the coverslip, cells were superfused (3.5 mL/min) with Tyrode's solution at 34°C. Cells were excited with light at 540 nm, and the resulting fluorescence emission intensity from a selected area of a single myocyte was measured simultaneously at 580 nm (I_{580}) and 640 nm (I_{640}), using a dual-emission photometer system (model D104C, Photon Technology International Inc), which contained an adjustable aperture and two multialkali photomultiplier tubes (type R928, Hamamatsu Photonics UK Ltd). Background fluorescence was measured using an identical aperture and subtracted from the signal. After current-voltage conversion, the acquired signals were digitized at 1.7 Hz and stored on computer hard disk, using pClamp software (Axon Instruments).

Calibration of Fluorescence Signal

The emission intensity ratio (I_{580}/I_{640}) was calculated and converted to a pH_i scale, using in situ calibration data obtained by exposing cells loaded with C-SNARF-1 to nigericin-containing calibration solutions of pH 5.8 to 8.0.[15] The calibration solutions consisted of 140 mmol/L KCl, 1 mmol/L $MgCl_2$, and 10 μmol/L nigericin and were buffered with 10 mmol/L of one of the following zwitterionic buffers: MES at pH 5.8, 6.2, and 6.4; PIPES at pH 6.6, 6.8, and 7.0; and HEPES at pH 7.2, 7.4, 7.7, and 8.0. All calibration solutions were adjusted to the correct pH with NaOH. Fig 1A shows a recording from a typical calibration experiment in a single cell. The data acquired from each such calibration experiment were normalized by dividing all I_{580}/I_{640} ratios by the ratio obtained in that cell at a pH_i of 7.0[16]; thus, the normalized I_{580}/I_{640} ratio at pH_i 7.0 was always 1. The calibration curve shown in Fig 1B was obtained by a nonlinear least squares fit of the normalized data from seven cells to the equation given below, with the curve constrained to pass through the point having the coordinates $(I_{580}/I_{640})/(I_{580}/I_{640})_{pH7}$=1.0, pH 7.0.[16]

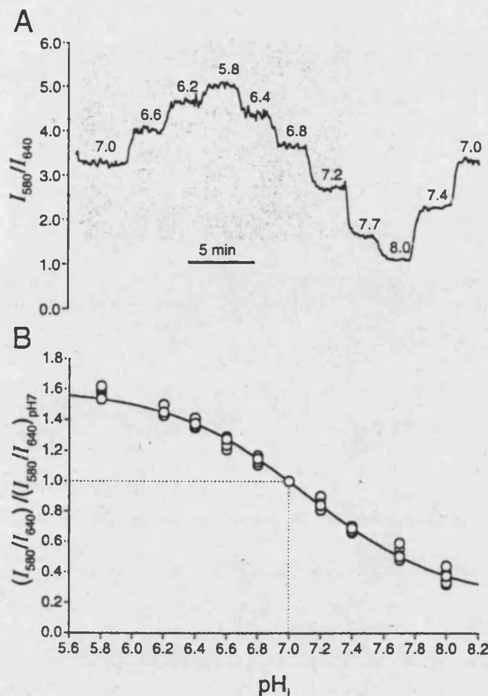

FIG 1. A, Representative recording of the C-SNARF-1 fluorescence emission intensity ratio (I_{580}/I_{640}) from a single cell during exposure to nigericin-containing calibration solutions of varying pH (numbers indicate pH). In this trace, the I_{580}/I_{640} ratio was averaged over 5-second intervals for clarity. B, Calibration curve constructed using normalized I_{580}/I_{640} ratio data from seven experiments (see text for details).

$$(1)\quad \frac{I_{580}/I_{640}}{(I_{580}/I_{640})_{pH7}}=1+a\left[\frac{10^{(pH-pK)}}{1+10^{(pH-pK)}}-\frac{10^{(7-pK)}}{1+10^{(7-pK)}}\right]$$

The best-fit values for pK and a were 7.12 and −1.38, respectively.

Estimation of β_i

β_i was estimated by stepwise removal of extracellular NH_4Cl, as described in detail previously.[17,18] Twelve cells from three different hearts were exposed to Tyrode's solution containing 20 mmol/L NH_4Cl for 1 to 2 minutes, followed by a stepwise reduction of extracellular NH_4^+ to 10, 5, 2.5, 1, and 0 mmol/L. At each step, calculated changes in $[NH_4^+]_i$ and measured changes in pH_i were used to estimate β_i, from the equation $\beta_i=\Delta[NH_4^+]_i/\Delta pH_i$. These experiments were carried out in the presence of 30 μmol/L HOE-694 in order to prevent acid extrusion via the Na^+-H^+ exchanger and 5 mmol/L Ba^{2+} to reduce NH_4^+ efflux through K^+ channels.[18] By plotting β_i as a function of pH_i from 51 determinations and subsequent linear least squares regression analysis, the following equation was obtained:

$$(2)\quad \beta_i=-34.9\cdot pH_i+273.5$$

Equation 2 was used in all subsequent experiments to estimate β_i at different pH_i values.

Calculation of Sarcolemmal Acid Efflux

Since all experiments were carried out in the nominal absence of bicarbonate, J_H was used as a direct index of sarcolemmal Na^+-H^+ exchanger activity.[18] J_H was estimated during recovery from an intracellular acidosis using the equation $J_H=\beta_i\cdot dpH_i/dt$ (where dpH_i/dt is the rate of recovery of pH_i), as previously described.[18] No corrections were made for cell volume–to–surface area ratio, since all experiments were carried out in the same cell type (ie, adult rat ventricular myocytes) in a randomized manner, with contemporary controls. Intracellular acidosis was induced by the washout (6 minutes) of 20 mmol/L NH_4Cl after its transient (2- to 3-minute) application. The pH_i trace during the recovery phase was fitted to a single exponential function, as previously described.[19] At pH_i intervals of 0.05 during this phase, J_H values were calculated from β_i (estimated as described above) and dpH_i/dt (calculated as the differential derivative of the exponential fit), thus enabling the construction of pH_i-versus-J_H relationships. A rightward shift of this curve (signifying a greater J_H at a given pH_i) was taken to indicate an increased Na^+-H^+ exchanger activity.

Estimation of Intracellular Ca^{2+}

$[Ca^{2+}]_i$ was estimated using the Ca^{2+}-sensitive fluorescent dye indo 1 (Cambridge BioScience), as described previously.[20] Aliquots of cells were loaded with indo 1, by incubating them for 20 minutes at room temperature in a 10 μmol/L solution of the acetoxymethyl ester. As in the studies with pH_i measurement, cells were superfused (3.5 mL/min) with Tyrode's solution at 34°C. The microepifluorescence approach used was also similar to that described above for C-SNARF-1, except that only a portion of a selected cell was illuminated (at 360 nm), and the fluorescence emission intensity was measured simultaneously at 405 and 485 nm, through the use of appropriate optical filters. The emission intensity ratio (I_{405}/I_{485}) was used as an index of $[Ca^{2+}]_i$, without calibration.[20]

Solutions

All chemicals were purchased from Sigma Chemical Co, unless stated otherwise. The thrombin receptor–activating peptide SFLLRN and the inactive control peptide FLLRN were gifts from Glaxo (Greenford, UK). The Na^+-H^+ exchanger inhibitor HOE-694 was a gift from Hoechst (Frankfurt, Germany).

All experiments were performed in HEPES-buffered Tyrode's solution. In Na^+-free Tyrode's solution, NaCl was replaced with 137 mmol/L choline chloride, and pH was adjusted to 7.4 with KOH. NH_4Cl (20 mmol/L) was added directly to Tyrode's solution without osmotic compensation. Stock solutions of thrombin (T-6759, Sigma) and HOE-694 were prepared in deionized water; SFLLRN and FLLRN were dissolved directly in Tyrode's solution. The PKC inhibitors GF109203X (Calbiochem) and chelerythrine were dissolved in dimethyl sulfoxide and subsequently diluted with Tyrode's solution to obtain appropriate stock solutions. All agents were added to superfusion solutions, at the appropriate concentrations, shortly before the beginning of experiments.

Experimental Protocols

Experiments were performed using the protocols described below, unless stated otherwise. Basal pH_i was noted after 5 to 10 minutes of superfusion with normal Tyrode's solution (pH 7.4). To study the effects of thrombin and SFLLRN on resting pH_i, cells were then exposed to these agents for a further 5 minutes. To study the effects of these stimuli on sarcolemmal Na^+-H^+ exchanger activity, cells were subjected to intracellular acidosis by consecutive transient exposures to NH_4Cl. After the initial 6-minute period of NH_4Cl washout (first acid pulse), cells were superfused with normal Tyrode's solution for an additional 6 minutes to allow further recovery of pH_i before the second transient exposure to NH_4Cl (second acid pulse). In control cells, both acid pulses occurred under identical conditions. When studying the effects of thrombin, SFLLRN, or FLLRN, these agents were present throughout the second pulse (ie, during exposure to and washout of NH_4Cl). When studying the effects of thrombin and SFLLRN in the presence of GF109203X or chelerythrine, these agents were included in all solutions from 6 minutes before the second pulse to the end of the experiment. J_H-versus-pH_i curves

were constructed using data obtained during the pH_i recovery phases following both acid pulses. This double-pulse protocol was necessitated by the intercell variability in J_H even at identical pH_i values (eg, see Figs 4, 5, and 7) and enabled paired data analysis. At the end of every experiment, the cell under study was exposed to nigericin-containing calibration solution at pH 7.0, thus enabling normalization of the I_{580}/I_{640} ratios. Equation 1 and the fitted values for pK and the constant *a* were then used to calculate pH_i from the normalized I_{580}/I_{640} values.

To study the effects of thrombin and SFLLRN on $[Ca^{2+}]_i$, each cell was consecutively exposed to (1) field stimulation at 0.5 Hz (to enable the recording of Ca^{2+} transients), (2) Tyrode's solution with or without 5 U/mL of thrombin or 25 μmol/L of SFLLRN, and (3) Tyrode's solution with 10 mmol/L of caffeine (at an increased superfusion rate of 12 to 15 mL/min), with the indo 1 emission intensity ratio (I_{405}/I_{485}) monitored throughout.

Statistical Analysis

Experiments within each study subsection were carried out in a randomized manner, with contemporary controls. Gaussian-distributed variables were expressed as mean±SEM. To assess changes in J_H within groups (ie, between first and second acid pulses), a paired *t* test was used. For intergroup comparisons, data were subjected to one-way ANOVA. If a difference among mean values was established, further analysis was carried out using either Dunnett's test (to compare every group with the control group) or Tukey's test (to compare every group with every other). A value of $P<.05$ was considered significant.

Results

Thrombin Receptor Expression in Ventricular Myocytes

Fig 2 illustrates the results of reverse-transcription PCR analysis of mRNA from myocyte-enriched preparations. No signal was obtained in the absence of reverse transcriptase (lane 2), confirming the absence of contamination. The intact PCR product (lane 3) was 946 bp in size, as expected from the known thrombin receptor cDNA sequence, and matched exactly that obtained by PCR amplification of thrombin receptor cDNA (lane 6). Cleavage of the PCR products by *Eco*RV gave two bands of the sizes 585 and 361 bp (lanes 4 and 7, respectively), as expected from the rat thrombin receptor cDNA sequence, regardless of the source of the template cDNA. These results suggest that mRNA coding for the cloned thrombin receptor was expressed in this cell preparation. The identity of the intact reverse-transcription PCR product was further confirmed by sequence analysis (data not shown), which revealed it to be homologous with the published rat thrombin receptor cDNA sequence.[11]

Effects of Thrombin Receptor Stimulation on Resting pH_i

Neither thrombin (5 U/mL) nor SFLLRN (25 μmol/L) produced a significant change in resting pH_i (n=3 per group). In control cells and those exposed to thrombin or SFLLRN, basal pH_i was 7.27±0.06, 7.31±0.08, and 7.24±0.07 (*P*=NS), respectively. After 3 minutes of exposure to vehicle, thrombin, or SFLLRN, pH_i remained at similar values, measuring 7.23±0.07, 7.25±0.09, and 7.20±0.06 (*P*=NS), respectively.

Regulation of Sarcolemmal Na^+-H^+ Exchanger Activity via the Thrombin Receptor

The Table shows basal and minimal pH_i values in the various study groups, obtained just before and immediately after the first and second acid pulses. Within each

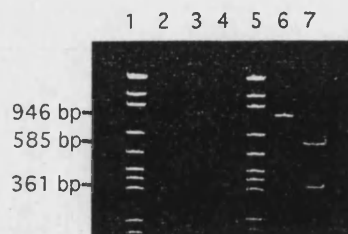

FIG 2. Reverse-transcription PCR analysis of thrombin receptor mRNA expression. Acrylamide gel electrophoresis was used to analyze the products from PCR amplification of either cDNA obtained by reverse transcription of mRNA from myocyte-enriched preparations (lanes 3 and 4) or rat thrombin receptor cDNA (lanes 6 and 7). Lanes 3 and 6 show intact PCR products, and lanes 4 and 7 show products obtained after exposure to *Eco*RV. Lane 2 shows negative control (no reverse transcriptase), and lanes 1 and 5 show pGEM DNA markers (Promega) of the sizes (from the top) 2645, 1605, 1198, 676, 517, 460, 396, 350, 222, and 179 bp. The sizes of the PCR products were 946 bp without exposure to *Eco*RV and 585 and 361 bp after exposure to *Eco*RV, as predicted from the rat thrombin receptor cDNA sequence.

study subsection, there was no significant difference between groups in basal or minimal pH_i at either time point. The overall mean value for basal pH_i before exposure to any acid load or pharmacological intervention, taking into account data from all cells included in this part of the study, was 7.25±0.01 (n=93). This value is comparable to basal pH_i values previously reported for rat ventricular myocytes maintained in HEPES-buffered medium.[21-23]

Fig 3 shows representative recordings of pH_i during two consecutive acid pulses in single myocytes as well as the pH_i-versus-J_H relationships constructed using data from several such experiments. In control experiments (Fig 3A), the profiles of pH_i recovery from intracellular acidosis were similar after both pulses; consequently, the acid efflux curves were superimposed. Thus, at any given pH_i, J_H values were similar at both time points during the experimental protocol, showing that temporal changes in Na^+-H^+ exchanger activity do not occur in the absence of agonist stimulation.

When cells were exposed to 5 U/mL of thrombin during the second acid pulse, pH_i recovery from intracellular acidosis was accelerated (Fig 3B). This effect was reflected by a rightward shift of the pH_i-versus-J_H curve such that over the pH_i range of 6.80 to 7.10, J_H was significantly greater in the presence of thrombin. In order to investigate the dose dependence of the effects of thrombin, such experiments were performed with three concentrations of thrombin, ranging from 0.2 to 5 U/mL. Fig 4 shows J_H at an identical pH_i of 6.9 after both the first acid pulse (in the absence of thrombin) and the second acid pulse (in the presence of thrombin). Thrombin increased J_H in a dose-dependent manner, with statistically significant increases of 62% and 87% at 1 and 5 U/mL, respectively.

The effect of the synthetic thrombin receptor–activating peptide SFLLRN was also examined to determine whether this could mimic the Na^+-H^+ exchanger stimulatory effect of thrombin, thereby supporting an involvement of the cloned receptor. As with thrombin, SFLLRN produced a dose-dependent rightward shift of the pH_i-versus-J_H curve. Consequently, J_H at pH_i 6.9 was increased in a dose-dependent manner, resulting in a statistically significant increase of 73% at 25 μmol/L (Fig 5). Indeed, with this

Mean Values for Basal pH_i (Measured Just Before NH_4Cl Application) and Minimal pH_i (Measured Immediately After NH_4Cl Washout) During the First and Second Acid Pulses

		First Acid Pulse		Second Acid Pulse	
Study Group	**n**	**Basal pH_i**	**Minimal pH_i**	**Basal pH_i**	**Minimal pH_i**
Effects of thrombin and thrombin receptor–activating peptide					
Control	11	7.23±0.04	6.70±0.04	7.18±0.04	6.69±0.05
Thrombin (0.2 U/mL)	7	7.22±0.05	6.73±0.06	7.11±0.03	6.73±0.05
Thrombin (1 U/mL)	6	7.18±0.06	6.59±0.09	7.03±0.04	6.50±0.07
Thrombin (5 U/mL)	5	7.24±0.04	6.73±0.06	7.11±0.06	6.65±0.09
SFLL (1 μmol/L)	4	7.25±0.06	6.71±0.08	7.13±0.06	6.70±0.08
SFLL (5 μmol/L)	4	7.21±0.04	6.69±0.09	7.10±0.05	6.71±0.09
SFLL (25 μmol/L)	8	7.27±0.03	6.63±0.05	7.14±0.04	6.62±0.05
FLL (25 μmol/L)	5	7.28±0.03	6.66±0.09	7.14±0.03	6.65±0.07
Role of PKC					
Control	6	7.23±0.02	6.68±0.04	7.22±0.05	6.71±0.03
Thrombin (5 U/mL)	5	7.27±0.06	6.65±0.05	7.25±0.04	6.67±0.06
SFLL (25 μmol/L)	5	7.24±0.07	6.60±0.03	7.18±0.04	6.66±0.05
GF alone	5	7.30±0.03	6.68±0.05	7.18±0.03	6.73±0.04
Thrombin (5 U/mL)+GF	5	7.22±0.04	6.55±0.04	7.27±0.04	6.66±0.05
SFLL (25 μmol/L)+GF	5	7.36±0.04	6.68±0.03	7.27±0.03	6.75±0.03
CH alone	4	7.27±0.06	6.70±0.05	7.20±0.07	6.72±0.07
Thrombin (5 U/mL)+CH	4	7.22±0.05	6.68±0.07	7.19±0.04	6.71±0.05
SFLL (25 μmol/L)+CH	4	7.24±0.07	6.72±0.06	7.22±0.05	6.73±0.07

SFLL indicates SFLLRN; FLL, FLLRN; GF, GF109203X (100 nmol/L); and CH, chelerythrine (5 μmol/L). Values are mean±SEM.

concentration, J_H was increased significantly throughout the pH_i range of 6.70 to 7.05. In contrast, 25 μmol/L of the inactive control peptide FLLRN did not produce any significant increase in J_H throughout this pH_i range (data not shown).

Role of Intracellular Ca^{2+}

The intracellular Ca^{2+} study was carried out to determine whether thrombin and SFLLRN (at the concentrations shown to increase sarcolemmal Na^+-H^+ exchanger activity) could also increase $[Ca^{2+}]_i$ within an appropriate time frame, thereby implicating a role for Ca^{2+} in the relevant signaling mechanism(s). Fig 6 shows a representative recording of the indo 1 emission intensity ratio from a single cell, during consecutive exposure to field stimulation, 5 U/mL of thrombin, and 10 mmol/L of caffeine. Although predictable changes in the signal were observed in response to both field stimulation and exposure to caf-

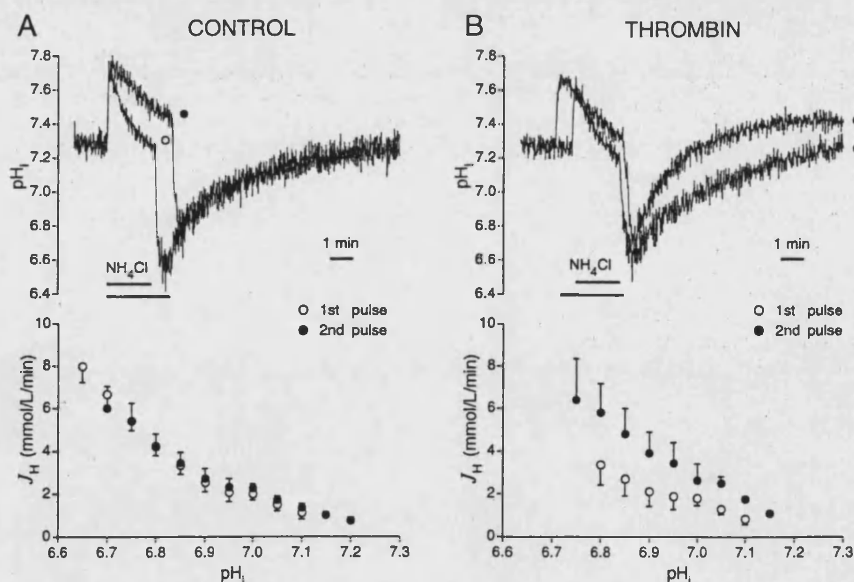

FIG 3. Representative pH_i recordings (top) and pH_i-vs-J_H curves (bottom) obtained during two consecutive acid pulses in control cells (A, n=11) and cells in which 5 U/mL thrombin was present throughout the second pulse (B, n=5). Thrombin accelerated recovery from acidosis and resulted in a rightward shift in the pH_i-vs-J_H relationship.

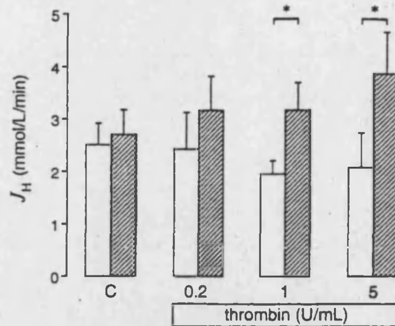

FIG 4. J_H values at an identical pH_i of 6.9 after consecutive acid pulses in control cells (C, n=11) and in cells that were exposed to thrombin (0.2 U/mL, n=7; 1 U/mL, n=6; and 5 U/mL, n=5) throughout the second pulse. Open columns indicate the first pulse; hatched columns, the second pulse. *$P<.05$.

feine (at the beginning and end of the protocol), there was no detectable change in the signal during 3 minutes of exposure to thrombin. Similar observations were made during exposure to 25 μmol/L of SFLLRN or Tyrode's solution alone (n=3 per group).

Role of PKC

The PKC inhibitor study was carried out to determine the role of PKC in thrombin receptor–mediated stimulation of sarcolemmal Na^+-H^+ exchanger activity. To this end, the effects of thrombin and SFLLRN on the pH_i-versus-J_H relationship were reexamined in the presence of one of two PKC inhibitors, GF109203X[24] or chelerythrine,[25] at concentrations shown in our preliminary studies[26] to be sufficient to inhibit phorbol ester–induced stimulation of sarcolemmal Na^+-H^+ exchanger activity. The results are summarized in Fig 7. GF109203X (100 nmol/L) and chelerythrine (5 μmol/L) had no significant effect on J_H in control cells. Consistent with our earlier observations, in the absence of the PKC inhibitors, both 5 U/mL of thrombin and 25 μmol/L of SFLLRN produced significant increases in J_H of 74% and 81%, respectively. However, in the presence of either GF109203X or chelerythrine, thrombin and SFLLRN were no longer able to produce a significant increase in J_H.

Effect of Na^+-H^+ Exchanger Inhibition

The Na^+-H^+ exchanger inhibitor study was carried out to confirm that the accelerated recovery of pH_i following intracellular acidosis (and hence the rightward shift of the pH_i-versus-J_H relationship) in the presence of thrombin or SFLLRN was mediated exclusively through activation of the sarcolemmal Na^+-H^+ exchanger. Cells (n=3 per group) were once again subjected to consecutive acid pulses, this time both in the presence of 5 U/mL of thrombin or 25 μmol/L of SFLLRN and with 10 μmol/L of HOE-694 (a novel Na^+-H^+ exchanger inhibitor[27]) also present during the second pulse. As illustrated by the representative recordings in Figs 8A and 9A, a rapid recovery of pH_i was observed after the first acid pulse in both cases. However, there was little recovery of pH_i after the second pulse, in the presence of HOE-694, despite the continued presence of thrombin or SFLLRN. These observations confirm that in the nominally bicarbonate-free conditions used, the sarcolemmal Na^+-H^+ exchanger is the primary mechanism of pH_i recovery from acidosis and that activation of other acid equivalent efflux mechanisms is unlikely to play a significant role in mediating the actions of thrombin and SFLLRN.

Role of Background Acid Loading

The acid-loading experiment was carried out, using a protocol that has been described previously,[28] to confirm that the accelerated recovery of pH_i following intracellular acidosis in the presence of thrombin or SFLLRN was not due to an inhibition of background acid-loading mechanisms (eg, metabolic acid production) under low pH_i conditions. Cells (n=3 per group) were subjected to an acid pulse in the nominal absence of extracellular Na^+ (to block Na^+-dependent acid efflux mechanisms), and 5 U/mL of thrombin or 25 μmol/L of SFLLRN was applied after the induction of intracellular acidosis. As illustrated by the representative recordings in Figs 8B and 9B, under these conditions, background acid loading was not observed, and neither thrombin nor SFLLRN had any significant effect on pH_i.

Discussion

The present study provides evidence, for the first time, that thrombin is capable of activating the sarcolemmal Na^+-H^+ exchanger in isolated adult rat ventricular myocytes. This effect of thrombin is mimicked by the synthetic thrombin receptor–activating peptide SFLLRN, thus indicating the involvement of a receptor-mediated mechanism. The Na^+-H^+ exchanger stimulatory actions of both thrombin and SFLLRN are abolished by selective inhibitors of PKC, suggesting an important role for this enzyme in the intracellular signaling mechanisms downstream from thrombin receptor activation.

Assessment of Na^+-H^+ Exchanger Activity

In the present study, an agonist-induced rightward shift of the pH_i-versus-J_H relationship (in the absence of bicarbonate-dependent pH_i regulatory mechanisms) has been taken to indicate activation of the Na^+-H^+ exchanger. Similar approaches have been used in various cell types to study changes in plasma membrane Na^+-H^+ exchanger activity (eg, in response to pharmacological manipulation[16] or oncogenic transformation[29]). Of particular relevance to the present study, shifts in the pH_i-versus-J_H relationship have been used previously in isolated ventric-

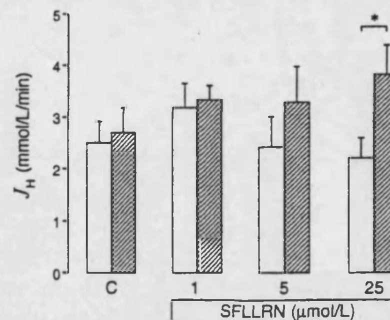

FIG 5. J_H values at an identical pH_i of 6.9 after consecutive acid pulses in control cells (C, n=11) and in cells that were exposed to SFLLRN (1 μmol/L, n=4; 5 μmol/L, n=4; and 25 μmol/L, n=8) throughout the second pulse. Open columns indicate the first pulse; hatched columns, the second pulse. *$P<.05$.

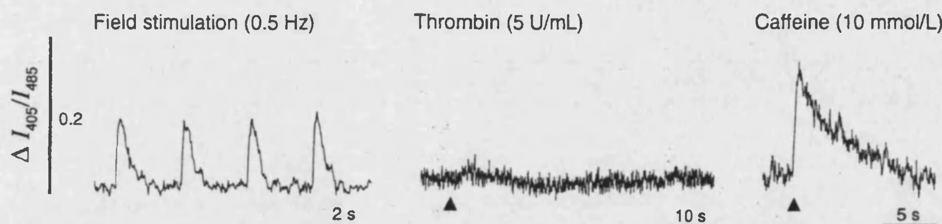

FIG 6. Recording of the indo 1 fluorescence emission intensity ratio (I_{405}/I_{485}) from a single cell during consecutive exposure to field stimulation (left), 5 U/mL of thrombin (middle), and 10 mmol/L of caffeine (right). In the middle and right panels, arrows indicate the start of superfusion with solution containing thrombin or caffeine. The recording is representative of three experiments following an identical protocol.

ular myocytes as indicators of altered sarcolemmal Na^+-H^+ exchanger activity in response to extracellular agonists.[18,22] Recently, Wu et al[21] have suggested that even in nominally bicarbonate-free medium, bicarbonate-dependent pH_i regulatory mechanisms may be operative, thereby complicating the interpretation of data with regard to Na^+-H^+ exchanger activity. Nevertheless, under the conditions used in the present study, mechanisms other than sarcolemmal Na^+-H^+ exchange are unlikely to have contributed significantly to acid equivalent efflux, since pH_i recovery from acidosis was blocked completely in the presence of the Na^+-H^+ exchanger inhibitor HOE-694. Furthermore, thrombin and SFLLRN did not appear to alter background acid loading within the relevant pH_i range. Thus, it is reasonable to assume that any rightward shift of the pH_i-versus-J_H relationship in response to thrombin receptor activation, by either thrombin or SFLLRN, was indeed a reflection of increased sarcolemmal Na^+-H^+ exchanger activity.

Actions of Thrombin on Cardiac Myocytes

The role of thrombin in modulating cardiac myocyte function has received little attention to date. Steinberg et al[10] were the first to study the effects of thrombin on mammalian myocytes, by showing that it could modulate phosphoinositide metabolism and cytosolic Ca^{2+} in cultured neonatal rat ventricular cells. Subsequently, the same group showed that thrombin-induced phosphoinositide hydrolysis in these cultured cells could be enhanced by hypoxia.[30] Recently, Glembotski et al[11] have demonstrated that the thrombin receptor is expressed by cultured neonatal rat ventricular myocytes, thereby providing a mechanistic basis for the cellular effects of thrombin in this preparation. With regard to adult mammalian cardiac myocytes, there is evidence that thrombin can activate the L-type Ca^{2+} channel in guinea pig cells[31] and induce lysophosphatidylcholine accumulation in rabbit cells,[32] with the latter effect mimicked by a thrombin receptor–activat-

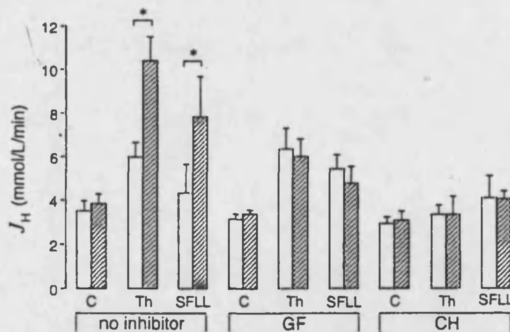

FIG 7. J_H values at an identical pH_i of 6.9 after consecutive acid pulses in control cells (C) and in cells that were exposed to 5 U/mL of thrombin (Th) or 25 μmol/L of SFLLRN (SFLL) throughout the second pulse, in the absence of PKC inhibitor (left) or in the presence of either 100 nmol/L of GF109203X (GF, middle) or 5 μmol/L of chelerythrine (CH, right). Open columns indicate the first pulse; hatched columns, the second pulse. Respective group sizes for C, Th, and SFLL were as follows: n=6, 5, and 5 in the absence of PKC inhibitor; n=5, 5, and 5 in the presence of GF; and n=4, 4, and 4 in the presence of CH. *$P<.05$.

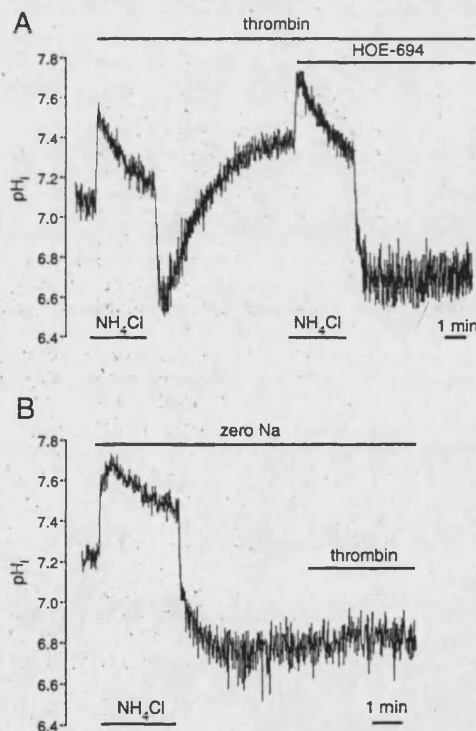

FIG 8. A, Representative pH_i recording (n=3) from a single myocyte during two consecutive acid pulses in the presence of thrombin (5 U/mL). During the second pulse, HOE-694 (10 μmol/L) was also present. B, Representative pH_i recording from a single myocyte (n=3) during an acid pulse in the absence of extracellular Na^+. Thrombin (5 U/mL) was applied for 3.5 minutes, as indicated, after the induction of intracellular acidosis.

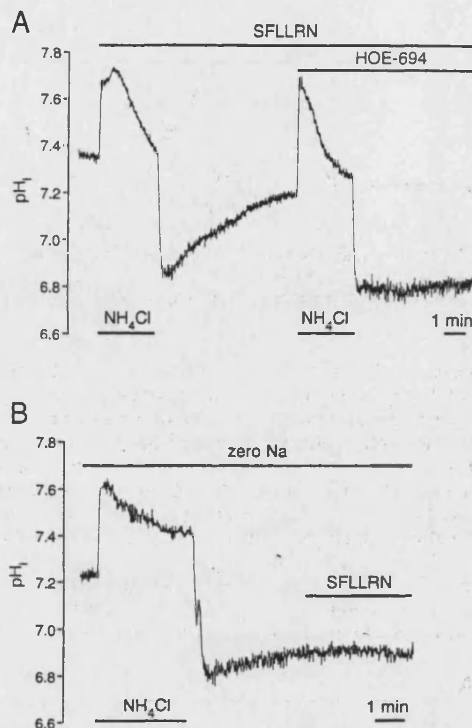

FIG 9. A, Representative pH_i recording (n=3) from a single myocyte during two consecutive acid pulses in the presence of SFLLRN (25 μmol/L). During the second pulse, HOE-694 (10 μmol/L) was also present. B, Representative pH_i recording from a single myocyte (n=3) during an acid pulse in the absence of extracellular Na^+. SFLLRN (25 μmol/L) was applied for 3.5 minutes, as indicated, after the induction of intracellular acidosis.

ing peptide.[32] However, the present study is the first to provide molecular as well as physiological evidence for thrombin receptor expression in adult ventricular myocytes.

It is distinctly possible that contaminating cells of nonmyocyte origin (eg, fibroblasts, endothelial cells, or smooth muscle cells) may have contributed to the thrombin receptor mRNA expression detected in our myocyte-enriched cell preparation by reverse-transcription PCR analysis. However, the stimulatory effects of thrombin and SFLLRN on sarcolemmal Na^+-H^+ exchanger activity (monitored in individual myocytes) are strongly suggestive of functional thrombin receptor expression by the myocyte fraction. It may be argued that such effects on cardiac myocytes could have a paracrine basis, through the thrombin receptor–mediated release (from contaminating nonmyocyte cells) of an unknown Na^+-H^+ exchanger stimulatory factor. However, this is unlikely for several reasons. First, all visible cells that adhered to the coverslip at the bottom of the cell chamber had the morphological characteristics of cardiac myocytes. Second, cell density in the chamber was very low, ensuring considerable dilution of any paracrine factor that is released from contaminating nonmyocyte cells. Finally, the cells in the chamber were continuously superfused at 3.5 mL/min (equivalent to a complete change of the chamber volume every 1.7 seconds); thus, any released paracrine factor would be rapidly removed. Nevertheless, definitive confirmation of thrombin receptor expression by cardiac myocytes awaits analysis by immunocytochemistry and/or in situ hybridization.

Regulation of Sarcolemmal Na^+-H^+ Exchanger Activity via the Thrombin Receptor

Thrombin has been shown to increase the activity of the plasma membrane Na^+-H^+ exchanger in other cell types of the cardiovascular system, such as platelets,[7] endothelial cells,[8] and vascular smooth muscle cells.[9] Our observations show that thrombin increases the activity of the sarcolemmal Na^+-H^+ exchanger also in adult rat ventricular myocytes and that this effect occurs in a dose-dependent manner. Furthermore, by demonstrating that the Na^+-H^+ exchanger stimulatory effect of thrombin can be mimicked by SFLLRN, our study strongly suggests the involvement of the cloned thrombin receptor.[4] It is important to note that thrombin receptor stimulation increased J_H (ie, acid efflux via the Na^+-H^+ exchanger) throughout a pH_i range of ≈6.70 to 7.10. Despite this, however, thrombin receptor stimulation did not alter resting pH_i over a 5-minute period. This is probably because the actual J_H achieved at pH_i values approaching basal pH_i (mean value of 7.25) was very low (<1 mmol/L/min), even in the presence of thrombin or SFLLRN.

Signaling Mechanisms Downstream From Receptor Stimulation

The thrombin receptor is a member of the seven-transmembrane-domain receptor family, and its stimulation has been linked with G protein–mediated activation of phospholipase C and, subsequently, PKC.[33] Indeed, in several cell types, thrombin-induced activation of the Na^+-H^+ exchanger has been shown to be mediated, at least in part, via PKC activation.[7,9] Our demonstration that the thrombin receptor–mediated stimulation of sarcolemmal Na^+-H^+ exchanger activity is abolished by both GF109203X and chelerythrine, two potent and selective inhibitors of PKC,[24,25] suggests a key role for this enzyme in the relevant intracellular signaling pathway in rat ventricular myocytes. This is consistent with previous studies in this cell type that have shown that agents capable of activating PKC (eg, phorbol esters,[22,26] α_1-adrenergic agonists,[22,26] and endothelin[23]) are potent activators of the sarcolemmal Na^+-H^+ exchanger.

Which of the isoforms of PKC are involved in thrombin receptor–mediated stimulation of sarcolemmal Na^+-H^+ exchanger activity cannot be deduced on the basis of the present study. The lack of a rapid effect on $[Ca^{2+}]_i$ by thrombin and SFLLRN (at concentrations sufficient to increase sarcolemmal Na^+-H^+ exchanger activity), taken together with the marked inhibitory effects of GF109203X and chelerythrine, might suggest a role for Ca^{2+}-independent isoforms of PKC in thrombin receptor–mediated stimulation of exchanger activity. In this regard, the Ca^{2+}-independent novel PKC isoforms δ and ϵ have been detected as the most abundant isoforms of this enzyme in adult rat ventricular myocardium (for recent review, see Sugden and Bogoyevitch[34]) and have been shown to be readily activated by exposure to a number of extracellular stimuli.[35,36] However, it should be noted that regardless of the identity of the isoform(s) involved, PKC is unlikely to regulate sarcolemmal Na^+-H^+ exchanger activity by direct phosphorylation of the exchanger.[37]

It is important to note also that the lack of effect of thrombin receptor stimulation on $[Ca^{2+}]_i$ in our quiescent myocytes is contrary to observations made in platelets[7] and vascular smooth muscle cells.[9] This apparent discrepancy is likely to have arisen because of the insignificant role played by phosphoinositide hydrolysis products in inducing Ca^{2+} release from intracellular stores in mammalian cardiac myocytes,[38,39] relative to other cell types.[40] The dominant mechanism of Ca^{2+} release from the sarcoplasmic reticulum in cardiac myocytes is widely accepted to be Ca^{2+}-induced release via ryanodine receptors,[39,41] which itself might be subject to some modulation by phosphoinositide hydrolysis products.[38,39] Therefore, the possibility cannot be discounted that thrombin receptor stimulation may affect $[Ca^{2+}]_i$ regulation in nonquiescent myocardium. Indeed, recent evidence[42] suggests that in spontaneously contracting or electrically driven neonatal rat ventricular myocytes, thrombin receptor activation by a high concentration (300 µmol/L) of SFLLRN can increase both systolic and diastolic $[Ca^{2+}]_i$.

Physiological/Pathophysiological Relevance

Glembotski et al[11] have shown that in cultured neonatal rat ventricular myocytes, activation of the thrombin receptor induces the phenotypic and morphological characteristics of cellular hypertrophy. Although other extracellular agonists that can induce a hypertrophic response in this preparation (eg, phenylephrine[43] and endothelin[44]) also share the ability to activate the sarcolemmal Na$^+$-H$^+$ exchanger,[3] it is currently unclear whether this action plays a role in the initiation of the hypertrophic response.

As noted earlier, the sarcolemmal Na$^+$-H$^+$ exchanger has been implicated as a key determinant of the severity of ischemia/reperfusion-induced cardiac dysfunction, including arrhythmias.[1,2] Therefore, it is possible that thrombin-induced activation of the sarcolemmal Na$^+$-H$^+$ exchanger could modulate the outcome of ischemia and reperfusion. In this regard, we have shown that although reperfusion-induced arrhythmias are suppressed by interventions designed to inhibit the Na$^+$-H$^+$ exchanger,[45,46] they are exacerbated by stimuli that can activate the exchanger.[47] Of particular relevance to the present study, Goldstein et al[48] have shown that the incidence of malignant ventricular arrhythmias during acute ischemia is greater after thrombotic coronary occlusion than after nonthrombotic balloon occlusion, implicating an arrhythmogenic role for factors (such as thrombin) that are associated with thrombus formation. More recently, the same group suggested that during myocardial ischemia, activation of the thrombin receptor may contribute to arrhythmogenesis by inducing an increase in intracellular Na$^+$,[49] an observation that is consistent with Na$^+$-H$^+$ exchanger activation. An arrhythmogenic role for thrombin during ischemia/reperfusion, possibly via Na$^+$-H$^+$ exchanger activation, could have clinical significance, since intracoronary thrombosis is the commonest cause of acute ischemia in patients with coronary artery disease.[50]

Potential Limitations of Study

Although the concentrations of thrombin (1 and 5 U/mL) that we have shown to significantly activate the sarcolemmal Na$^+$-H$^+$ exchanger in freshly isolated adult rat ventricular myocytes are within the range proposed to occur in the vicinity of intracoronary thrombi,[48] they are nevertheless higher than those previously shown to elicit cellular responses in cultured neonatal ventricular myocytes from the same species.[10,11] In contrast, the concentration of SFLLRN that significantly increased sarcolemmal Na$^+$-H$^+$ exchanger activity in the present study was comparable to that shown recently to induce physiological responses in cultured neonatal rat ventricular myocytes.[51] This may be a reflection of partial receptor proteolysis during our collagenase-based cell isolation procedure, since it is known that the cleaved thrombin receptor becomes desensitized to further activation by thrombin.[52] Indeed, we have preliminary evidence (data not shown) that in myocytes isolated using a combination of protease and collagenase (rather than collagenase alone), the Na$^+$-H$^+$ exchanger is no longer activatable by thrombin. It would be of interest to determine whether in these cells SFLLRN retains the ability to increase the exchanger's activity. It would also be of value to determine whether the maximal responses elicited by thrombin versus SFLLRN in our cells, in terms of stimulation of sarcolemmal Na$^+$-H$^+$ exchanger activity, are comparable.

The sequence of the thrombin receptor–activating peptide SFLLRN is based on the human thrombin receptor sequence and differs by one amino acid from the corresponding sequence of the rat thrombin receptor (SFFLRN). Therefore, it may be argued that this could have led to an underestimation of the effects of SFLLRN in rat ventricular myocytes. Contrary to this, however, it has been shown recently[53] that the thrombin receptors of rat vascular smooth muscle cells can be activated with comparable potency by synthetic receptor–activating peptides based on either the human or the rat thrombin receptor sequence.

It should be noted that the findings of the present study do not preclude an involvement of changes in $[Ca^{2+}]_i$ in the signaling pathway(s) underlying thrombin receptor–mediated stimulation of sarcolemmal Na$^+$-H$^+$ exchanger activity. In this regard, in the present study, the effects of thrombin and SFLLRN on $[Ca^{2+}]_i$ were examined only under conditions in which pH_i was unaffected. To ascertain the potential role of changes in $[Ca^{2+}]_i$, it would be necessary to determine whether thrombin and SFLLRN modulate $[Ca^{2+}]_i$ under conditions in which they also increase Na$^+$-H$^+$ exchanger activity (ie, in the presence of acute intracellular acidosis).

Concluding Comments

In conclusion, the present study indicates that adult rat ventricular myocytes express a functional thrombin receptor, whose stimulation leads to increased activity of the sarcolemmal Na$^+$-H$^+$ exchanger. PKC appears to play a key role in the intracellular signaling mechanisms(s) downstream from thrombin receptor stimulation, although direct phosphorylation of the Na$^+$-H$^+$ exchanger by this enzyme is unlikely to be involved. The physiological and/or pathophysiological significance of thrombin receptor–mediated regulation of sarcolemmal Na$^+$-H$^+$ exchanger activity remains to be determined.

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Regulation of Sarcolemmal Na^+/H^+ Exchanger Activity by Angiotensin II in Adult Rat Ventricular Myocytes

Opposing Actions via AT_1 Versus AT_2 Receptors

Suba Gunasegaram, Robert S. Haworth, David J. Hearse, Metin Avkiran

Abstract—Increased sarcolemmal Na^+/H^+ exchanger activity has been implicated as a mediator of the cardiac actions of angiotensin II. We studied the receptor subtypes and signaling pathways involved in the regulation of sarcolemmal Na^+/H^+ exchanger activity by angiotensin II in adult rat ventricular myocytes. Cells were loaded with the pH-sensitive fluoroprobe carboxy-seminaphthorhodafluor-1, and acid efflux rates estimated during recovery from intracellular acidosis were used to quantify exchanger activity. Sarcolemmal Na^+/H^+ exchanger activity was not affected by angiotensin II alone but was increased by angiotensin II plus PD123319 (AT_2 antagonist). In contrast, angiotensin II plus losartan (AT_1 antagonist) or CGP42112A (AT_2 agonist) did not affect exchanger activity. The increase in Na^+/H^+ exchanger activity induced by angiotensin II plus PD123319 was blocked by losartan, PD98059 (extracellular signal–regulated kinase inhibitor), GF109203X (protein kinase C inhibitor), and tyrphostin AG1478 (epidermal growth factor receptor kinase inhibitor). Extracellular signal-regulated kinase phosphorylation and activity, measured by immunoblot analysis and an immune-complex kinase assay, respectively, were increased significantly by angiotensin II plus PD123319; these increases were blocked by losartan and PD98059. The increase in extracellular signal–regulated kinase phosphorylation induced by angiotensin II plus PD123319 was blocked also by GF109203X and tyrphostin AG1478. These data show that AT_1 stimulation increases sarcolemmal Na^+/H^+ exchanger activity in adult rat ventricular myocytes and that this response requires extracellular signal-regulated kinase activation through a protein kinase C– and epidermal growth factor receptor–mediated mechanism. The positive effect of AT_1 stimulation on Na^+/H^+ exchanger activity is counteracted by simultaneous AT_2 stimulation through a mechanism that does not involve direct inhibition of the exchanger or attenuation of extracellular signal–regulated kinase activation. (*Circ Res*. **1999;85:919-930.**)

Key Words: angiotensin ■ myocyte ■ Na^+/H^+ exchanger ■ signal transduction ■ extracellular signal–regulated kinase

The sarcolemmal Na^+/H^+ exchanger (NHE) of cardiac myocytes consists of the ubiquitous NHE1 isoform of this multigene family[1] and plays an important role in mediating recovery of intracellular pH (pH_i) from acidosis.[2] Although sarcolemmal NHE activity is regulated primarily by pH_i and is increased in response to acidosis, it is also subject to modulation by a number of neurohormonal stimuli,[3] such as α_1-adrenergic agonists[4] endothelin 1[5] and thrombin.[6] These agonists appear to stimulate sarcolemmal NHE activity, through their respective G_q protein–coupled receptors, by increasing the exchanger's affinity for intracellular H^+, which is the primary mechanism underlying receptor-mediated regulation of NHE1.[7]

Several recent studies have suggested that angiotensin II also may stimulate sarcolemmal NHE activity and that a relative intracellular alkalosis that arises from increased H^+ extrusion through the exchanger may underlie the positive inotropic action of this peptide.[8,9] However, studies with ventricular myocytes isolated from adult rat and rabbit hearts have revealed that (1) H^+ efflux rate via the exchanger is increased only moderately by angiotensin II and only over a very limited pH_i range (≈6.95 to 7.00),[9] which is contrary to observations made with other mediators that stimulate exchanger activity via G_q protein–coupled receptors,[4,6] and (2) the degree of intracellular alkalosis and the magnitude of the positive inotropic effect induced by angiotensin II are markedly smaller than those induced by endothelin 1,[8] another vasoactive peptide that has been shown to stimulate the exchanger.[5] Thus, the role of angiotensin II as an important modulator of sarcolemmal NHE activity appears open to question.

In light of the above, it is interesting to note that (1) both AT_1 and AT_2 subtypes of the angiotensin receptor are expressed in the ventricular myocardium of many species, including rat,[10] rabbit,[11] and human,[12,13] and (2) in a variety of cell types in culture, AT_1 and AT_2 mediate opposing actions,

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Mean Values for Basal pH_i (Measured Just Before NH_4Cl Application) and Minimal pH_i (Measured Immediately After NH_4Cl Washout) During the First and Second Acid Pulses

		First Acid Pulse		Second Acid Pulse	
Study Group	n*	Basal pH_i	Minimal pH_i	Basal pH_i	Minimal pH_i
Effect of simultaneous stimulation of AT_1 and AT_2					
Control	8	7.25±0.07	6.65±0.05	7.22±0.05	6.66±0.08
ANG 10	8	7.25±0.04	6.68±0.08	7.20±0.04	6.65±0.15
ANG 100	8	7.22±0.03	6.70±0.04	7.18±0.09	6.68±0.05
ANG 1000	8	7.28±0.05	6.68±0.09	7.25±0.09	6.65±0.08
Effect of selective stimulation of AT_1					
Control	8	7.28±0.05	6.75±0.03	7.25±0.06	6.79±0.04
ANG 100	8	7.25±0.10	6.68±0.05	7.20±0.05	6.65±0.03
ANG 100+PD 10	8	7.30±0.09	6.65±0.09	7.25±0.06	6.60±0.06
ANG 100+PD 30	8	7.25±0.06	6.58±0.05	7.28±0.04	6.60±0.07
ANG 100+PD 100	8	7.29±0.03	6.67±0.08	7.25±0.05	6.69±0.09
PD 100	8	7.33±0.09	6.70±0.15	7.30±0.15	6.75±0.08
Control	7	7.19±0.09	6.60±0.05	7.00±0.06	6.55±0.05
ANG 10+PD 100	7	7.28±0.06	6.65±0.07	7.20±0.07	6.62±0.05
ANG 30+PD 100	7	7.20±0.11	6.65±0.09	7.15±0.09	6.63±0.08
ANG 100+PD 100	7	7.25±0.09	6.70±0.08	7.22±0.10	6.75±0.07
Control	10	7.25±0.05	6.78±0.09	7.21±0.09	6.80±0.06
ANG 100+PD 100	10	7.18±0.09	6.73±0.06	7.20±0.05	6.75±0.05
ANG 100+PD 100+LOS 10	10	7.28±0.09	6.75±0.04	7.25±0.04	6.69±0.09
ANG 100+PD 100+LOS 30	10	7.19±0.15	6.64±0.15	7.20±0.04	6.65±0.17
ANG 100+PD 100+LOS 100	10	7.28±0.19	6.70±0.09	7.25±0.06	6.75±0.13
PD 100+LOS 100	10	7.19±0.09	6.73±0.06	7.24±0.05	6.70±0.06
Effect of selective stimulation of AT_2					
Control	9	7.18±0.05	6.75±0.07	7.20±0.07	6.80±0.05
ANG 100	9	7.28±0.09	6.74±0.08	7.25±0.05	6.75±0.06
ANG 100+LOS 10	9	7.18±0.15	6.68±0.17	7.28±0.03	6.70±0.07
ANG 100+LOS 30	9	7.18±0.13	6.65±0.15	7.15±0.17	6.65±0.14
ANG 100+LOS 100	9	7.23±0.06	6.79±0.09	7.20±0.09	6.80±0.13
LOS 100	9	7.25±0.04	6.73±0.05	7.22±0.08	6.70±0.06
Control	8	7.28±0.09	6.65±0.15	7.22±0.11	6.68±0.09
CGP 20	8	7.25±0.05	6.62±0.09	7.22±0.09	6.65±0.06
Role of the ERK pathway					
Control	8	7.25±0.04	6.64±0.03	7.22±0.09	6.60±0.05
ANG 100+PD 100	8	7.19±0.05	6.68±0.05	7.25±0.15	6.68±0.09
ANG 100+PD 100+MEKI 10	8	7.25±0.04	6.60±0.08	7.27±0.15	6.60±0.10
ANG 100+PD 100+MEKI 50	8	7.23±0.08	6.65±0.15	7.20±0.09	6.70±0.05
MEKI 50	8	7.30±0.15	6.70±0.09	7.28±0.05	6.75±0.05
Upstream regulators of AT_1-mediated actions					
Control	7	7.23±0.06	6.65±0.07	7.22±0.08	6.64±0.06
GF 1	7	7.25±0.08	6.63±0.09	7.23±0.07	6.63±0.18
AG 250	7	7.23±0.08	6.62±0.15	7.20±0.09	6.68±0.09
ANG 100+PD 100	7	7.24±0.06	6.65±0.15	7.21±0.09	6.68±0.09
ANG 100+PD 100+GF 1	7	7.22±0.17	6.68±0.19	7.18±0.18	6.65±0.09
ANG 100+PD 100+AG 250	7	7.25±0.09	6.65±0.09	7.23±0.15	6.64±0.08

ANG indicates angiotensin II; PD, PD123319 (AT_2-selective antagonist); LOS, losartan (AT_1-selective antagonist); CGP, CGP42112A (AT_2-selective agonist); MEKI, PD98059 (MEK inhibitor); GF, GF109203X (PKC inhibitor); and AG, tyrphostin AG1478 (EGF receptor kinase inhibitor). Numbers in first column indicate concentration in nmol/L, except for MEKI and GF, which are in μmol/L.

*Number of myocytes per group; in each protocol, the cells were obtained from 6 to 10 separate hearts.

particularly in growth regulation.[14–16] Furthermore, in some preparations,[17,18] AT_2 stimulation has been shown to counteract AT_1-mediated activation of extracellular signal–regulated kinases 1 and 2 (ERK1/ERK2). Because this pathway of the mitogen activated protein kinase signaling cascade is a key mediator of growth factor–induced stimulation of NHE1,[19] it is possible that AT_1 and AT_2 may mediate opposing actions also in the regulation of sarcolemmal NHE activity.

In an effort to gain a better understanding of angiotensin II–mediated regulation of sarcolemmal NHE activity, the objectives of the present study were to determine, in adult rat ventricular myocytes, whether (1) simultaneous stimulation of AT_1 and AT_2 by angiotensin II alters sarcolemmal NHE activity, (2) selective stimulation of AT_1 or AT_2 by angiotensin II alters sarcolemmal NHE activity, (3) the ERK pathway is involved in any regulation of the exchanger by angiotensin II, and (4) protein kinase C (PKC) and/or the epidermal growth factor (EGF) receptor are upstream mediators of any effects of angiotensin II on ERK and sarcolemmal NHE activity.

Materials and Methods

Studies in Adult Myocytes

Cell Isolation

Ventricular myocytes were isolated from hearts of adult male Wistar rats by enzymatic digestion[4,6,20,21] for the study of drug effects on sarcolemmal NHE activity or cellular ERK phosphorylation and activity.

Determination of Sarcolemmal NHE Activity

A microepifluorescence technique was used to record pH_i in single myocytes loaded with cSNARF-1, and the rate of acid efflux (J_H), calculated at pH_i intervals of 0.05 during recovery from intracellular acidosis, was used as the indicator of NHE activity.[4,6,20,21] Cells (n=7 to 10 per group, obtained from 6 to 10 hearts in each protocol) were subjected to acidosis by transient exposure to NH_4Cl (first acid pulse), which was repeated ≈15 minutes later (second acid pulse).[4,6] Within each protocol, there was no significant difference between groups in basal or minimal pH_i (Table). In control cells, both acid pulses occurred in the absence of drug. When the effects of angiotensin II (Sigma) were studied, this was present during the second pulse. When used, the AT_2-selective antagonist PD123319 (Research Biochemicals International) and/or the AT_1-selective antagonist losartan (gift from Merck Sharp and Dohme, Inc) were present from 3 minutes before the second acid pulse. When studying the effects of angiotensin II plus PD123319 in the presence of a kinase inhibitor (mitogen-activated protein kinase kinase [MEK] inhibitor PD98059, PKC inhibitor GF109203X, or EGF receptor kinase inhibitor tyrphostin AG1478; Calbiochem-Novabiochem Corp), this was present from 10 minutes before the second acid pulse.

Determination of Cellular ERK Phosphorylation and Activity

Angiotensin II–mediated regulation of ERK was studied by the following 2 approaches: (1) determination, by Western analysis, of ERK phosphorylation on both threonine and tyrosine residues of the regulatory TEY motif, and (2) measurement, by an immune-complex kinase assay, of ERK activity. Drug exposure protocols were identical to those used to study sarcolemmal NHE activity.

Determination of Cellular AT_2 Expression

AT_2 expression was determined by Western analysis, using a rabbit polyclonal AT_2 antibody[22,23] (gift from Dr R.M. Carey, University of Virginia Health Sciences Center, Charlottesville, VA). Membrane protein from HEK293 cells stably transfected with AT_2 was a gift from Dr A.J. Balmforth (University of Leeds, UK).

Studies in Neonatal Myocytes

Ventricular myocytes isolated from hearts of 2-day-old Wistar rats of mixed sex were cultured in 6-well plates (on coverslips for microepifluorescence studies) for 2 to 3 days and transferred to serum-free medium 24 hours before use. Drug effects on cellular ERK phosphorylation and sarcolemmal NHE activity were determined as described for adult cells.

Statistical Analysis

Data are presented as mean±SEM. To assess changes in J_H within groups (ie, between the first and second acid pulses), a paired *t* test was used. For intergroup comparisons of the change in J_H at pH_i 6.90 ($\Delta J_{H6.9}$) or of ERK phosphorylation/activity, data were subjected to ANOVA, followed by the Dunnett test (to compare every group with the control group) or Student-Newman-Keuls test (to compare every group with every other). $P<0.05$ was considered significant.

An expanded Materials and Methods section is available online at http://www.circresaha.org.

Results

Studies in Adult Myocytes

Effect of Simultaneous Stimulation of AT_1 and AT_2 on Sarcolemmal NHE Activity

Angiotensin II at 10 to 1000 nmol/L had no significant effect on J_H throughout the pH_i range 6.75 to 7.10 (Figure 1). Therefore, $\Delta J_{H6.9}$ did not differ significantly from control (0.5±0.6 mmol/L · min^{-1}) in the groups that received 10 (0.2±0.8 mmol/L · min^{-1}), 100 (0.2±0.4 mmol/L · min^{-1}), or 1000 (0.5±0.3 mmol/L · min^{-1}) nmol/L angiotensin II. These data indicate that simultaneous stimulation of AT_1 and AT_2 by angiotensin II does not significantly affect sarcolemmal NHE activity. Consistent with this, exposure of myocytes to 100 nmol/L angiotensin II for 10 minutes did not significantly affect resting pH_i (7.28±0.03 and 7.29±0.04, respectively, before and after angiotensin II; n=3).

Effect of Selective Stimulation of AT_1 on Sarcolemmal NHE Activity

Figure 2 shows representative recordings of pH_i during the consecutive acid pulses in single myocytes as well as the J_H-versus-pH_i relationships constructed using data from 8 such experiments in each of the 3 key groups of this subsection of the study. In control experiments (Figure 2A), the profiles of pH_i recovery from intracellular acidosis were similar after both pulses; consequently, the J_H-versus-pH_i curves were superimposed, indicating that temporal changes in NHE activity do not occur in the absence of drug exposure. Similar results were obtained when cells were exposed to 100 nmol/L angiotensin II during the second acid pulse (Figure 2B), as described above. However, when cells were pretreated with 100 nmol/L PD123319, 100 nmol/L angiotensin II accelerated pH_i recovery from intracellular acidosis and produced a rightward shift of the J_H-versus-pH_i curve, such that over the pH_i range 6.75 to 7.00, J_H was significantly greater in the presence of angiotensin II (Figure 2C).

Figure 3A shows J_H at pH_i 6.90 ($J_{H6.9}$) during the first and the second acid pulses in the various study groups and illustrates the dose-dependent action of PD123319 in revealing the NHE-stimulatory effect of angiotensin II. As shown,

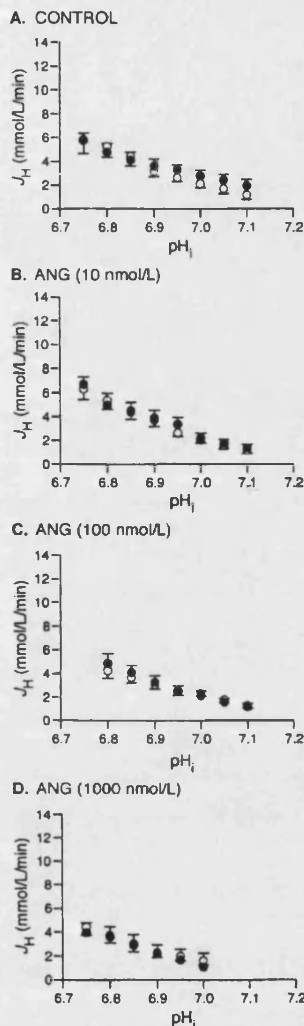

Figure 1. Effect of simultaneous stimulation of AT_1 and AT_2 on sarcolemmal NHE activity in adult rat ventricular myocytes. J_H-vs-pH_i curves obtained during the first (○) and second (●) acid pulses are shown in control cells (A) and in cells that were exposed to angiotensin II at 10 (B), 100 (C), or 1000 (D) nmol/L throughout the second pulse (n=8 cells per group, obtained from 6 hearts). ANG indicates angiotensin II.

100 nmol/L angiotensin II significantly increased $J_{H6.9}$ only in the presence of pretreatment with 30 or 100 nmol/L PD123319; importantly, 100 nmol/L PD123319 alone did not significantly affect $J_{H6.9}$, indicating that the increase in NHE activity did not arise from a direct exchanger-stimulatory action of the AT_2 antagonist. In these experiments, although $\Delta J_{H6.9}$ was not significantly different from control in cells that received angiotensin II alone or PD123319 alone, PD123319 dose-dependently revealed a stimulatory response to angiotensin II (Figure 3B). The ability of angiotensin II to increase sarcolemmal NHE activity only in the presence of the AT_2 antagonist suggests that this response may arise from selective AT_1 stimulation.

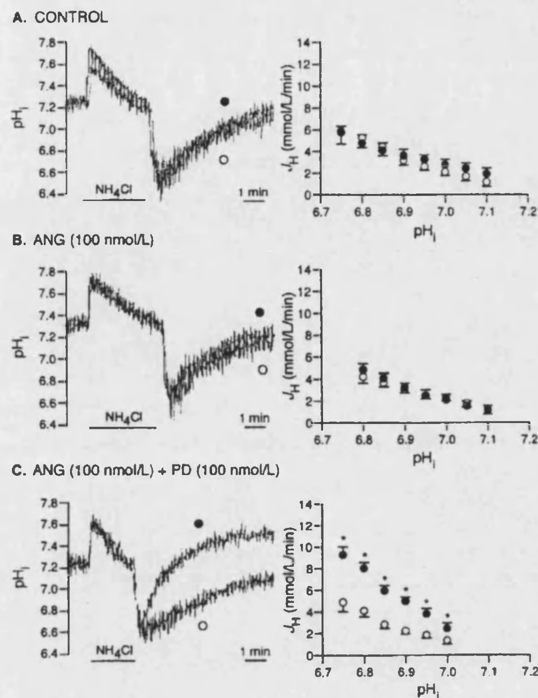

Figure 2. Effect of selective stimulation of AT_1 on recovery from intracellular acidosis and pH_i sensitivity of sarcolemmal NHE activity in adult rat ventricular myocytes. Representative pH_i recordings (left panels) and J_H-vs-pH_i curves (right panels) obtained during the first (○) and second (●) acid pulses are shown in control cells (A) and in cells that were exposed to 100 nmol/L angiotensin II throughout the second pulse in the absence (B) or presence (C) of 100 nmol/L PD123319. *$P<0.05$ vs first pulse (n=8 cells per group, obtained from 9 hearts). ANG indicates angiotensin II; PD, PD123319.

To determine the dose-dependency of the NHE stimulatory effect of angiotensin II in the presence of AT_2 blockade, we also studied the effects of various concentrations of angiotensin II (10, 30, or 100 nmol/L) in combination with 100 nmol/L PD123319. As shown in Figure 4A, under these conditions, angiotensin II produced a significant increase in $J_{H6.9}$ at all 3 concentrations. The dose dependency of the response is illustrated in Figure 4B, which shows that $\Delta J_{H6.9}$ was significantly greater than control in the groups that received 30 or 100 nmol/L angiotensin II in combination with 100 nmol/L PD123319.

To confirm that the NHE stimulatory effect of the PD123319/angiotensin II combination that was observed in the studies described above was indeed mediated via AT_1, we sought to determine whether this effect could be inhibited by losartan, an AT_1-selective antagonist. As shown in Figure 5A, the combination of 100 nmol/L PD123319 and 100 nmol/L angiotensin II once again significantly increased $J_{H6.9}$, confirming our earlier findings. This effect, however, was partially inhibited by 10 nmol/L losartan and was completely abolished by 30 and 100 nmol/L losartan. As shown in Figure 5B, relative to control, $\Delta J_{H6.9}$ was significantly increased by

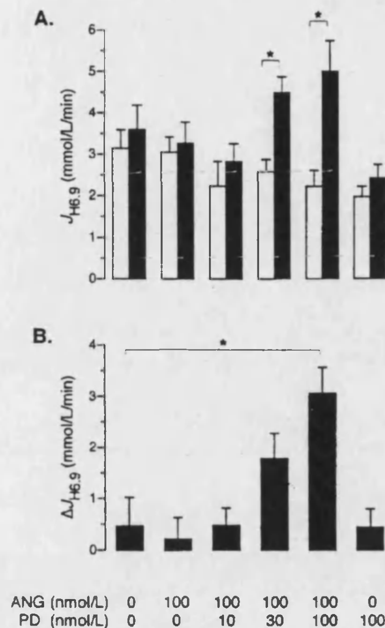

Figure 3. Effect of selective stimulation of AT_1 on sarcolemmal NHE activity in adult rat ventricular myocytes. $J_{H6.9}$ values during the first (□) and second (■) acid pulses (A) and $\Delta J_{H6.9}$ values during the second pulse relative to the first (B) are shown in control cells and in cells that were exposed to combinations of angiotensin II (100 nmol/L) and PD123319 (10, 30, or 100 nmol/L) during the second pulse. *$P<0.05$ between indicated groups (n=8 cells per group, obtained from 9 hearts). ANG indicates angiotensin II; PD, PD123319.

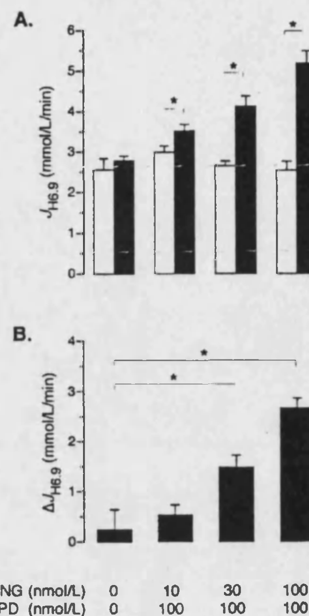

Figure 4. Effect of selective stimulation of AT_1 on sarcolemmal NHE activity in adult rat ventricular myocytes. $J_{H6.9}$ values during the first (□) and second (■) acid pulses (A) and $\Delta J_{H6.9}$ values during the second pulse relative to the first (B) are shown in control cells and in cells that were exposed to combinations of angiotensin II (10, 30, or 100 nmol/L) and PD123319 (100 nmol/L) during the second pulse. *$P<0.05$ between indicated groups (n=7 cells per group, obtained from 6 hearts). ANG indicates angiotensin II; PD, PD123319.

the PD123319/angiotensin II combination, but this effect was inhibited, in a dose-dependent manner, by losartan. The combination of 100 nmol/L PD123319 and 100 nmol/L losartan was without effect on J_H in the absence of angiotensin II. Furthermore, the inhibitory effect of losartan on the response to the PD123319/angiotensin II combination was sustained throughout the pH_i range 6.75 to 7.05 (data not shown). These findings provide further support for the hypothesis that selective stimulation of AT_1 increases sarcolemmal NHE activity.

Effect of Selective Stimulation of AT_2 on Sarcolemmal NHE Activity

The inability of angiotensin II to increase sarcolemmal NHE activity unless AT_2 is blocked suggests that stimulation of this receptor subtype opposes AT_1-mediated activation of the exchanger. To determine whether this negative regulatory effect is mediated by a direct inhibition of the exchanger, we next determined the effect of selective stimulation of AT_2 on sarcolemmal NHE activity. This was achieved by exposing cells either to 100 nmol/L angiotensin II in the presence of increasing concentrations (10 to 100 nmol/L) of losartan or to the AT_2-selective agonist CGP42112A (20 nmol/L). These experiments revealed that neither angiotensin II in combination with losartan nor CGP42112A had any effect on J_H throughout the pH_i range studied (data not shown). These findings indicate that the negative effect of AT_2 stimulation on AT_1-mediated activation of the sarcolemmal NHE is likely to occur not by direct inhibition of the exchanger but by inhibition of the signaling pathway(s) that mediate the AT_1 response.

AT_2 Expression in Adult Rat Ventricular Myocytes

The pharmacological data presented above suggest that AT_1 and AT_2 mediate opposing actions in the regulation of sarcolemmal NHE activity in adult rat ventricular myocytes. Contrary to this, however, some radioligand binding studies suggest that AT_2 may be absent in adult rat myocardium.[24,25] To determine whether AT_2 is expressed at protein level in the adult rat ventricular myocytes used in the present study, we used Western analysis with an AT_2 antibody that has been recently characterized by Wang et al.[23] The immunoblot illustrated in Figure 6 reveals that this antibody detected a protein of ≈44 kDa, which is believed to represent AT_2,[23] in protein extracts from 3 independent adult rat ventricular myocyte preparations, as well as in a membrane protein sample from HEK293 cells stably transfected with AT_2. This finding is consistent with the recent immunocytochemical data of Wang et al[23] and earlier radioligand binding studies in rat ventricular tissue[10,26] and supports the existence of functional AT_2 in adult rat ventricular myocytes.

Role of the ERK Pathway in the Regulation of Sarcolemmal NHE Activity by Angiotensin II

In studying the role of the ERK pathway in the regulation of sarcolemmal NHE activity by angiotensin II, we initially

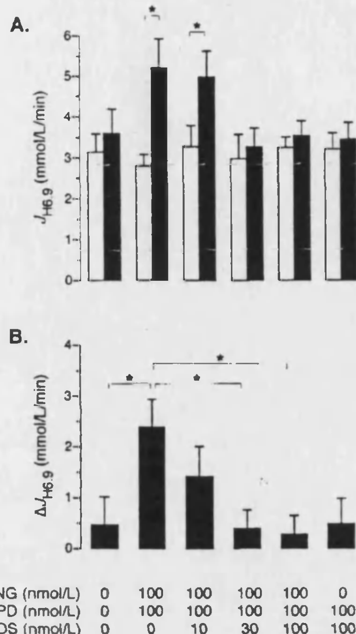

Figure 5. Effect of losartan on AT_1-mediated stimulation of sarcolemmal NHE activity in adult rat ventricular myocytes. $J_{H6.9}$ values during the first (□) and second (■) acid pulses (A) and $\Delta J_{H6.9}$ values during the second pulse relative to the first (B) are shown in control cells and in cells that were exposed to various combinations of angiotensin II (100 nmol/L), PD123319 (100 nmol/L), and losartan (10, 30, or 100 nmol/L) during the second pulse. *$P<0.05$ between indicated groups (n=10 cells per group, obtained from 9 hearts). ANG indicates angiotensin II; PD, PD123319, and LOS, losartan.

examined whether combinations of angiotensin II and PD123319 produce progressive activation of this pathway in parallel with their effects on sarcolemmal NHE activity. As shown in Figure 7, in the presence of 100 nmol/L PD123319, angiotensin II produced a dose-dependent increase in ERK1/ERK2 phosphorylation, which mirrored its dose-dependent effects on sarcolemmal NHE activity (see Figure 4B). This finding is consistent with a causal association between activation of the ERK pathway and stimulation of NHE activity.

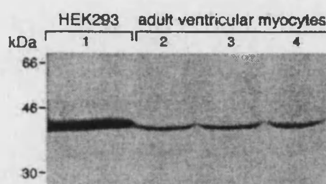

Figure 6. AT_2 expression in adult rat ventricular myocytes. Autoradiogram is from a Western blot using a polyclonal AT_2 antibody and illustrates AT_2 expression (at 44 kDa) in a membrane sample from HEK293 cells stably transfected with the human AT_2 (lane 1) and in protein extracts from 3 independent adult rat ventricular myocyte preparations (lanes 2 to 4). Forty micrograms of protein was loaded per lane, and the primary antibody was used at 1:500 dilution.

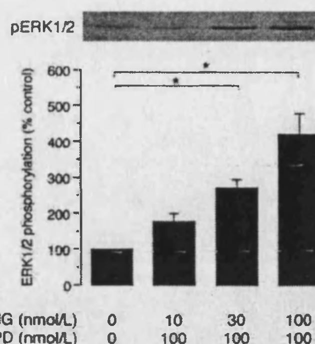

Figure 7. Effect of angiotensin II on cellular ERK1/ERK2 phosphorylation in adult rat ventricular myocytes. ERK1/ERK2 phosphorylation is shown in control cells and in cells that were exposed to combinations of angiotensin II (10, 30, or 100 nmol/L) and PD123319 (100 nmol/L). The autoradiogram shows a representative Western blot (phosphorylated ERK1/ERK2; pERK1/2). *$P<0.05$ between indicated groups (n=4 experiments, using cells obtained from 4 hearts). ANG indicates angiotensin II; PD, PD123319.

To determine whether activation of the ERK pathway is a necessary step in AT_1-mediated stimulation of sarcolemmal NHE activity, we then tested whether PD98059 (which selectively inhibits MEK, the upstream activator of ERK1/ERK2)[27,28] inhibits this response. As shown in Figure 8A, $J_{H6.9}$ was again significantly increased by the PD123319/angiotensin II combination; however, this effect was abolished in the presence of 10 or 50 μmol/L PD98059, although even the higher concentration of the MEK inhibitor was without significant effect when given alone. Thus, as shown in Figure 8B, $\Delta J_{H6.9}$ was significantly greater than control in the group that received the PD123319/angiotensin II combination but was unaffected by this combination in the presence of PD98059 or by PD98059 alone. Indeed, $\Delta J_{H6.9}$ in response to the PD123319/angiotensin combination was significantly smaller in the presence of either concentration of the MEK inhibitor than in their absence. This finding suggests that AT_1-mediated stimulation of the sarcolemmal NHE requires ERK activation.

To confirm the role of the ERK pathway in AT_1-mediated NHE stimulation, we next tested whether selective AT_1 stimulation does indeed increase cellular ERK1/ERK2 phosphorylation and activity in our system and whether the interventions that we have shown to inhibit AT_1-mediated NHE stimulation (such as losartan and PD98059) inhibit any such increase. Additionally, we compared the effects of selective AT_1 stimulation versus simultaneous AT_1/AT_2 stimulation, to determine whether the different effects of these stimuli on sarcolemmal NHE activity could arise from different magnitudes of ERK activation. Figure 9 shows the effects of the various interventions on myocyte ERK1/ERK2 phosphorylation (Figure 9A) and activity (Figure 9B). As shown, ERK phosphorylation and activity were both increased significantly by selective AT_1 stimulation, and these effects were abolished by losartan and by PD98059 (losartan or PD98059 alone did not significantly affect ERK activity; data not shown). The parallel effects of these interventions on

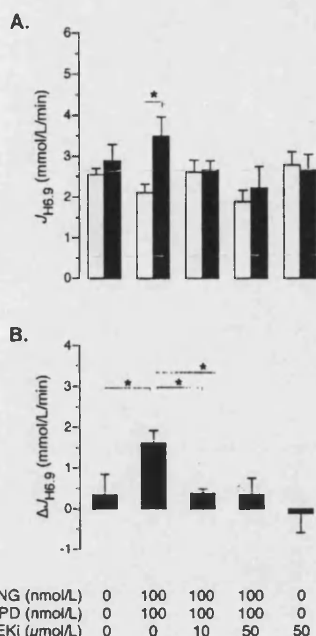

Figure 8. Effect of MEK inhibition on AT_1-mediated stimulation of sarcolemmal NHE activity in adult rat ventricular myocytes. $J_{H6.9}$ values during the first (□) and second (■) acid pulses (A) and $\Delta J_{H6.9}$ values during the second pulse relative to the first (B) are shown in control cells and in cells that were exposed to various combinations of angiotensin II (100 nmol/L), PD123319 (100 nmol/L), and PD98059 (10 or 50 µmol/L) during the second pulse. $^*P<0.05$ between indicated groups (n=8 cells per group, obtained from 8 hearts). ANG indicates angiotensin II; PD, PD123319, and MEKi, PD98059 (MEK inhibitor).

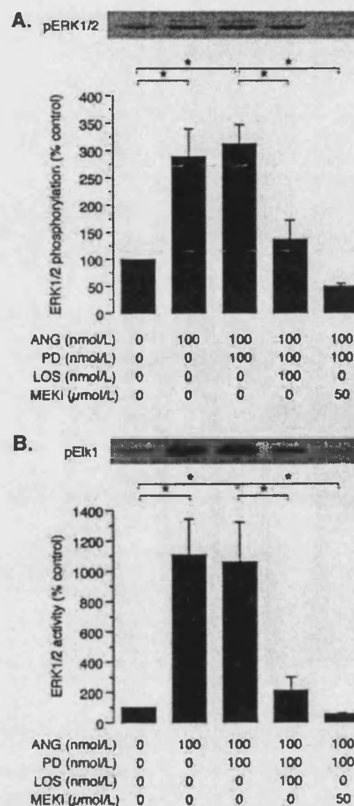

Figure 9. Effect of angiotensin II on cellular ERK1/ERK2 phosphorylation and activity in adult rat ventricular myocytes. ERK1/ERK2 phosphorylation (A) and activity (B) are shown in control cells and in cells that were exposed to various combinations of angiotensin II (100 nmol/L), PD123319 (100 nmol/L), losartan (100 nmol/L), and PD98059 (50 µmol/L). Autoradiograms show representative Western blots (phosphorylated ERK1/ERK2 [pERK1/2]; phosphorylated Elk1 [pElk1]). $^*P<0.05$ between indicated groups (n=4 experiments with each assay, using cells obtained from 8 hearts). ANG indicates angiotensin II; PD, PD123319; and MEKi, PD98059 (MEK inhibitor).

ERK phosphorylation/activity (Figure 9) and sarcolemmal NHE activity (see Figures 5 and 8) support the hypothesis that ERK activation is required for AT_1-mediated stimulation of the exchanger. Notably, however, ERK activation by simultaneous AT_1 and AT_2 stimulation was similar in magnitude to that by selective AT_1 stimulation in both assays (Figure 9A and 9B). These data suggest that the ability of AT_2 stimulation to oppose AT_1-mediated NHE activation does not arise from an attenuation of ERK1/ERK2 activation.

Upstream Regulators of AT_1-Mediated Actions

Previous studies suggest that AT_1-mediated activation of the ERK pathway is PKC dependent in cultured neonatal rat ventricular myocytes,[29] but protein tyrosine kinase dependent (via EGF receptor transactivation) in adult rat vascular smooth muscle cells[30] and neonatal rat cardiac fibroblasts.[31] To delineate the roles of these pathways in AT_1-mediated activation of the ERK pathway in adult rat ventricular myocytes, they were exposed to angiotensin II plus PD123319 in the presence of 1 µmol/L GF109203X (PKC inhibitor) or 250 nmol/L tyrphostin AG1478 (EGF receptor kinase inhibitor). As shown in Figure 10, both kinase inhibitors abolished the increase in ERK phosphorylation induced by angiotensin II plus PD123319, suggesting roles for both PKC and the EGF receptor as upstream mediators of the ERK response to AT_1 stimulation. This finding led us to determine the effects of GF109203X and tyrphostin AG1478 also on AT_1-mediated stimulation of sarcolemmal NHE activity. Figure 11 illustrates that, in parallel with their inhibitory effects on ERK activation (Figure 10), GF109203X and tyrphostin AG1478 also inhibited AT_1-mediated NHE activation. This is consistent with our conclusion above that ERK activation is necessary for AT_1-mediated stimulation of the exchanger.

Studies in Neonatal Myocytes

Because angiotensin receptor expression in rat myocardium is subject to developmental regulation,[10] the effects of angiotensin II on ERK and NHE activity may differ between neonatal and adult myocytes. To ascertain whether this is the case, we determined the effects of angiotensin II, given alone or together with PD123319, on cellular ERK and sarcolemmal NHE activity also in neonatal rat ventricular myocytes

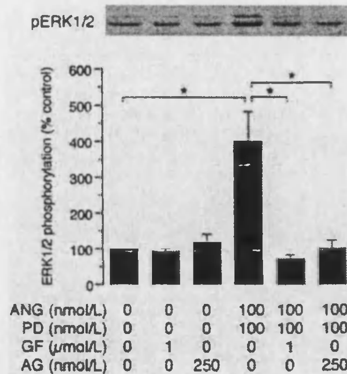

Figure 10. Effects of PKC and EGF receptor kinase inhibition on AT_1-mediated stimulation of cellular ERK1/ERK2 phosphorylation in adult rat ventricular myocytes. ERK1/ERK2 phosphorylation is shown in control cells and in cells that were exposed to various combinations of angiotensin II (100 nmol/L), PD123319 (100 nmol/L), GF109203X (1 μmol/L), and tyrphostin AG1478 (250 nmol/L). Autoradiogram shows a representative Western blot (phosphorylated ERK1/ERK2; pERK1/2). *$P<0.05$ between indicated groups (n=4 experiments, using cells obtained from 4 hearts). ANG indicates angiotensin II; PD, PD123319; GF, GF109203X; and AG, tyrphostin AG1478.

maintained in culture. Figure 12A shows that, in this preparation, angiotensin II increased cellular ERK activity to a similar extent regardless of the presence or absence of the AT_2-selective antagonist. This is consistent with the previous findings of Sadoshima et al[32] in a similar neonatal myocyte preparation and with our observations in adult ventricular myocytes as described above (Figure 9). Distinct from our observations in adult myocytes (Figure 3), however, angiotensin II induced no increase in sarcolemmal NHE activity in neonatal myocytes, in the presence of up to 100 nmol/L PD123319 (Figure 12B).

Discussion

The present study provides the first evidence in adult ventricular myocytes that AT_1 and AT_2 mediate opposing actions in determining a physiological response to angiotensin II, namely an increase in sarcolemmal NHE activity. To delineate the roles of AT_1 and AT_2 in the regulation of sarcolemmal NHE activity, we have used the endogenous agonist angiotensin II (which has similar affinity for both receptor subtypes) in combination with PD123319 (an AT_2-selective antagonist) and/or losartan (an AT_1-selective antagonist). Our data show that, in freshly isolated adult rat ventricular myocytes, angiotensin II increases sarcolemmal NHE activity only in the presence of AT_2 blockade and that this effect is inhibited by AT_1 blockade. These observations (which are distinct from those made in rat aortic smooth muscle cells[33]) suggest that, in adult rat ventricular myocytes, (1) AT_1 stimulation increases sarcolemmal NHE activity, and (2) this effect is opposed by AT_2 stimulation. In view of the signaling pathways that AT_1 shares in common with other G_q protein–coupled receptors that mediate increased sarcolemmal NHE activity in this cell type (such as α_1-adrenergic,[4] endothelin,[5] and thrombin[6] receptors), it is probable that AT_2 may atten-

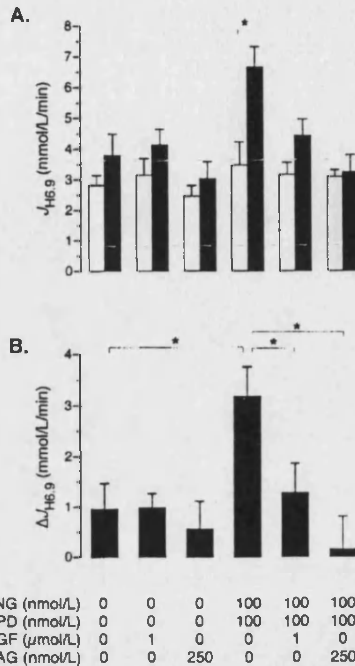

Figure 11. Effects of PKC and EGF receptor kinase inhibition on AT_1-mediated stimulation of sarcolemmal NHE activity in adult rat ventricular myocytes. $J_{H6.9}$ values during the first (□) and second (■) acid pulses (A) and $\Delta J_{H6.9}$ values during the second pulse relative to the first (B) are shown in control cells and in cells that were exposed to various combinations of angiotensin II (100 nmol/L), PD123319 (100 nmol/L), GF109203X (1 μmol/L), and tyrphostin AG1478 (250 nmol/L) during the second pulse. *$P<0.05$ between indicated groups (n=7 cells per group, obtained from 10 hearts). ANG indicates angiotensin II; PD, PD123319; GF, GF109203X; and AG, tyrphostin AG1478.

uate exchanger activation by other neurohormonal stimuli also, although confirmation of this awaits further investigation.

On a more fundamental level, our data support the existence of functional AT_1 and AT_2 on adult rat ventricular myocytes, an issue that has been subject to question.[34,35] Although it may be argued that the regulation of sarcolemmal NHE activity by angiotensin II in the present study could have a paracrine basis through the release from contaminating nonmyocyte cells of unknown NHE-regulatory factor(s), this is unlikely for several reasons. First, all visible cells that adhered to the coverslip at the bottom of the cell chamber had the morphological characteristics of cardiac myocytes. Second, cell density in the chamber was very low, ensuring considerable dilution of any paracrine factor(s) that are released from contaminating nonmyocyte cells. Finally, the myocytes under study were continuously superfused at 3.5 mL/min (equivalent to a complete change of the chamber volume every 2 seconds), thereby ensuring that any paracrine factor(s) released into the superfusion medium would be rapidly removed. Thus, it is highly likely that, in the present study, angiotensin receptor–mediated regulation of sarcolemmal NHE activity was mediated through the interaction of angiotensin II with its receptors on the myocytes themselves.

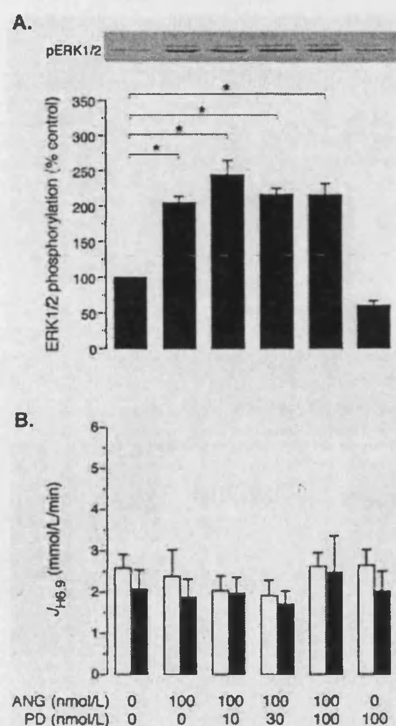

Figure 12. Effects of angiotensin II on cellular ERK1/ERK2 phosphorylation (A) and sarcolemmal NHE activity (B) in neonatal rat ventricular myocytes. ERK1/ERK2 phosphorylation (A; n=3 experiments, using cells obtained from 3 different preparations) and $J_{H6.9}$ values during the first (□) and second (■) acid pulses (B; n=12 cells per group, obtained from 8 separate preparations) are shown in control cells and in cells that were exposed to combinations of angiotensin II (100 nmol/L) and PD123319 (10, 30, or 100 nmol/L). Autoradiogram in panel A shows a representative Western blot (phosphorylated ERK1/ERK2; pERK1/2). *$P<0.05$ between indicated groups. ANG indicates angiotensin II; PD, PD123319.

Although this is the first report of opposing actions of angiotensin receptor subtypes in adult cardiac myocytes, analogous observations have been reported previously with regard to angiotensin II–mediated regulation of several physiological processes of cardiovascular relevance. The first such reports showed that AT_2 mediates an antiproliferative effect and counteracts the growth-promoting action of AT_1 stimulation in vascular smooth muscle cells[18] and coronary endothelial cells.[14] More recently, evidence has been obtained that AT_2 stimulation opposes AT_1-mediated hypertrophic effects in neonatal rat ventricular myocytes,[16] induction of new protein synthesis in adult rat hearts,[36] and chronotropic effects in adult mouse hearts.[37] Further information on the roles of AT_2 in cardiovascular regulation will undoubtedly arise from studies in transgenic mice, in which the AT_2 gene has been either disrupted[38,39] or overexpressed,[37] which have already revealed that AT_2 attenuates AT_1-mediated pressor responses to angiotensin II.[37–39] Taken together, these findings (1) challenge the notion that AT_1 is the primary mediator of the cardiovascular actions of angiotensin II (see also recent review by Matsubara[40]) and (2) illustrate that cardiovascular responses to angiotensin II may vary significantly depending on the relative availability of AT_1 and AT_2. Indeed, in the present study, angiotensin II in the presence of an equimolar concentration of PD123319 increased sarcolemmal NHE activity in adult myocytes but failed to do so in neonatal myocytes. This may reflect the greater density of AT_2 in neonatal myocardium,[10] although roles for developmental changes in other pertinent signaling mechanisms cannot be excluded.

In the present study, we have also investigated the mechanisms through which the opposing actions of AT_1 versus AT_2 on sarcolemmal NHE activity in adult ventricular myocytes may be mediated. The obvious possibility that AT_2-mediated pathways may directly inhibit sarcolemmal NHE activity, thereby resulting in functional antagonism of the AT_1-mediated response, was excluded on the basis that selective AT_2 stimulation was without effect on exchanger activity. This finding indicates that the ability of AT_2 stimulation to oppose NHE activation via AT_1 is likely to be mediated by inhibition of the signaling pathway(s) that mediate the AT_1 response. Although NHE1 is subject to regulation by multiple signaling pathways in various cell types (see recent reviews in References 41 and 42), we elected to concentrate initially on the role of the ERK pathway, on the basis of several recent findings. First, work in cultured fibroblasts has shown that the ERK pathway is a critical mediator of NHE1 activation by exogenous stimuli such as thrombin,[19] which we have shown to increase sarcolemmal NHE activity in adult rat ventricular myocytes.[6] Second, AT_1 stimulation has been shown to induce ERK activation in cultured neonatal ventricular myocytes,[32] as well as in other cell types.[17,43] Finally, AT_2 stimulation appears to oppose AT_1-mediated ERK activation in various cell types in culture, such as vascular smooth muscle cells[18] and brain neuronal cells.[17]

In our studies, (1) PD98059, a pharmacological inhibitor of the ERK pathway, inhibited the AT_1-mediated increase in sarcolemmal NHE activity; (2) selective AT_1 stimulation, which increased sarcolemmal NHE activity, also produced increases in ERK1/ERK2 phosphorylation and activity; and (3) losartan and PD98059, which inhibited the AT_1-mediated increase in sarcolemmal NHE activity, also inhibited the AT_1-mediated increases in ERK1/ERK2 phosphorylation and activity. Such findings strongly suggest that ERK activation is mechanistically involved in AT_1-mediated stimulation of sarcolemmal NHE activity in adult rat ventricular myocytes. Furthermore, our observation in adult myocytes that simultaneous AT_1 and AT_2 stimulation produced an increase in myocyte ERK1/ERK2 phosphorylation and activity similar to that of selective AT_1 stimulation suggests that (1) AT_2-mediated inhibition of the AT_1-mediated increase in sarcolemmal NHE activity in this cell type does not arise from an attenuation of ERK activation and (2) ERK activation is not sufficient to increase sarcolemmal NHE activity. The latter conclusion is supported also by our observations in neonatal ventricular myocytes, in which angiotensin II induced significant increases in ERK1/ERK2 phosphorylation without parallel increases in sarcolemmal NHE activity, even in the presence of up to 100 nmol/L PD123319. It appears, therefore, that, although ERK activation is necessary for

AT_1-mediated stimulation of sarcolemmal NHE activity, it is not the sole signaling mechanism that mediates this response.

In the broader context of angiotensin receptor–mediated regulation of myocardial ERK activity, it is important to note that our common finding in adult and neonatal rat ventricular myocytes, that simultaneous AT_1 and AT_2 stimulation and selective AT_1 stimulation both produce a similar activation of the ERK pathway, is consistent with earlier data. Thus, Masaki et al[37] and Sadoshima et al[32] have previously shown that, in wild-type adult mouse hearts and in neonatal rat ventricular myocytes, respectively, angiotensin II alone produces a significant increase in ERK1/ERK2 activity that is comparable in magnitude with that produced by the combination of angiotensin II and PD123319.

In the present study, we have also investigated the potential roles of PKC and the EGF receptor as upstream mediators of the effects of AT_1 stimulation on ERK and NHE activity in adult rat ventricular myocytes. Our observation that the PKC inhibitor GF109203X and the EGF receptor kinase inhibitor tyrphostin AG1478 could each inhibit AT_1-mediated activation of both ERK and NHE suggests that, in this cell type, PKC activation and EGF receptor transactivation are both necessary to achieve such responses. Notably, analogous findings with regard to the regulation of myocyte ERK activity by a different stimulus have recently been reported by Seko et al,[44] who showed that activation of the ERK pathway by pulsatile stretch in neonatal myocytes could be blocked equally effectively by the PKC inhibitor calphostin C and by the tyrosine kinase inhibitor genistein. One possible mechanism that might explain our data is a requirement for PKC activity for AT_1-mediated transactivation of the EGF receptor. Such a requirement has been shown to exist in HEK293 cells for transactivation of the EGF receptor by another member of the G_q protein–coupled receptor family, namely, the m1 muscarinic receptor.[45] Determination of the role of PKC in any AT_1-mediated transactivation of the EGF receptor in adult rat ventricular myocytes requires further study.

Our present results do not allow delineation of the mechanism(s) through which AT_2 stimulation opposes the AT_1-mediated increase in sarcolemmal NHE activity. One potential mechanism is AT_2-mediated inhibition of exchanger-stimulatory AT_1 signaling downstream from ERK1/ERK2 activation. In this regard, although ERK1/ERK2 can phosphorylate in vitro a fusion protein that contains the carboxyl-terminal region of NHE1,[46] the work of Bianchini et al[19] suggests that ERK-mediated stimulation of NHE1 activity in intact cells is not by direct phosphorylation but most likely involves intermediary proteins. A candidate NHE-regulatory intermediary protein, the regulation of which by AT_1 and AT_2 in adult rat ventricular myocytes warrants investigation, is the 90-kDa ribosomal S6 kinase, given that this enzyme lies downstream of ERK1/ERK2 in vascular smooth muscle cells and has been proposed as a putative NHE1 kinase.[47] Another potential mechanism that is consistent with our findings is an AT_2-mediated activation of distinct signaling pathways that do not affect basal NHE activity but oppose ERK1/ERK2-mediated stimulation of the exchanger. Potential such pathways include those mediated via p38 kinase,[48] protein kinase D,[49] and a calcineurin homologous protein,[50] given that these pathways have been proposed to have negative NHE-regulatory roles in other cell types.

It is important to consider briefly the potential physiological/pathophysiological and therapeutic implications of the novel opposing actions of AT_1 and AT_2 reported here. First, our findings may help explain the relative inefficacy of angiotensin II in increasing sarcolemmal NHE activity (eg, see Matsui et al[9] versus Yokoyama et al[4]) and enhancing myocardial inotropic status.[8] They may also have relevance to the opposing effects of AT_1 versus AT_2 in the induction of myocyte hypertrophy,[16] given that upregulation of NHE has recently been associated with hypertrophy in both in vivo[51] and in vitro[52,53] models. Finally, in considering the potential therapeutic implications of our findings, it should be noted that sarcolemmal NHE activity is believed to be a critical determinant of the severity of cardiac injury and dysfunction, including the induction of ventricular fibrillation, in myocardial ischemia and reperfusion (for a recent review, see Avkiran[54]). On the basis of the present data, it is reasonable to speculate that selective antagonists of AT_1 may possess cardioprotective efficacy. This may arise from inhibition of sarcolemmal NHE activation during ischemic episodes, not only by angiotensin II itself (through blockade of positive-regulatory AT_1) but also by other neurohormonal stimuli (through enhanced stimulation of negative-regulatory AT_2), particularly given that treatment with AT_1 antagonists appears to increase the plasma angiotensin II concentration.[55] Indeed, in view of the evidence that the AT_2:AT_1 ratio is increased in the failing human heart,[12,13] it is possible that such a cardioprotective mechanism may have contributed to the reduction in sudden cardiac death that has recently been reported in heart failure patients treated with losartan.[56]

In conclusion, the present study has shown, for the first time, that AT_1 stimulation increases sarcolemmal NHE activity in adult rat ventricular myocytes and that this effect is opposed via AT_2-mediated pathways. Our work has also revealed that (1) ERK activation is necessary but not sufficient for AT_1-mediated NHE activation, (2) activation of ERK and NHE by AT_1 stimulation both occur via a PKC- and EGF receptor–mediated pathway, and (3) the AT_2-mediated inhibitory effect on AT_1-mediated stimulation of NHE activity does not occur via direct NHE inhibition or an attenuation of ERK activation. In view of the potential impact that changes in sarcolemmal NHE activity have on cardiac function in health and disease, further investigation of the molecular signaling mechanisms that mediate such regulation of the exchanger by angiotensin receptors appears warranted.

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α_1-Adrenergic Stimulation of Sarcolemmal Na^+-H^+ Exchanger Activity in Rat Ventricular Myocytes

Evidence for Selective Mediation by the α_{1A}-Adrenoceptor Subtype

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Abstract—α_1-Adrenoceptor (α_1-AR) stimulation increases sarcolemmal Na^+-H^+ exchanger (NHE) activity. The present study was designed to determine the role(s) of α_1-AR subtype(s) in mediating this response. As an index of NHE activity, acid efflux rates (J_Hs) were determined in single rat ventricular myocytes loaded with the pH-sensitive fluoroprobe carboxy-seminaphthorhodafluor-1 after 2 consecutive intracellular acid pulses in bicarbonate-free medium. J_H at pH_i 6.90 did not change significantly during the second pulse relative to the first in control cells but increased in a dose-dependent manner when the second pulse occurred in the presence of phenylephrine (nonselective α_1-AR agonist) or A61603 (α_{1A}-AR–selective agonist), with EC_{50} values of 1.24 μmol/L and 3.6 nmol/L, respectively (both agonists given together with 1 μmol/L atenolol). Stimulation of NHE activity by 10 μmol/L phenylephrine was inhibited in a dose-dependent manner by the competitive antagonists prazosin, WB4101, and 5-methylurapidil, with IC_{50} values of 12, 32, and 149 nmol/L, respectively. Analyses of the relative EC_{50} and IC_{50} values obtained (and K_i values estimated from the antagonist IC_{50}s) in relation to the relative potencies of these agents at native rat α_1-AR subtypes and their relative affinities for recombinant rat α_1-ARs suggest that α_1-adrenergic stimulation of sarcolemmal NHE activity is likely to be mediated selectively by the α_{1A}-AR. (*Circ Res.* **1998;82:1078-1085.**)

Key Words: Na^+-H^+ exchanger ■ α_1-adrenoceptor subtype ■ myocyte ■ receptor selectivity

The sarcolemmal NHE is a primary acid extrusion mechanism in cardiac myocardium[1,2] whose activity is subject to modulation by neurohormonal stimuli.[3] In this regard, there is now convincing evidence that α_1-adrenergic stimulation increases sarcolemmal NHE activity in ventricular myocytes.[4-6] However, the question of whether this response is mediated by a specific α_1-AR subtype(s) has not been addressed.

In recent years, there has been considerable confusion regarding the classification of α_1-AR subtypes, particularly over the relationship between pharmacologically identified native α_1-ARs and the cloned α_1-ARs. However, much of this confusion has now been resolved (see Ford et al[7] and Graham et al[8]), such that the classification currently recommended by the International Union of Pharmacology[9] identifies α_1-AR subtypes as α_{1A}-, α_{1B}-, and α_{1D}-ARs, which correspond respectively to the recombinant subtypes previously referred to as α_{1c}-, α_{1b}-, and α_{1d}-ARs (the last of which has also been referred to as the α_{1a}- or $\alpha_{1a/d}$-AR). Within the context of the present study, it is important to note that α_{1A}- and α_{1B}-AR transcripts represent the dominant subtypes that are expressed in adult rat myocardium (81% of total in isolated myocytes[10] and 97% of total in whole hearts[11]). A similar pattern of expression may exist at the protein level also, on the basis of radioligand binding studies in adult rat myocardium that have shown (1) the presence of 2 high-affinity binding sites with the characteristics of α_{1A}- and α_{1B}-ARs[12,13] and (2) the absence of a high-affinity binding site for BMY7378, an α_{1D}-AR–selective antagonist.[14]

Determination of the role of α_{1A}-AR versus α_{1B}-AR subtypes in mediating physiological responses to α_1-adrenergic stimulation in myocardium (as in other tissues) is complicated by the paucity of highly selective ligands (ie, ligands that possess at least a 100- to 1000-fold higher affinity for one subtype relative to the other).[8] Studies with recombinant α_1-ARs have confirmed that endogenous catecholamines and the synthetic agonist phenylephrine do not discriminate between α_{1A}- and α_{1B}-ARs.[15,16] In contrast, the recently described potent α_1-adrenergic agonist A61603 appears to exhibit considerable selectivity for the α_{1A}-AR.[17] The alkylating agent CEC inactivates primarily the α_{1B}-AR but can produce partial inactivation of the other subtypes also, particularly with prolonged exposure at high concentration.[15,16] Of available competitive antagonists, prazosin is nonselective, but WB4101 and 5-methylurapidil exhibit ≥25-fold greater affinity for α_{1A}- versus α_{1B}-ARs (although WB4101 exhibits high affinity also for α_{1D}-ARs).[15,16] From the above, it is clear that characterization of α_1-AR subtype–mediated responses in intact myocardium or isolated myocytes may be achieved only through the methodical application of multiple agents.

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Selected Abbreviations and Acronyms

AR = adrenoceptor
C-SNARF-1 = carboxy-seminaphthorhodafluor-1
CEC = chloroethylclonidine
J_H = rate of acid efflux
$J_{H6.9}$ = J_H at pH_i 6.9
NHE = Na^+-H^+ exchanger

The objective of the present study was to determine the role(s) of α_1-AR subtype(s) in mediating α_1-adrenergic stimulation of sarcolemmal NHE activity. Toward this aim, we have used isolated ventricular myocytes from the adult rat heart in conjunction with a microepifluorescence-based assay for sarcolemmal NHE activity. In preliminary experiments, we studied the relative effects of CEC versus WB4101 on phenylephrine-induced stimulation of NHE activity to determine the likelihood of a receptor subtype-selective response. The results of the preliminary experiments led us to formulate the hypothesis that α_1-adrenergic stimulation of sarcolemmal NHE activity is mediated selectively by the α_{1A}-AR subtype. In order to test this hypothesis rigorously, we then carried out extensive dose-response studies with 2 agonists (phenylephrine and A61603) and 3 antagonists (prazosin, WB4101, and 5-methylurapidil; all in conjunction with phenylephrine) and compared the relative EC_{50} and IC_{50} values obtained (and the K_i values estimated from the antagonist IC_{50}s) with the relative potencies and affinities of these agents at native and recombinant rat α_1-AR subtypes.

Materials and Methods

This investigation was performed in accordance with the Home Office Guidance on the Operation of the Animals (Scientific Procedures) Act 1986, published by Her Majesty's Stationery Office, London, UK.

Determination of Sarcolemmal NHE Activity in Isolated Ventricular Myocytes

Sarcolemmal NHE activity was determined in single ventricular myocytes from the rat heart using a microepifluorescence-based approach that we have used in previous studies.[18-20] In brief, adult male Wistar rats (200 to 250 g body weight) were anesthetized by inhalation of diethyl ether, and hearts were excised and perfused (37°C) in the Langendorff mode for four sequential periods as follows: (1) with Tyrode's solution (mmol/L: NaCl 137, KCl 5.4, $CaCl_2$ 1.8, $MgCl_2$ 0.5, HEPES 10, and glucose 10, adjusted to pH 7.4 at 34°C with NaOH) for 5 minutes, (2) with nominally Ca^{2+}-free Tyrode's solution (mmol/L: NaCl 135, KCl 5.4, NaH_2PO_4 0.33, $MgCl_2$ 1.0, HEPES 10, and glucose 10, adjusted to pH 7.2 at 34°C with NaOH) for 5.5 minutes, (3) with nominally Ca^{2+}-free Tyrode's solution containing collagenase (Worthington type 1, 94 U/mL) for 8.5 minutes, and (4) with storage buffer (mmol/L: KOH 78, KCl 30, KH_2PO_4 30, $MgSO_4$ 3, EGTA 0.5, HEPES 10, glutamic acid 50, taurine 20, and glucose 10, adjusted to pH 7.2 at 34°C with KOH) for 5 minutes. All solutions were gassed with 100% O_2. After the perfusion procedure, the ventricles were removed and chopped into several pieces in storage buffer. The tissue fragments were then gently agitated to facilitate cell dispersion, and the cell suspension (>80% rod-shaped cells[18]) was maintained in storage buffer at 25°C for at least 1 hour before use in the microepifluorescence studies.

pH_i was monitored in single ventricular myocytes using the pH-sensitive fluorescent dye C-SNARF-1. Cells loaded with C-SNARF-1 were placed on a glass coverslip in a 100 μL chamber and continuously superfused (3.5 mL/min) with Tyrode's solution (34°C) of the composition described above. Since cells were maintained in bicarbonate-free medium throughout the experimental protocol, J_H calculated during recovery from intracellular acidosis (see below) could be used as an indicator of sarcolemmal NHE activity.[18-20]

Experimental Protocols

Experiments were performed according to a protocol involving 2 consecutive acid pulses, as we have described previously.[18] After 5 to 10 minutes of superfusion with normal Tyrode's solution (pH 7.4), cells (n=7 to 10 per group) were subjected to intracellular acidosis by transient (3-minute) exposure to 20 mmol/L NH_4Cl (first acid pulse). After a 6-minute period of NH_4Cl washout, cells were superfused with Tyrode's solution for an additional 6 minutes before a second transient exposure to NH_4Cl (second acid pulse). In control cells, both acid pulses occurred under identical conditions. When studying the effects of phenylephrine (0.1 to 100 μmol/L) or A61603 (0.1 to 300 nmol/L), the α_1-AR agonist was present throughout the second pulse (ie, during exposure to and washout of NH_4Cl) together with 1 μmol/L atenolol (to preclude β_1-AR–mediated effects). When studying the effects of phenylephrine (100 μmol/L) in the presence of the alkylating agent CEC (3 μmol/L) or the competitive α_1-AR antagonist WB4101 (3 μmol/L) in our preliminary studies, these agents were included in all solutions from 6 minutes before the second pulse to the end of the experiment. When the inhibition curves were constructed for prazosin (0.1 to 300 nmol/L), WB4101 (1 to 1000 nmol/L), and 5-methylurapidil (3 to 3000 nmol/L) versus phenylephrine (10 μmol/L), the antagonists were included in all solutions from 3 minutes before the second pulse. J_H was calculated at pH_i intervals of 0.05 during the recovery phases after both acid pulses.[18] Each cell received only a single drug intervention.

Drugs

Drugs were purchased from Sigma Chemical Co and were dissolved directly in Tyrode's solution, unless stated otherwise. Prazosin, WB4101, and 5-methylurapidil were purchased from Research Biochemicals International (via Semat Technical) and were dissolved in deionized water, ethanol, and dimethyl sulfoxide, respectively. A61603 was a gift from Abbott Laboratories (Abbott Park, Ill) and was dissolved in 0.3 mmol/L ascorbate. All stock solutions were diluted (≥1:1000) in Tyrode's solution to obtain the appropriate concentrations shortly before the beginning of experiments (concomitant controls received the appropriate vehicle).

Data Analysis

Experiments within each study subsection were carried out in a randomized manner. Data are expressed as mean±SEM. Within individual groups, the paired *t* test was used to assess changes in J_H at identical pH_i levels between the first and second acid pulses ($P<0.05$ was considered significant). Agonist dose-response and antagonist inhibition curves were constructed by measuring as the agonist response the change in $J_{H6.9}$ during the second pulse (ie, in the presence of agonist) relative to the first (ie, in the absence of agonist). The curves were fitted to a variable slope logistic equation, and EC_{50} and IC_{50} values were determined using Prism 2.0 for Macintosh software (GraphPad).

Results

Effects of CEC and WB4101 on Phenylephrine-Induced Stimulation of Sarcolemmal NHE Activity

The objective of this preliminary study was to determine whether the NHE-stimulatory effect of 100 μmol/L phenylephrine (a concentration shown previously to significantly increase sarcolemmal exchanger activity in ventricular myocytes from both guinea pig[5] and rat[6]) could be inhibited by pretreatment with either CEC or WB4101. Figure 1 shows

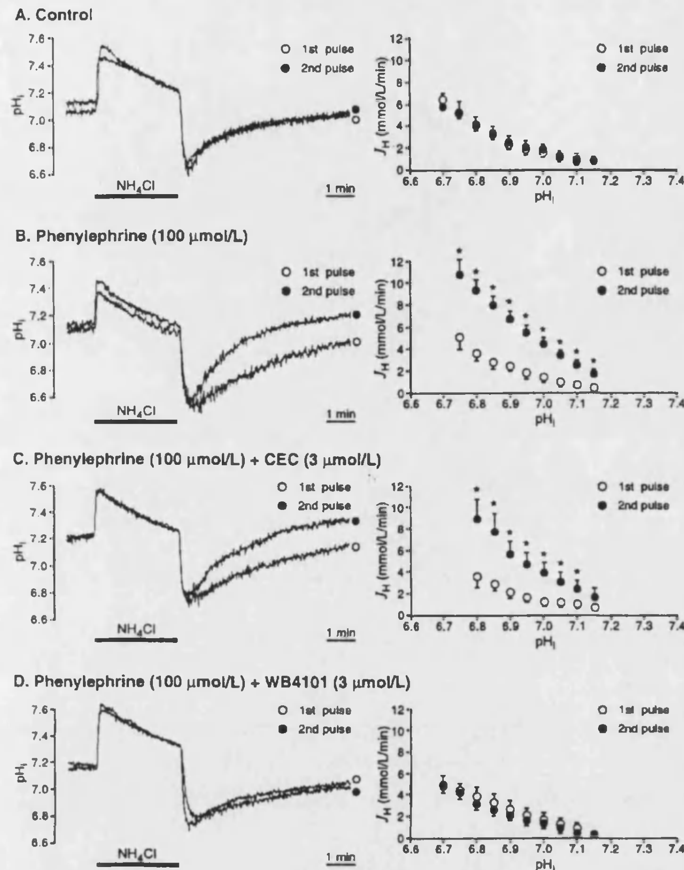

Figure 1. Representative pH_i recordings (left) and J_H-vs-pH_i curves (right) obtained during 2 consecutive acid pulses in control cells (A) and in cells that were exposed to 100 µmol/L phenylephrine throughout the second pulse in the absence (B) or presence of 3 µmol/L CEC (C) or 3 µmol/L WB4101 (D). In all cases, phenylephrine was administered together with 1 µmol/L atenolol. *$P<0.05$ vs first pulse (n=7 cells per group).

representative recordings of pH_i from single myocytes, as well as quantitative data in the form of J_H-versus-pH_i curves, in 4 study groups. In control cells, in which the consecutive acid pulses occurred under identical conditions, the profiles of pH_i recovery from acidosis and the J_H-versus-pH_i curves were similar during both acid pulses (Figure 1A), indicating that temporal changes in NHE activity do not occur in the absence of agonist stimulation. In contrast, exposure to 100 µmol/L phenylephrine plus 1 µmol/L atenolol during the second pulse accelerated recovery from intracellular acidosis and produced an upward shift of the J_H-versus-pH_i curve (Figure 1B). At $pH_i \leq 7.15$, J_H was significantly greater in the presence of 100 µmol/L phenylephrine, indicating increased sarcolemmal NHE activity in response α_1-adrenergic stimulation (in agreement with earlier reports[4-6]). When cells were pretreated with 3 µmol/L CEC before exposure to 100 µmol/L phenylephrine, the NHE-stimulatory response to the α_1-AR agonist was retained (Figure 1C); in contrast, when cells were pretreated with 3 µmol/L WB4101, the response to phenylephrine was abolished (Figure 1D). This finding suggests that the α_{1B}-AR may not be the primary mediator of the NHE-stimulatory action of phenylephrine and implicates a role for the α_{1A}-AR.

Relative Potencies of Phenylephrine and A61603 in Stimulating Sarcolemmal NHE Activity

As a first step in testing rigorously the hypothesis that α_1-adrenergic stimulation of sarcolemmal NHE activity is mediated selectively by the α_{1A}-AR subtype, we determined and compared the dose-response characteristics of phenylephrine and A61603. Figure 2A shows the effects of the 2 agonists on $J_{H6.9}$ during the second acid pulse; as can be seen, both phenylephrine and A61603 increased $J_{H6.9}$ in a dose-dependent manner. The agonist dose-response curves derived from these data (Figure 2B) revealed EC_{50} values of 1.24 µmol/L and 3.6 nmol/L for phenylephrine and A61603, respectively, indicating a 340-fold greater potency for the latter agonist.

Relative Inhibitory Potencies of Prazosin, WB4101, and 5-Methylurapidil Against Phenylephrine-Induced Stimulation of Sarcolemmal NHE Activity

To further examine the role of the α_{1A}-AR in stimulating sarcolemmal NHE activity, we also determined the inhibitory potencies of the competitive α_1-AR antagonists prazosin, WB4101, and 5-methylurapidil against the stimulatory effect induced by phenylephrine at the 10 µmol/L concentration. In

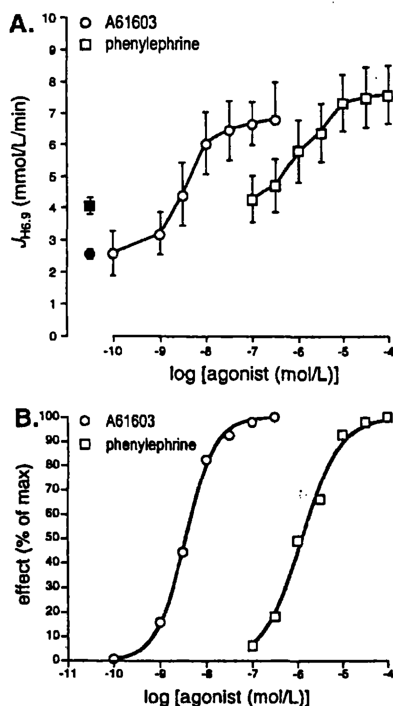

Figure 2. Panel A shows $J_{H6.9}$ during the second acid pulse in the presence of various concentrations of phenylephrine or A61603 (n=7 to 10 cells per concentration). Solid symbols indicate the mean $J_{H6.9}$ during the first acid pulse, in the absence of agonist, in the same cells. In all cases, phenylephrine or A61603 was administered together with 1 μmol/L atenolol. Panel B shows the agonist dose-response curves, which revealed EC_{50} values of 1.24 μmol/L for phenylephrine and 3.6 nmol/L for A61603.

concomitant experiments with phenylephrine alone (n=27 cells), $J_{H6.9}$ was 3.80±0.45 mmol/L per minute during the first pulse and was increased by 98% to 7.51±0.52 mmol/L per minute during the second pulse. Figure 3 shows the effects of the agonist on $J_{H6.9}$ during the second pulse, in the presence of various concentrations of prazosin (Figure 3A), WB4101 (Figure 3B), or 5-methylurapidil (Figure 3C). As illustrated, the NHE-stimulatory response to phenylephrine was inhibited in a dose-dependent manner by all 3 antagonists. The antagonist inhibition curves derived from these data (Figure 3D) revealed IC_{50} values of 12, 32, and 149 nmol/L for prazosin, WB4101, and 5-methylurapidil, respectively. These IC_{50} values and the phenylephrine EC_{50} (1.24 μmol/L) were then entered into a functional equivalent of the Cheng-Prusoff equation (Craig[21]) to obtain estimates of antagonist K_i values for comparison with published K_i values (from radioligand binding studies) at recombinant rat α_1-AR subtypes (Table 1).

Discussion

α_1-AR Subtype(s) Mediating α_1-Adrenergic Stimulation of Sarcolemmal NHE Activity

In previous studies investigating adrenergic modulation of sarcolemmal NHE activity, a stimulatory role for the α_1-AR was concluded on the basis of (1) the ability of α_1-AR agonists, such as phenylephrine[5,6] or 6-fluoronorepinephrine[4] (usually in the presence of a β_1-AR antagonist), to increase NHE activity and (2) the ability of α_1-AR antagonists, such as prazosin,[4,5] to inhibit such NHE-stimulatory effects. However, because of the lack of selectivity of the agents used, it is not possible to draw any conclusions from these studies regarding the identity of the α_1-AR subtype(s) involved in the NHE-stimulatory response.

In the present study, the novel α_{1A}-AR-selective agonist A61603 exhibited a 340-fold greater potency than phenylephrine in stimulating sarcolemmal NHE activity. Relative to phenylephrine, A61603 has been shown to possess 330-fold greater potency in inducing contraction of the rat vas deferens (an α_{1A}-AR–mediated response), only 40-fold greater potency in inducing contraction of the rat spleen (an α_{1B}-AR–mediated response), and 33-fold less potency in inducing contraction of rat aortic rings (an α_{1D}-AR–mediated response).[17] The close agreement between the relative potencies of A61603 and phenylephrine in inducing contraction of the rat vas deferens (as reported by Knepper et al[17]) and stimulating sarcolemmal NHE activity (as shown in the present study) is supportive of our hypothesis that α_1-adrenergic stimulation of sarcolemmal NHE activity is mediated by the α_{1A}-AR subtype. Furthermore, the absolute EC_{50} values for A61603 and phenylephrine obtained in the present study (3.6 nmol/L and 1.24 μmol/L, respectively) are comparable to those reported by Knepper et al[17] for α_{1A}-AR–mediated contraction of the rat vas deferens (6.2 nmol/L and 2.05 μmol/L, respectively) but not for α_{1B}-AR–mediated contraction of the rat spleen (380 nmol/L and 15.70 μmol/L, respectively) or α_{1D}-AR–mediated contraction of rat aortic rings (6.55 μmol/L and 198 nmol/L, respectively).

In interpreting our data with prazosin, WB4101, and 5-methylurapidil, the relative affinities of these antagonists for recombinant α_1-AR subtypes, expressed in α_1-AR–deficient cells, need to be taken into consideration. Such consideration is hindered, however, by the variability in the K_i values reported for these antagonists at recombinant receptors expressed in cell lines such as COS-7 and Rat-1 (Table 2), which may arise (at least in part) from differences in the species of origin of the receptors (eg, see Shibata et al[22]). In this regard, only Laz et al[15] have reported K_i values for prazosin, WB4101, and 5-methylurapidil (the 3 antagonists used in the present study) at recombinant α_1-AR subtypes exclusively of rat origin. Therefore, in view of the commonality in species, we have chosen to use the these values for comparison with those estimated in the present study. As shown in Table 1, both the absolute and the relative K_i values for prazosin, WB4101, and 5-methylurapidil estimated in the present study are in good agreement with those reported for these antagonists at recombinant α_{1A}-ARs but not α_{1B}- or α_{1D}-ARs of rat origin. This observation provides additional support for our hypothesis that α_1-adrenergic stimulation of sarcolemmal NHE activity is mediated selectively by the α_{1A}-AR subtype.

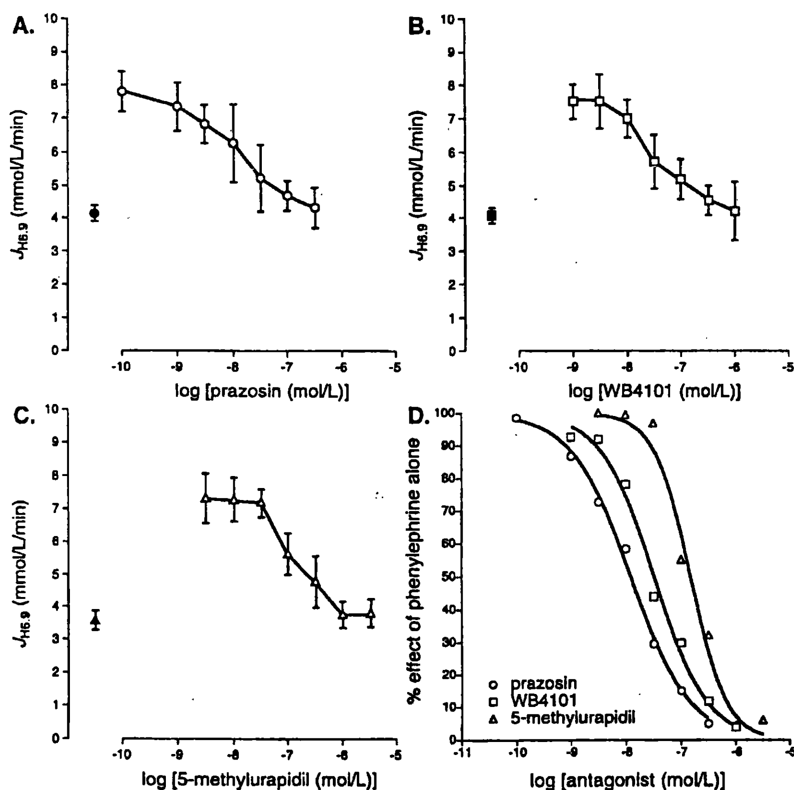

Figure 3. Panels A through C show $J_{H6.9}$ during the second acid pulse in the presence of 10 μmol/L phenylephrine in cells that received various concentrations of prazosin (A), WB4101 (B), or 5-methylurapidil (C) (n=7 to 9 cells per concentration). Solid symbols indicate the mean $J_{H6.9}$ during the first acid pulse, in the absence of agonist or antagonist, in the same cells. In all cases, phenylephrine was administered together with 1 μmol/L atenolol. Panel D shows the antagonist inhibition curves, which revealed IC_{50} values of 12 nmol/L for prazosin, 32 nmol/L for WB4101, and 149 nmol/L for 5-methylurapidil.

Potential Physiological/Pathophysiological Significance of Findings

Myocardial α_1-ARs have been implicated as mediators of a variety of physiological and pathophysiological responses to adrenergic stimulation (for reviews, see Fedida et al[23] and Terzic et al[24]). α_{1A}-AR–mediated stimulation of sarcolemmal NHE activity is likely to contribute to at least some of these responses, such as enhanced contractility (see Capogrossi[25] versus Pucéat[26]) and increased susceptibility to ischemia- and reperfusion-induced dysfunction,[27] with the latter most probably resulting from the exacerbation of intracellular Na^+ and Ca^{2+} accumulation.[28] With regard to ischemia and reperfusion, α_1-AR stimulation has also been implicated in the induction of ischemic preconditioning in rat myocardium.[29] Since ischemic preconditioning is associated with reduced intracellular acidosis during the prolonged ischemia,[30] it is possible that α_{1A}-AR–mediated stimulation of sarcolemmal NHE activity may contribute to this phenomenon. However, Gabel et al[31] have shown recently that H^+ efflux during prolonged ischemia is not increased in preconditioned hearts. Furthermore, any stimulation of sarcolemmal NHE activity is unlikely to contribute to the cardioprotective mechanism(s) of ischemic preconditioning, since we have shown such protection to be retained (and indeed enhanced) in the presence of NHE inhibition.[20]

α_{1A}-AR–mediated stimulation of exchanger activity may be involved in the induction of hypertrophy by α_1-adrenergic agonists, since the α_{1A}-AR subtype has been implicated as the mediator of this response in cultured myocytes[32] and since other stimuli that can induce such a response (eg, thrombin[33] and endothelin[34]) also share the ability to increase sarcolemmal NHE activity.[18,35] On a related note, Rokosh et al[36] have shown recently that hypertrophy of rat myocardium is associated with transcriptional induction of the α_{1A}-AR subtype, suggesting that α_{1A}-AR–mediated responses (such as stimulation of sarcolemmal NHE activity) may assume greater significance in hypertrophied myocardium. Finally, our findings may have particular relevance to cardiac α_1-adrenergic (patho)physiology in humans, since the α_{1A}-AR appears to be the predominant subtype expressed in human ventricular myocardium.[37]

TABLE 1. Antagonist K_i Values

	Estimated K_i,* nmol/L	Reported K_i,† nmol/L		
		α_{1A}-AR	α_{1B}-AR	α_{1D}-AR
Prazosin	1.3 (1)	0.3 (1)	0.2 (1)	0.3 (1)
WB4101	3.6 (3)	0.9 (3)	22 (110)	2 (7)
5-Methylurapidil	16.4 (13)	4.0 (13)	118 (590)	40 (133)

Values in parentheses indicate K_i relative to prazosin.

*Estimated from the antagonist IC_{50} and agonist (phenylephrine) EC_{50} values (see Figures 2 and 3) and the functional equivalent of the Cheng-Prusoff equation (Craig[21]), in which $K_i = IC_{50}/[1+(A/EC_{50})]$, with A denoting the agonist concentration used (10 μmol/L).

†Reported by Laz et al[15] for recombinant α_1-AR subtypes of rat origin, expressed in COS-7 cells.

TABLE 2. Antagonist K_i Values at Recombinant α_1-AR Subtypes

	α_{1A}-AR		α_{1B}-AR		α_{1D}-AR		Expression System	Reference*
	K_i, nmol/L	Origin	K_i, nmol/L	Origin	K_i, nmol/L	Origin		
Prazosin	0.37	Bovine	0.56	Rat	0.33	Rat	COS-7	38
	0.27	Bovine	0.41	Hamster	0.33	Rat	COS-7/HeLa	39
	0.30	Rat	0.20	Rat	0.30	Rat	COS-7	15
	0.18	Bovine	0.07	Hamster	0.19	Rat	HeLa	40
	NA		0.14	Hamster	NA		CHO	41
	0.20	Human	0.19	Hamster	0.16	Rat	COS-7	42
	0.21	Bovine	0.05	Hamster	0.10	Rat	LTK	17
	0.59	Human	0.55	Human	0.33	Human	LM (tk−)	43
	0.13	Human	0.32	Human	0.04	Human	Rat-1	44
	0.50	Bovine	0.13	Hamster	0.08	Rat	Rat-1	44
	0.39	Human	0.07	Human	0.16	Human	COS-7	45
	2.20	Bovine	0.38	Hamster	0.34	Rat	Rat-1	46
	0.19	Bovine	0.06	Hamster	0.10	Rat	LTK−	47
	0.17	Human	0.25	Human	0.07	Human	CHO	22
	0.19	Rat	0.38	Hamster	0.35	Rat	Rat-1	48
	0.13	Human	0.08	Human	0.09	Human	Rat-1	48
Mean	0.40±0.13		0.24±0.04		0.20±0.03			
WB4101	0.68	Bovine	28.6	Rat	2.10	Rat	COS-7	38
	0.62	Bovine	38.0	Hamster	2.00	Rat	COS-7/HeLa	39
	0.90	Rat	22.0	Rat	2.00	Rat	COS-7	15
	0.21	Rat	6.2	Hamster	14.10	Rat	COS-1	16
	0.08	Bovine	3.7	Hamster	0.43	Rat	HeLa	40
	NA		5.2	Hamster	NA		CHO	41
	0.23	Human	4.5	Hamster	0.31	Rat	COS-7	42
	0.39	Human	4.6	Human	0.91	Human	LM (tk−)	43
	1.25	Human	10.0	Human	1.25	Human	Rat-1	44
	0.63	Bovine	7.9	Hamster	1.25	Rat	Rat-1	44
	0.40	Human	1.6	Human	0.59	Human	COS-7	45
	1.38	Bovine	6.9	Hamster	1.35	Rat	Rat-1	46
	0.04	Bovine	0.6	Hamster	0.12	Rat	LTK−	47
	0.21	Human	3.5	Human	0.26	Human	CHO	22
	0.17	Rat	6.9	Hamster	1.94	Rat	Rat-1	48
	0.29	Human	2.4	Human	0.47.	Human	Rat-1	48
Mean	0.50±0.11		9.5±2.7		1.94±0.89			
5-Methylurapidil	6.8	Bovine	340	Hamster	330	Rat	COS-7/HeLa	39
	4.0	Rat	118	Rat	40	Rat	COS-7	15
	3.4	Rat	430	Hamster	282	Rat	COS-1	16
	1.2	Bovine	62	Hamster	18	Rat	HeLa	40
	4.1	Human	160	Hamster	32	Rat	COS-7	42
	NA		180	Hamster	NA		CHO	41
	0.6	Bovine	42	Hamster	14	Rat	LTK	17
	2.1	Human	174	Human	12	Human	LM (tk−)	43
	3.2	Human	251	Human	63	Human	Rat-1	44
	2.0	Bovine	316	Hamster	79	Rat	Rat-1	44
	2.0	Human	23	Human	30	Human	COS-7	45
	2.6	Bovine	91	Hamster	62	Rat	Rat-1	46
	0.6	Bovine	38	Hamster	14	Rat	LTK−	47
	0.9	Human	39	Human	10	Human	CHO	22
	0.8	Rat	100	Hamster	50	Rat	Rat-1	48
	1.0	Human	34	Human	12	Human	Rat-1	48
Mean	2.4±0.4		150±31		70±25			

Origin refers to the species of origin of the recombinant receptor, and expression system refers to the cell type(s) in which receptors were expressed. NA indicates not applicable.

*In cited articles, recombinant receptors of the α_{1A}-AR subtype may be referred to as α_{1c}-ARs, the α_{1B}-AR subtype as α_{1b}-ARs, and the α_{1D}-AR subtype as α_{1a}-, α_{1d}-, or $\alpha_{1a/d}$-ARs.

Concluding Comments

By the application of an established microepifluorescence-based assay for sarcolemmal NHE activity and a variety of pharmacological tools to stimulate or block α_1-AR subtypes, the present study has shown that α_1-adrenergic stimulation of the exchanger is likely to be mediated selectively by the α_{1A}-AR subtype. Since this pathway may be of significance in mediating a variety of physiological and pathophysiological responses to α_1-adrenergic stimuli, definitive confirmation of the role of the α_{1A}-AR in regulating sarcolemmal NHE activity (perhaps through targeted disruption of α_{1A}-AR expression) appears desirable.

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Roles of Mitogen-Activated Protein Kinases and Protein Kinase C in α_{1A}-Adrenoceptor–Mediated Stimulation of the Sarcolemmal Na^+-H^+ Exchanger

Andrew K. Snabaitis, Hiroyuki Yokoyama, Metin Avkiran

Abstract—Activation of the sarcolemmal Na^+-H^+ exchanger (NHE) has been implicated as a mechanism of inotropic, arrhythmogenic, antiacidotic, and hypertrophic effects of α_1-adrenoceptor (AR) stimulation. Although such regulation of sarcolemmal NHE activity has been shown to be selectively mediated through the α_{1A}-AR subtype, distal signaling mechanisms remain poorly defined. We investigated the roles of various kinase pathways in α_{1A}-AR–mediated stimulation of sarcolemmal NHE activity in adult rat ventricular myocytes. As an index of NHE activity, *trans*-sarcolemmal acid efflux rate (J_H) was determined through microepifluorescence in single cells, during recovery from intracellular acidosis in bicarbonate-free conditions. Extracellular signal-regulated kinase (ERK), p38-mitogen-activated protein kinase (MAPK), and $p90^{rsk}$ activities were indexed on the basis of analysis of their phosphorylation status. In control cells, there was no change in J_H in response to vehicle. Phenylephrine and A61603, an α_{1A}-AR subtype–selective agonist, increased J_H, as well as cellular ERK and $p90^{rsk}$ activities. Neither agonist affected p38 activity, which was increased with sorbitol. The MAPK kinase inhibitor PD98059 abolished phenylephrine- and A61603-induced increases in J_H and cellular ERK and $p90^{rsk}$ activities. In contrast, the PKC inhibitor GF109203X abolished phenylephrine- and A61603-induced increases in J_H but failed to prevent the increases in ERK and $p90^{rsk}$ activities. Our findings suggest that α_{1A}-AR–mediated stimulation of sarcolemmal NHE activity in rat ventricular myocytes requires activation of the ERK (but not the p38) pathway of the MAPK cascade and that the ERK-mediated effect may occur via $p90^{rsk}$. Activation of PKC is also required for α_{1A}-AR–mediated NHE stimulation, but such regulation occurs through an ERK-independent pathway. (***Circ Res*. 2000;86:214-220.**)

Key Words: Na^+-H^+ exchange ■ receptors, adrenergic ■ signal transduction ■ protein kinases

The sarcolemmal Na^+-H^+ exchanger (NHE) consists of the ubiquitous NHE-1 isoform of the multigene NHE family[1] and is an important H^+ extrusion mechanism that contributes to the integrated control of intracellular pH (pH_i) in cardiac myocytes.[2] Although sarcolemmal NHE activity is regulated primarily by pH_i and is markedly increased in response to acidosis,[2] it is also subject to modulation by several stimuli that act via G_q protein–coupled receptors (G_qPCRs), such as α_1-adrenergic agonists,[3] endothelin,[4] thrombin,[5] and angiotensin II.[6] These stimuli increase sarcolemmal NHE activity by enhancing the affinity of the exchanger for intracellular H^+, which is the primary mechanism underlying receptor-mediated regulation of NHE-1.[7]

Of the various G_qPCR signaling pathways that regulate sarcolemmal NHE activity, those that are activated by α_1-adrenoceptors (α_1-ARs) warrant attention because they are likely to mediate important physiological and pathophysiological responses. In this regard, increased sarcolemmal NHE activity and consequent increases in pH_i, intracellular Na^+, or both have been suggested to be causally involved in the positive inotropic,[8] arrhythmogenic,[9] and hypertrophic[10,11] consequences of myocardial α_1-AR stimulation. Furthermore, α_1-AR–mediated stimulation of sarcolemmal NHE activity may contribute to the antiacidotic effect during ischemia of ischemic or pharmacological preconditioning.[12] In an effort to delineate the molecular mechanisms that underlie α_1-adrenergic stimulation of sarcolemmal NHE activity, we recently demonstrated that such regulation of the exchanger is mediated selectively through the α_{1A}-AR subtype.[13] Nevertheless, pertinent signaling pathways distal to the G_qPCR remain controversial (eg, see Wallert and Fröhlich[3] versus Pucéat et al[14] on the role of protein kinase C [PKC]) and incompletely characterized.

The results of recent studies in noncardiac cells suggest that intracellular signals transduced via the extracellular signal-regulated kinase (ERK)[15–17] and p38[18] pathways of the mitogen-activated protein kinase (MAPK) cascade may be important contributors to G_qPCR-mediated regulation of NHE-1 activity. Furthermore, G_qPCR (including α_1-AR) stimulation has been shown to activate both ERK and p38 in

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isolated rat hearts[19] and cultured neonatal rat ventricular myocytes,[20,21] through mechanisms that may involve PKC.[21,22] However, the potential roles and interactions of ERK, p38, and PKC in α_{1A}-AR–mediated regulation of sarcolemmal NHE activity have not been investigated.

The present study was undertaken to determine the involvement of ERK, p38, and PKC pathways in α_{1A}-AR–mediated stimulation of sarcolemmal NHE activity in freshly isolated adult rat ventricular myocytes. To achieve this, we used established techniques for the determination of NHE and various kinase activities, in conjunction with 2 agonists of distinct α_1-AR subtype selectivity and specific kinase inhibitors. Our data suggest that α_{1A}-AR–mediated stimulation of sarcolemmal NHE activity in adult rat ventricular myocytes requires activation of the ERK (but not the p38) pathway of the MAPK cascade. Activation of PKC is also required for this response, but PKC and ERK appear to be independent regulators of NHE activity in response to α_{1A}-AR stimulation.

Materials and Methods

This investigation was performed in accordance with the Home Office "Guidance on the Operation of the Animals (Scientific Procedures) Act 1986," published by HMSO (London).

Isolation of Ventricular Myocytes

Ventricular myocytes were isolated from the hearts of adult male Wistar rats (weight 200 to 250 g) through enzymatic digestion for the study of drug effects on sarcolemmal NHE[5,6,13,23] or cellular kinase[6] activity.

Determination of Sarcolemmal NHE Activity

Sarcolemmal NHE activity was determined in single myocytes loaded with the pH-sensitive fluoroprobe cSNARF-1, through the use of a microepifluorescence technique.[5,6,13,23] Cells were maintained in bicarbonate-free medium (34°C) throughout each experiment, thus enabling the rate of acid efflux (J_H) to be used as the indicator of sarcolemmal NHE activity. To quantify drug-induced changes in NHE activity, J_H values were determined at pH_i intervals of 0.05 during recovery from intracellular acidosis.

Determination of Cellular MAPK and $p90^{rsk}$ Activities

MAPK activities were determined through the detection of dual phosphorylation of ERK1/2 and p38 on the Thr and Tyr residues of their regulatory Thr-Xaa-Tyr motifs, by Western analysis with dual phosphospecific antibodies (New England Biolabs).[6] The activity of $p90^{rsk}$ was determined through the detection of Ser381 phosphorylation with a phosphospecific antibody (New England Biolabs). To confirm equal protein loading, we used nonphosphospecific antibodies for ERK2 (Santa Cruz Biotechnology), p38 (Santa Cruz Biotechnology), and $p90^{rsk}$ (Transduction Laboratories). Specific protein bands were detected with enhanced chemiluminescence and autoradiography, and phosphorylation status was quantified with laser densitometry.

Experimental Protocols

For the determination of drug effects on NHE activity, myocytes (10 per group, obtained from 7 to 9 separate hearts in each protocol) were subjected to intracellular acidosis through transient (3 minutes) exposure to 20 mmol/L NH_4Cl (first acid pulse), which was repeated ≈15 minutes later (second acid pulse).[5,6,13] In control cells, both acid pulses occurred in the absence of any drug. When the effects of phenylephrine (Sigma), a non–subtype-selective α_1-AR agonist, or A61603 (gift from Abbott Laboratories), an α_{1A}-AR subtype–selective agonist, were studied, this was present during the second pulse. When the effects of either agonist in the presence of the MAPK kinase (MEK) inhibitor PD98059 (Calbiochem-Novabiochem) or the PKC inhibitor GF109203X (Calbiochem-Novabiochem) were studied, the inhibitor was present from 10 minutes before the second acid pulse. Drug vehicles were included in superfusion solutions, as appropriate. For determination of the effects on kinase activity, myocytes in suspension were exposed to the same drugs with the use of identical concentrations and exposure times (4 experiments with each protocol, with cells from 4 separate hearts).

Statistical Analysis

Values are given as mean±SEM. Experiments in each microepifluorescence protocol were randomized, with contemporary controls. A paired t test was used to assess changes in J_H between the first and second acid pulses. For an intergroup comparison of the change in J_H at pH_i 6.90 ($\Delta J_{H6.9}$) or of protein kinase phosphorylation, data were subjected to ANOVA; further analysis was made with Dunnett's test to compare each treatment group with the control group. $P<0.05$ was considered significant.

Results

Role of ERK1/2 in α_{1A}-AR–Mediated Stimulation of Sarcolemmal NHE Activity

Figure 1 shows the J_H-versus-pH_i relationships obtained after 2 consecutive acid pulses in the 6 groups of this protocol, which addressed the role of ERK1/2 in α_{1A}-AR–mediated stimulation of sarcolemmal NHE activity. During the second acid pulse, control cells (Figure 1A) continued to receive agonist-free superfusate, whereas the other groups were exposed to phenylephrine (Figure 1B) or A61603 (Figure 1C) in the absence or presence of PD98059. The concentrations of phenylephrine and A61603 were selected on the basis of our dose-response studies[13] and were those that produced near-maximal stimulation of sarcolemmal NHE activity. In control cells, the J_H-versus-pH_i curves obtained after both acid pulses were superimposed (Figure 1A, left), indicating that temporal changes in NHE activity do not occur in the absence of drug exposure. In these cells, PD98059 alone had no effect on the J_H-versus-pH_i curve (Figure 1A, right). Consistent with our previous data that α_{1A}-AR stimulation increases sarcolemmal NHE activity,[13] phenylephrine and A61603 both produced rightward shifts of the J_H-versus-pH_i curve such that over the range of pH_i 6.80 to 7.20, J_H was significantly greater in the presence of either agonist (Figures 1B and 1C, left). However, in the presence of PD98059, neither phenylephrine nor A61603 produced a significant shift in the J_H-versus-pH_i curve (Figures 1B and 1C, right). Figure 3A shows $\Delta J_{H6.9}$ values in the 6 study groups and allows a comparison of the effects of the different stimuli on sarcolemmal NHE activity. As illustrated, in the absence of PD98059, $\Delta J_{H6.9}$ was significantly greater in cells that received phenylephrine or A61603. In contrast, in the presence of PD98059, there was no significant difference in $\Delta J_{H6.9}$ between control cells and those exposed to either α_1-AR agonist. Because PD98059 inhibits Raf-mediated activation of MEK1/2,[24] which in turn activates ERK1/2,[25] these data suggest that activation of the ERK pathway is a necessary step in α_{1A}-AR–mediated stimulation of sarcolemmal NHE activity.

Role of PKC in α_{1A}-AR–Mediated Stimulation of Sarcolemmal NHE Activity

Figure 2 shows the J_H-versus-pH_i relationships obtained in this protocol, which was analogous to that described earlier

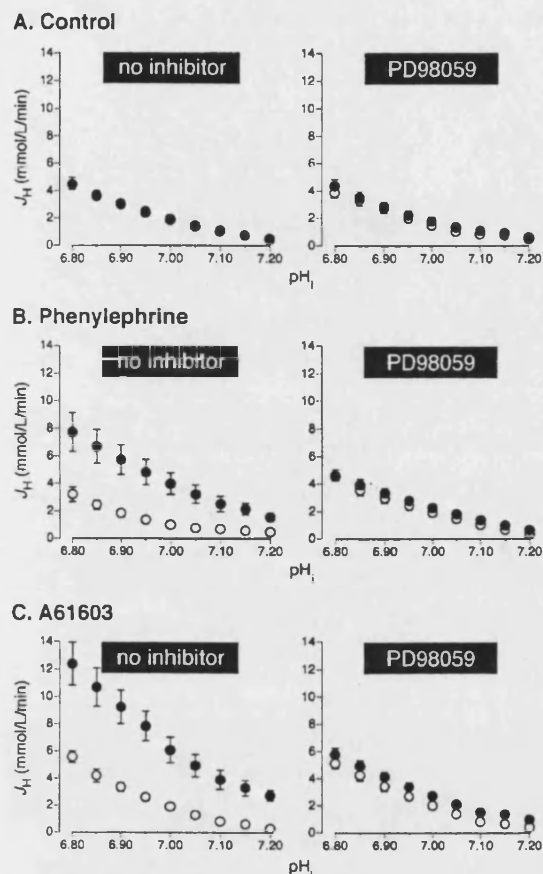

Figure 1. Effect of MEK inhibition on α_{1A}-AR–mediated stimulation of sarcolemmal NHE activity in adult rat ventricular myocytes. J_H-versus pH_i curves obtained during first (○) and second (●) acid pulses are shown in control cells (A) and in cells exposed to 10 μmol/L phenylephrine (B) or 30 nmol/L A61603 (C) during second pulse in absence or presence of MEK inhibitor PD98059 (50 μmol/L) (10 cells per group, obtained from 7 hearts).

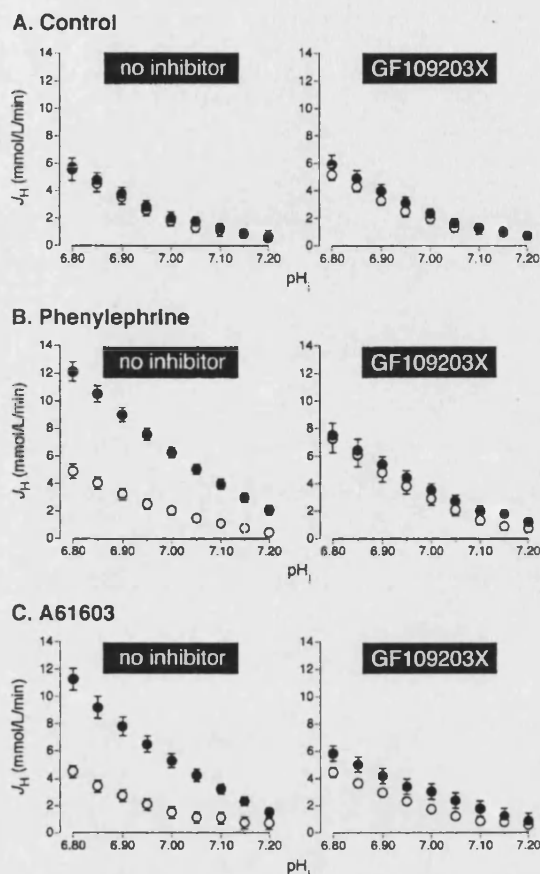

Figure 2. Effect of PKC inhibition on α_{1A}-AR–mediated stimulation of sarcolemmal NHE activity in adult rat ventricular myocytes. J_H-versus pH_i curves obtained during first (○) and second (●) acid pulses are shown in control cells (A) and in cells exposed to 10 μmol/L phenylephrine (B) or 30 nmol/L A61603 (C) during second pulse in absence or presence of PKC inhibitor GF109203X (1 μmol/L) (10 cells per group, obtained from 9 hearts).

except that it tested the role of the PKC pathway. In control cells, the J_H-versus-pH_i curves obtained after both acid pulses were again superimposed (Figure 2A, left). GF109203X had no effect on the J_H-versus-pH_i curve in control cells (Figure 2A, right) but abolished the rightward shifts of the curve induced by phenylephrine (Figure 2B) or A61603 (Figure 2C). As illustrated in Figure 3B, in the absence of GF109203X, $\Delta J_{H6.9}$ was again significantly greater in cells that received phenylephrine or A61603, reflecting α_{1A}-AR–mediated stimulation of sarcolemmal NHE activity. In contrast, in the presence of GF109203X, there was no significant change in $\Delta J_{H6.9}$ in response to either α_1-AR agonist. Because GF109203X is a selective inhibitor of PKC,[26] these data suggest that PKC is a critical component of the distal signaling pathways of the α_{1A}-AR that mediate the stimulation of sarcolemmal NHE activity.

Regulation of Sarcolemmal NHE Activity via PKC and ERK1/2: Contiguous or Independent Pathways?

These data suggest that in α_{1A}-AR–mediated stimulation of sarcolemmal NHE activity, both PKC and ERK1/2 are critical components of the signaling pathways distal to the G_qPCR. This situation could arise if (1) PKC and ERK1/2 are proximal and distal components of a contiguous signaling pathway or (2) PKC and ERK1/2 mediate independent signaling pathways, but activation of both is necessary to achieve the full response. To address this issue, we determined the effects on ERK activity of α_1-AR stimulation in the absence or presence of GF109203X. Figure 4 shows that in parallel with their effects on sarcolemmal NHE activity, phenylephrine and A61603 produced significant increases in ERK activity. GF109203X failed to prevent significant increases in ERK activity in response to phenylephrine and

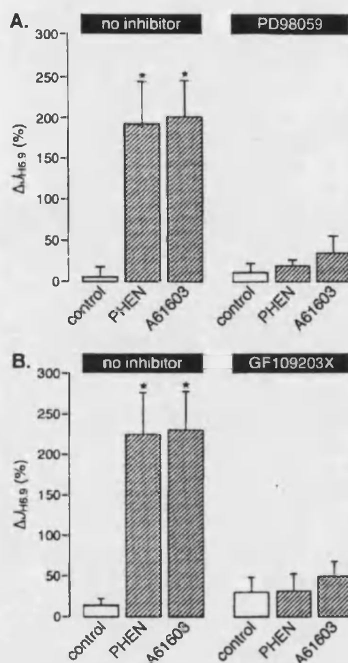

Figure 3. Effect of MEK or PKC inhibition on α_{1A}-AR–mediated increases in sarcolemmal NHE activity in adult rat ventricular myocytes. Change in J_H at pH_i 6.9 ($\Delta J_{H6.9}$) during second acid pulse relative to first is shown in control cells and in cells exposed to 10 μmol/L phenylephrine or 30 nmol/L A61603 during second pulse in absence or presence of MEK inhibitor PD98059 (50 μmol/L) (A) or PKC inhibitor GF109203X (1 μmol/L) (B). *$P<0.05$ vs control (10 cells per group, obtained from 7 to 9 hearts).

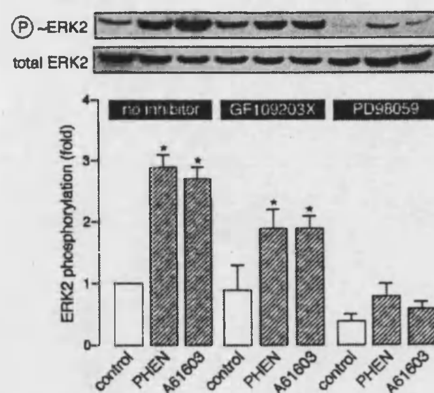

Figure 4. Effect of α_1-AR stimulation, in absence or presence of PKC or MEK inhibition, on ERK activity in adult rat ventricular myocytes. Cells were exposed to vehicle (control), 10 μmol/L phenylephrine (PHEN), or 30 nmol/L A61603 in absence or presence of PKC inhibitor GF109203X (1 μmol/L) or MEK inhibitor PD98059 (50 μmol/L). Autoradiograms show representative Western blots with phosphospecific (top) and nonphosphospecific (bottom) ERK antibodies (see Materials and Methods). *$P<0.05$ vs control (4 experiments, with cells from 4 hearts).

A61603, whereas PD98059 abolished ERK activation by each agonist. The distinct effects of the 2 kinase inhibitors on ERK activation (Figure 4), despite their common ability to prevent α_{1A}-AR–mediated stimulation of sarcolemmal NHE activity (Figure 3), allow the following conclusions to be made: (1) in adult rat ventricular myocytes, α_1-AR–mediated activation of ERK1/2 occurs, to a large extent, through PKC-independent mechanisms, and (2) activation of both pathways is required for α_{1A}-AR–mediated stimulation of sarcolemmal NHE activity.

The lack of an effect of GF109203X on ERK activation might reflect the absence of PKC-mediated ERK regulation or the dissociation of such regulation from the α_1-AR–mediated response. To address this issue, we determined the effects on ERK activity of direct PKC activation by phorbol 12-myristate 13-acetate (PMA). As shown in Figure 5, PMA produced a significant increase in ERK activity, indicating that PKC-mediated ERK activation is functional in adult rat ventricular myocytes. Figure 5 also shows that GF109203X abolished PMA-induced ERK activation, thus confirming that the concentration used was sufficient to block PKC-mediated responses.

Role of p38 in α_{1A}-AR–Mediated Stimulation of Sarcolemmal NHE Activity

In some cardiac preparations,[19,21] α_1-AR stimulation has been shown to activate p38, which has been implicated in G_qPCR-mediated regulation of plasma membrane NHE activity in rat vascular smooth muscle cells.[18] Therefore, we tested whether p38 could also be involved in α_{1A}-AR–mediated regulation of sarcolemmal NHE activity in adult rat ventricular myocytes. However, as illustrated in Figure 6, neither phenylephrine nor A61603 produced a significant increase in p38 activity. In contrast, osmotic stress, induced by exposure to 0.5 mol/L sorbitol and used as a positive control, produced a significant increase in p38 activity (Figure 6). The common inability of the α_1-AR agonists to increase p38 activity at concentrations that were sufficient to increase sarcolemmal NHE activity precludes a role for the p38 pathway in α_{1A}-AR–mediated regulation of the exchanger.

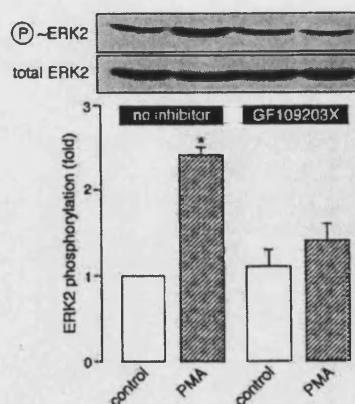

Figure 5. Effect of PKC activation on ERK activity in adult rat ventricular myocytes. Cells were exposed to vehicle (control) or 30 nmol/L PMA in absence or presence of PKC inhibitor GF109203X (1 μmol/L). Autoradiograms show representative Western blots with phosphospecific (top) and nonphosphospecific (bottom) ERK antibodies (see Materials and Methods). *$P<0.05$ vs control (4 experiments, with cells from 4 hearts).

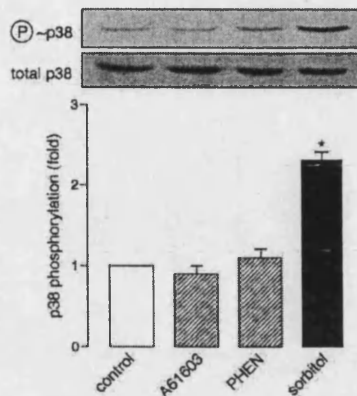

Figure 6. Effect of α_1-AR stimulation versus osmotic stress on p38 activity in adult rat ventricular myocytes. Cells were exposed to vehicle (control), 30 nmol/L A61603, 10 μmol/L phenylephrine (PHEN), or 0.5 mol/L sorbitol. Autoradiograms show representative Western blots with phosphospecific (top) and nonphosphospecific (bottom) p38 antibodies (see Materials and Methods). *$P<0.05$ vs control (4 experiments, with cells from 4 hearts).

Downstream Effectors of ERK1/2

The 90-kDa ribosomal S6 kinase ($p90^{rsk}$), which is activated by ERK1/2, has been shown to phosphorylate the regulatory domain of NHE-1[17,27,28] and may mediate serum- or endothelin-induced stimulation of NHE activity in cultured fibroblasts[27] and neonatal rat ventricular myocytes.[28] To determine whether $p90^{rsk}$ could be a downstream effector in ERK-mediated regulation of the sarcolemmal NHE in adult rat ventricular myocytes, we determined the effects of α_1-AR stimulation on the activity of this kinase. As shown in Figure 7, both phenylephrine and A61603 significantly increased $p90^{rsk}$ activity. The activation of $p90^{rsk}$ by α_1-AR stimulation was abolished by PD98059 but unaffected by GF109203X, suggesting that such activation occurred via an ERK-dependent but PKC-independent pathway. This is consistent with an effector role for $p90^{rsk}$ in ERK-mediated regulation of the sarcolemmal NHE, in response to α_{1A}-AR stimulation.

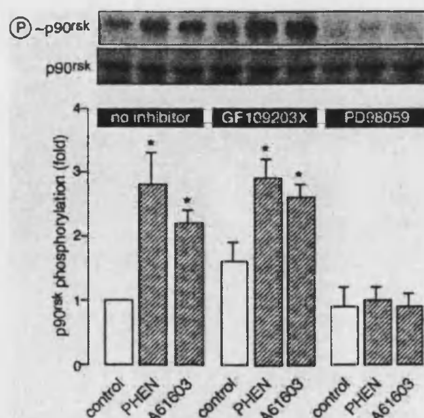

Figure 7. Effect of α_1-AR stimulation, in absence or presence of PKC or MEK inhibition, on $p90^{rsk}$ activity in adult rat ventricular myocytes. Cells were exposed to vehicle (control), 10 μmol/L phenylephrine (PHEN), or 30 nmol/L A61603 in absence or presence of PKC inhibitor GF109203X (1 μmol/L) or MEK inhibitor PD98059 (50 μmol/L). Autoradiograms show representative Western blots with phosphospecific (top) and nonphosphospecific (bottom) $p90^{rsk}$ antibodies (see Materials and Methods). *$P<0.05$ vs control (4 experiments, with cells from 4 hearts).

Discussion

Our main novel findings, which were obtained in adult rat ventricular myocytes, are that (1) inhibition of either MEK (the upstream activator of ERK1/2) or PKC abolishes stimulation of sarcolemmal NHE activity by the α_1-AR agonists phenylephrine and A61603; (2) inhibition of MEK, but not PKC, abolishes ERK activation by both agonists; (3) activity of $p90^{rsk}$, a putative NHE-1 kinase, is regulated in parallel with that of ERK1/2; and (4) phenylephrine and A61603 do not activate p38.

The ability of the MEK inhibitor PD98059 to abolish phenylephrine- and A61603-induced increases in the activities of both sarcolemmal NHE and cellular ERK1/2 provides the first evidence that ERK activation is a critical step in α_{1A}-AR–mediated stimulation of the exchanger in adult rat ventricular myocytes. This finding, considered together with our recent work in the same system on the regulation of sarcolemmal NHE activity via the angiotensin II type 1 (AT_1) receptor[6] and other pertinent data from noncardiac cells[15–17] and cultured neonatal rat ventricular myocytes,[28,29] suggests that the ERK pathway is a critical regulator of NHE-1 activity in response to multiple stimuli in various cell types. Furthermore, the parallel changes observed in NHE, ERK1/2, and $p90^{rsk}$ activities in response to α_1-AR stimulation, in the absence or presence of the MEK and PKC inhibitors, are consistent with an effector role for $p90^{rsk}$ in ERK-mediated regulation of the sarcolemmal NHE. In this regard, recent studies have revealed that the regulatory domain of NHE-1 is a substrate for $p90^{rsk}$[17,27,28] and that phosphorylation of NHE-1 by p90rsk at Ser703 stimulates exchanger activity.[27]

Our finding that PKC inhibition by GF109203X also abolishes α_{1A}-AR–mediated stimulation of sarcolemmal NHE activity supports earlier data from Wallert and Fröhlich,[3] who studied the effects on exchanger activity of the α_1-AR agonist 6-fluoronorepinephrine, and from studies with other G_qPCR agonists, such as endothelin,[4] thrombin,[5] and angiotensin II.[6] In another pertinent study,[14] however, GF109203X was shown not to inhibit phenylephrine-induced stimulation of sarcolemmal NHE activity. In that study,[14] phenylephrine was used at a concentration of 100 μmol/L, which is 10-fold greater than that used in our present work. Furthermore, this concentration is $\geq$80-fold greater than the EC_{50} value of phenylephrine for stimulation of sarcolemmal NHE activity[13] or phosphoinositide hydrolysis[30] in adult rat ventricular myocytes and for translocation of PKCϵ in neonatal rat ventricular myocytes.[20] To determine whether the difference in agonist concentration could account for the contrasting effects of GF109203X in our study and that by Pucéat et al,[14] we carried out additional experiments with a 10-fold greater concentration (100 μmol/L) of phenylephrine. In these experiments, GF109203X failed to inhibit the stimulation of sarcolemmal NHE activity by phenylephrine, which in-

creased $J_{H6.9}$ from 4.1±0.5 to 9.5±0.8 mmol · L^{-1} · min^{-1} (P<0.05) when administered alone and from 3.5±0.6 to 8.4±1.2 mmol · L^{-1} · min^{-1} (P<0.05) when administered after pretreatment with GF109203X (8 cells per group, from 3 hearts). This suggests that in the presence of a supramaximal α_1-AR agonist concentration, non–PKC-mediated pathways may be sufficient to effect increased sarcolemmal NHE activity. However, with agonist concentrations that are likely to be of greater physiological relevance, PKC activation appears to be a necessary component of the pertinent signaling pathways distal to the α_{1A}-AR.

The common ability of GF109203X and PD98059 to inhibit α_{1A}-AR–mediated stimulation of sarcolemmal NHE activity may suggest that PKC and ERK are proximal and distal components, respectively, of a contiguous NHE-regulatory signaling pathway. Indeed, our recent work in an identical system has shown that PKC and ERK1/2 participate in such a contiguous pathway in response to AT_1 receptor stimulation.[6] In our present work, however, ERK activation by the α_1-AR agonists was not prevented by GF109203X (Figure 4). This indicates that PKC and ERK1/2 mediate largely independent signaling pathways and that the activation of both pathways is necessary to achieve α_{1A}-AR–mediated stimulation of sarcolemmal NHE activity. With regard to the inability of GF109203X to prevent ERK activation by α_1-adrenergic stimulation, it is notable that α_{1A}-AR–mediated ERK activation has recently been reported to be PKC independent in PC12 cells stably transfected with this receptor subtype.[31] Furthermore, at a concentration of 1 μmol/L, GF109203X has been shown to produce only marginal inhibition of endothelin-induced ERK activation in neonatal rat ventricular myocytes.[21] This observation is similar to our present findings in adult rat ventricular myocytes, in which the same concentration of GF109203X reduced the magnitude but did not prevent the occurrence of significant ERK activation by α_1-adrenergic stimulation (Figure 4). We did not test higher concentrations of GF109203X because 1 μmol/L was sufficient to abolish PMA-induced ERK activation (which confirms that it was sufficient to inhibit PKC-mediated responses) and due to concern for potential nonspecific effects.[26]

Significant ERK activation was achieved through the exposure of adult rat ventricular myocytes to PMA (Figure 5), which illustrates that PKC can function as a proximal activator of the ERK pathway in this cell type. However, our observation that GF109203X prevented ERK activation by PMA (Figure 5) but not that by phenylephrine or A61603 (Figure 4) indicates that the PKC-mediated mechanism is not the major mechanism of ERK activation in response to α_1-adrenergic stimulation. This contrasts with our recent findings regarding ERK activation via the AT_1 receptor[6] and suggests the existence of receptor-specific differences in the role of PKC in G_qPCR-mediated ERK activation.

In contrast to recent reports in neonatal rat ventricular myocytes[21] and intact adult rat hearts,[19] we found no activation of p38 in response to either α_1-AR agonist. This points toward a difference between neonatal and adult myocyte preparations in G_qPCR-mediated regulation of p38 activity, although it is unclear whether this reflects a maturational difference or arises from the maintenance of neonatal cells in culture. It should also be noted that in the earlier studies, neonatal myocytes[21] or isolated hearts[19] were exposed to 100 μmol/L phenylephrine, which produced peak p38 activation after 10 minutes[19,21] In contrast, in our study, myocytes were exposed to 10 μmol/L phenylephrine for 3 minutes (which was sufficient to stimulate the sarcolemmal NHE) before the assessment of p38 activity. These differences in agonist concentration and duration of exposure may contribute to the distinct findings. Regardless of these issues, the inability of phenylephrine and A61603 to alter p38 activity in the present study precludes a role for the p38 pathway in NHE-regulatory signaling mechanisms distal to the α_{1A}-AR in adult rat ventricular myocytes.

Our results have shown that in isolated adult rat ventricular myocytes, α_{1A}-AR–mediated stimulation of sarcolemmal NHE activity requires activation of the ERK (but not the p38) pathway of the MAPK cascade. Activation of PKC is also required for this response, but PKC and ERK are independent regulators of NHE activity in response to α_{1A}-AR stimulation. Stimulation of NHE activity by the ERK pathway is likely to occur via activation of $p90^{rsk}$, which phosphorylates the exchanger at Ser703 and may alter its interaction with accessory proteins that regulate exchanger activity.[27] Although the mechanism through which PKC contributes to α_{1A}-AR–mediated stimulation of NHE activity is unknown, PKC does not directly phosphorylate the regulatory domain of the exchanger,[32] and altered phosphorylation of accessory proteins may play an important role. In view of the potential physiological and pathophysiological significance of α_1-adrenergic stimulation of sarcolemmal NHE activity, further work is required to fully characterize the relevant signaling pathways.

Acknowledgments

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SPECIAL REPORT

Adenosine A_1 receptor stimulation inhibits α_1-adrenergic activation of the cardiac sarcolemmal Na^+/H^+ exchanger

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Sarcolemmal Na^+/H^+ exchanger (NHE) activity is increased by stimulation of G_q protein-coupled receptors (G_qPCRs), but the roles of other GPCRs are largely unknown. We determined the effects of N-[(1S,trans)-2-hydroxycyclopentyl]adenosine (GR79236), a selective agonist of the G_iPCR adenosine A_1 receptor, on sarcolemmal NHE activity in adult rat ventricular myocytes ($n=8-10$ per group). NHE activity was indexed by the H^+ efflux rate after intracellular acidification, measured by microepifluorescence. GR79236 alone (0.01–10 μM) had no effect on NHE activity. However, co-administration of GR79236 inhibited, in a concentration-dependent manner, the stimulation of NHE activity by the α_1-adrenoceptor agonist phenylephrine (10 μM). The inhibitory effect of GR79236 (10 μM) was abolished by (1) the selective A_1 antagonist 1,3-dipropyl-8-cyclopentyl-xanthine (0.1 μM), confirming an A_1 receptor-mediated action, and (2) pre-treatment with pertussis toxin (5 μg ml^{-1} for 60 min), indicating a G_i protein-mediated mechanism. Our data suggest the existence of inhibitory crosstalk between the G_iPCR adenosine A_1 receptor and the G_qPCR α_1-adrenoceptor in the regulation of sarcolemmal NHE activity.
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Keywords: G protein-coupled receptors; adenosine A_1 receptor; α_1-adrenoceptor; cardiac myocyte; Na^+/H^+ exchanger

Abbreviations: AR, adrenoceptor; CABG, coronary artery bypass graft; DPCPX, 1,3-dipropyl-8-cyclopentylxanthine; GPCR, G protein-coupled receptor; NHE, Na^+/H^+ exchanger; PAR1, protease-activiated receptor 1

Introduction The sarcolemmal Na^+/H^+ exchanger (NHE) is the ubiquitously expressed product of the NHE1 gene (Fliegel & Dyck, 1995) and is an important H^+ extrusion mechanism that contributes to the control of intracellular pH (pH_i) in cardiac myocytes (Leem *et al.*, 1999). Nevertheless, increased activity of the sarcolemmal NHE has been implicated in the development of myocardial injury and dysfunction during ischaemia and reperfusion, and NHE1-selective pharmacological inhibitors have been shown to be cardioprotective in this setting in numerous animal studies (Avkiran, 1999a). Recent clinical data suggest that one such inhibitor, cariporide, may provide cardioprotective benefit in patients with anterior myocardial infarction who receive early reperfusion by direct coronary angioplasty (Rupprecht *et al.*, 2000) and in high-risk patients who undergo global myocardial ischaemia and reperfusion during coronary artery bypass graft (CABG) surgery (Théroux *et al.*, 2000).

Sarcolemmal NHE activity is regulated primarily by pH_i and increases markedly in response to intracellular acidosis (Leem *et al.*, 1999); it is also subject to stimulation by agents that act *via* G_q protein-coupled receptors (G_qPCRs), such as α_1-adrenoceptor (α_1-AR) agonists, endothelin, thrombin and angiotensin II (reviewed by Avkiran, 1999b). However, there is little known about the regulation of sarcolemmal NHE activity by receptors outside the G_qPCR family.

Adenosine is an adenine nucleoside that has been shown to possess cardioprotective efficacy in animal models of myocardial ischaemia and reperfusion (Lasley & Mentzer, 1996). The therapeutic potential of adenosine has also recently been tested in humans, as an adjunct to thrombolysis in patients with acute myocardial infarction (Mahaffey *et al.*, 1999) and as an additive to cardioplegia in patients undergoing CABG surgery (Mentzer *et al.*, 1999), with encouraging results. Although adenosine can potentially afford myocardial protection during ischaemia and reperfusion through the stimulation of multiple adenosine receptors in a variety of cell types, direct stimulation of myocardial A_1 receptors appears to be an important component of such protection (Lasley & Mentzer, 1996). Indeed, hearts from transgenic mice with cardiac-specific overexpression of the A_1 receptor have been shown to exhibit reduced susceptibility to ischaemia and reperfusion-induced injury (Matherne *et al.*, 1997). The mechanisms through which the myocardial A_1 receptor, which is a member of the G_iPCR family, exerts protection are unclear.

Our recent work has shown that stimulation of another G_iPCR, the angiotensin AT_2 receptor, inhibits sarcolemmal NHE activation *via* the G_qPCR angiotensin AT_1 receptor (Gunasegaram *et al.*, 1999). The possibility exists that adenosine A_1 receptors may also initiate signalling events that negatively regulate sarcolemmal NHE activity. Therefore, the present study was undertaken to determine the effects of adenosine A_1 receptor stimulation by the selective agonist N-[(1S,trans)-2-hydroxycyclopentyl]adenosine (GR79236) (Gurden *et al.*, 1993) on sarcolemmal NHE activity, in the resting state and following G_qPCR stimulation. Preliminary results of the study have been published in abstract form (Yokoyama & Avkiran, 2000).

Methods This investigation was performed in accordance with the Home Office 'Guidance on the Operation of the Animals (Scientific Procedures) Act 1986', published by HMSO, London.

Isolation of ventricular myocytes Ventricular myocytes were isolated from hearts of adult (200–250 g body weight) male Wistar rats (B&K Universal, Hull, U.K.) by enzymatic

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digestion, as previously described (Yasutake *et al.*, 1996; Yokoyama *et al.*, 1998; Gunasegaram *et al.*, 1999; Snabaitis *et al.*, 2000).

Determination of sarcolemmal NHE activity Sarcolemmal NHE activity was determined in single myocytes loaded with the pH-sensitive fluoroprobe cSNARF-1, using an established microepifluorescence technique (Yasutake *et al.*, 1996; Yokoyama *et al.*, 1998; Gunasegaram *et al.*, 1999; Snabaitis *et al.*, 2000). Cells were superfused with bicarbonate-free Tyrode's solution (34°C) throughout each experiment and the rate of acid efflux (J_H) was used as the index of sarcolemmal NHE activity. J_H was determined at an intracellular pH (pH_i) of 6.90 ($J_{H6.9}$), during recovery from intracellular acidosis that was induced by transient exposure to NH_4Cl (see below).

Experimental protocols Myocytes were subjected to intracellular acidosis by a 3-min exposure to 20 mmol/l NH_4Cl (first acid pulse), which was repeated 15 min later (second acid pulse) (Yasutake *et al.*, 1996; Yokoyama *et al.*, 1998; Gunasegaram *et al.*, 1999; Snabaitis *et al.*, 2000). In control cells, both acid pulses occurred in the absence of any drug. When studying the effects of GR79236 (gift from GlaxoWellcome, Stevenage, U.K.) alone, this was present during the second acid pulse. When studying the effects of GR79236 on the response to phenylephrine or thrombin (both from Sigma, Poole, U.K.), the G_qPCR agonist was given during the second acid pulse and GR79236 was present from 6 min before this pulse. 1,3-Dipropyl-8-cyclopentylxanthine (DPCPX; Sigma), when used, was given concomitantly with GR79236. Stock solutions of drugs, except DPCPX, were dissolved in deionized water and were diluted ($\geqslant$1000 fold) in Tyrode's solution to obtain appropriate final concentrations; the DPCPX stock solution was dissolved in dimethylsulphoxide (final concentration 0.005%). Phenylephrine solutions contained 1 μM atenolol (Sigma), to preclude β_1-AR-mediated actions (Yokoyama *et al.*, 1998). When required, cells were pre-treated with 5 μg ml^{-1} pertussis toxin (Sigma) for 60 min, in order to inactivate G_i proteins (Obayashi *et al.*, 1997).

Statistical analysis Data are expressed as mean$\pm$s.e.mean. Each protocol comprised 4–8 study groups and experiments within it were carried out in a randomized manner (n=8–10 myocytes per group, obtained from 5–15 hearts). For intergroup comparison of the changes in $J_{H6.9}$ ($\Delta J_{H6.9}$) in response to vehicle or drug(s), data were subjected to ANOVA; if a significant difference was found, further analysis was by Dunnett's test, to compare each treatment group with the control group. $P<0.05$ was considered significant.

Results *Effects of GR79236 alone* $J_{H6.9}$ values during recovery from the first acid pulse in control cells and in those that received 0.01, 0.1, 1 or 10 μM GR79236 (n=10 cells per group, from 10 hearts) were 3.56$\pm$0.31, 3.28$\pm$0.45, 3.71$\pm$0.70, 4.36$\pm$0.77 and 3.06$\pm$0.41 mM min^{-1}, respectively (NS). There was no significant change in $J_{H6.9}$ during the second acid pulse in any group, with $\Delta J_{H6.9}$ values of 13$\pm$19, 18$\pm$25, 13$\pm$20, 4$\pm$12 and 10$\pm$31%, respectively (NS). These findings indicate that acute exposure to GR79236 does not significantly affect sarcolemmal NHE activity in the resting state.

Effects of GR79236 on the response to phenylephrine We next determined whether exposure to GR79236 affects the stimulation of sarcolemmal NHE activity by phenylephrine. $J_{H6.9}$ values during the first acid pulse did not differ significantly between the eight study groups (n=10 cells per group, from 15 hearts) and were as follows: 3.18$\pm$0.48 mM min^{-1} in control cells; 3.21$\pm$0.44 mM min^{-1} in cells that received phenylephrine (10 μM) alone; 2.5$\pm$0.29, 2.78$\pm$0.50, 2.73$\pm$0.69 and 3.33$\pm$0.27 mM min^{-1}, respectively, in cells that received phenylephrine (10 μM) in combination with 0.01, 0.1, or 10 μM GR79236; 3.80$\pm$0.69 and 3.13$\pm$0.49 mM min^{-1}, respectively, in cells that received 1 or 10 μM GR79236 alone. Figure 1 illustrates the $\Delta J_{H6.9}$ values in these groups. Consistent with our earlier findings (Yokoyama *et al.*, 1998; Snabaitis *et al.*, 2000), phenylephrine alone produced a large and significant increase in sarcolemmal NHE activity. GR79236 inhibited the NHE-stimulatory effect of phenylephrine in a concentration-dependent manner, such that the α_1-AR agonist no longer produced a significant increase in NHE activity when given in combination with 1 or 10 μM GR79236. Once again, 1 or 10 μM GR79236 alone failed to produce a significant change in sarcolemmal NHE activity. These data indicate that exposure to GR79236 inhibits α_1-AR-mediated stimulation of sarcolemmal NHE activity.

Reversal of the inhibitory effect of GR79236 by DPCPX In order to confirm that the inhibitory effect of GR79236 on the α_1-adrenergic response was mediated *via* the A_1 receptor, rather than through a non-specific action, we tested the reversibility of this effect by the selective A_1 receptor antagonist DPCPX. $J_{H6.9}$ values during the first acid pulse did not differ significantly between the five study groups (n=9 cells per group, from seven hearts) and ranged between 2.48$\pm$0.44 and 3.08$\pm$0.30 mM min^{-1}. Figure 2 illustrates the $\Delta J_{H6.9}$ values. As expected, 10 μM phenylephrine produced a significant increase in NHE activity and this response was again abolished when phenylephrine was given in combination with 10 μM GR79236. However, when GR79236 was given concomitantly with 0.1 μM DPCPX, the A_1 agonist was no longer able to inhibit α_1-AR-mediated stimulation of sarcolemmal NHE activity. DPCPX alone was without effect. These findings confirm that the inhibitory effect of GR79236 on α_1-adrenergic stimulation of sarcolemmal NHE activity was mediated *via* the A_1 receptor.

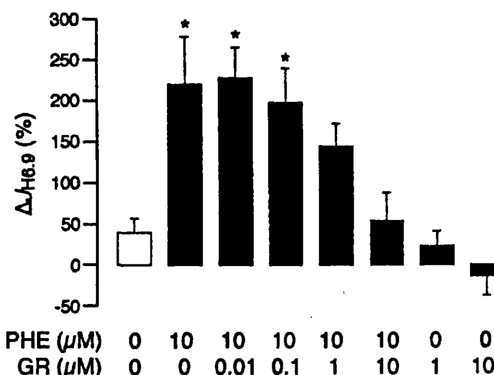

Figure 1 The change in H+ efflux rate at pH_i 6.90 ($\Delta J_{H6.9}$) in the control group and in response to phenylephrine (PHE) and GR79236 (GR), alone or in combination. The protocol comprised 80 myocytes (n=10 per group) obtained from 15 hearts. *$P<0.05$ vs control.

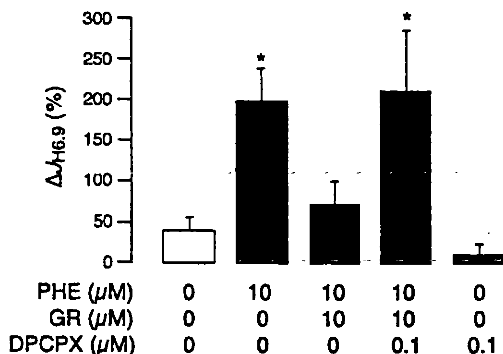

Figure 2 The change in H+ efflux rate at pH_i 6.90 ($\Delta J_{H6.9}$) in the control group and in response to phenylephrine (PHE), GR79236 (GR) and 1,3-dipropyl-8-cyclopentylxanthine (DPCPX), alone or in combination. The protocol comprised 45 myocytes ($n=9$ per group) obtained from seven hearts. *$P<0.05$ vs control.

Reversal of the inhibitory effect of GR79236 by pertussis toxin In order to probe the signalling mechanisms distal to the A_1 receptor that mediate the inhibitory effect of GR79236, we examined the consequences of inactivating the G_i protein, by a 60-min pertussis toxin pre-treatment. $J_{H6.9}$ values during the first acid pulse were similar between the eight study groups ($n=8$ cells per group, from 14 hearts) within this protocol and ranged between 2.81 ± 0.47 and 3.30 ± 0.40 mM min^{-1}. Figure 3 illustrates the $\Delta J_{H6.9}$ values. Our observations in cells pre-treated with vehicle for 60 min were essentially identical to those illustrated in Figure 1; 10 μM phenylephrine significantly increased sarcolemmal NHE activity and this response was abolished by 10 μM GR79236 (Figure 3, top panel). In contrast, in cells pre-treated with pertussis toxin for 60 min, 10 μM GR79236 was no longer able to inhibit the stimulation of sarcolemmal NHE activity by 10 μM phenylephrine (Figure 3, bottom panel). It appears therefore that G_i protein activation is a critical step in the signalling mechanisms downstream of the A_1 receptor that mediate the inhibitory effect of GR79236 on α_1-adrenergic stimulation of sarcolemmal NHE activity.

Effects of GR79236 on the response to thrombin Finally, we determined whether GR79236 could inhibit the stimulation of sarcolemmal NHE activity by another G_qPCR agonist, namely thrombin (Yasutake *et al.*, 1996). In this protocol also, $J_{H6.9}$ values during the first acid pulse were similar between the four study groups (range from 3.75 ± 0.75 to 4.13 ± 0.71 mM min^{-1}; $n=8$ cells per group, from five hearts). As expected from our earlier work (Yasutake *et al.*, 1996), relative to control ($\Delta J_{H6.9}$ $14 \pm 19\%$), 5 u ml^{-1} thrombin significantly increased sarcolemmal NHE activity ($\Delta J_{H6.9}$ $126 \pm 34\%$); however, this response was attenuated by 1 μM GR79236 ($\Delta J_{H6.9}$ $50 \pm 22\%$) and abolished by 10 μM GR79236 ($\Delta J_{H6.9}$ $26 \pm 16\%$). These data suggest that GR79236 can inhibit the stimulation of sarcolemmal NHE activity by multiple G_qPCR agonists.

Discussion Although the anti-β_1-adrenergic effect of adenosine A_1 receptor stimulation (thought to be mediated primarily through a G_i protein-mediated reduction in adenylyl cyclase activity) is well established, the present data represent the first demonstration of an anti-α_1-adrenergic effect of A_1 receptor stimulation in cardiac myocytes. Specifically, our data have

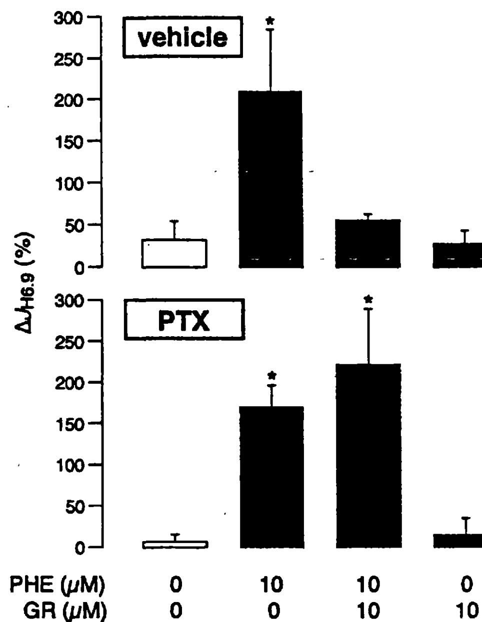

Figure 3 The change in H+ efflux rate at pH_i 6.90 ($\Delta J_{H6.9}$) in the control group and in response to phenylephrine (PHE) and GR79236 (GR), alone or in combination. The protocol comprised 64 myocytes ($n=8$ per group) obtained from 14 hearts. The cells were pre-treated for 60 min with either vehicle (top panel) or 5 μg ml^{-1} pertussis toxin (PTX; bottom panel). *$P<0.05$ vs control.

shown that the A_1 agonist GR79236 inhibits α_1-adrenergic stimulation of sarcolemmal NHE activity, a response that is mediated *via* the α_{1A}-AR (Yokoyama *et al.*, 1998). The inhibitory effect of GR79236 occurs *via* stimulation of the A_1 receptor (based on its reversibility by the A_1 antagonist DPCPX) and requires a functional G_i protein (based on its abolition following pertussis toxin pre-treatment). Importantly, the effect was not limited to inhibition of the α_1-adrenergic response, since GR79236 also inhibited the stimulation of sarcolemmal NHE activity by thrombin, a response that is mediated by the thrombin receptor (Yasutake *et al.*, 1996), now termed protease-activated receptor 1 (PAR1). Since both the α_{1A}-AR and PAR1 are members of the G_qPCR family, our data suggest the existence of a novel inhibitory crosstalk mechanism between the G_iPCR adenosine A_1 receptor and multiple G_qPCRs, at least in rat ventricular myocytes. If confirmed, such a mechanism may have implications beyond the regulation of sarcolemmal NHE activity.

In view of the important role that sarcolemmal NHE activity is believed to play in the development of myocardial injury and dysfunction during ischaemia and reperfusion (Avkiran, 1999a), inhibition of G_qPCR-mediated NHE activation is likely to contribute to the mechanisms underlying the cardioprotective effects of adenosine A_1 receptor stimulation, by GR79236 (Louttit *et al.*, 1999) and other interventions (Lasley & Mentzer, 1996). However, during ischaemia and reperfusion, sarcolemmal NHE activity may be stimulated not only by a variety of endogenous mediators (e.g. catecholamines, thrombin, endothelin) that act *via* G_qPCRs, but also by factors such as oxidant stress and exposure to lipid metabolites (Avkiran, 1999b). Therefore, it would be of interest to

determine whether A_1 receptor stimulation attenuates the stimulation of sarcolemmal NHE activity by these additional factors.

Of relevance to the present work, increased sarcolemmal NHE activity and consequent increases in pH_i and/or intracellular $[Na^+]$ have been suggested also to be causally involved in the positive inotropic and hypertrophic consequences of myocardial α_1-AR stimulation. In the light of our data, it is reasonable to expect that adenosine A_1 receptor stimulation may attenuate these effects. Indeed, there is preliminary evidence that, in rat ventricular myocytes stimulated at 0.5 Hz, GR79236 inhibits the positive effects of phenylephrine on (1) cell shortening under normal conditions, and (2) the recovery of cell shortening following intracellular acidosis (P. Krishnan and J.C. Kentish, King's College London, personal communication).

Our data suggest that the inhibitory effect of adenosine A_1 receptor stimulation on the α_1-adrenergic response is mediated *via* the G_i protein. However, the relevant mechanisms that are downstream of G_i protein activation are unclear. In view of the inability of GR79236 to significantly reduce NHE activity when given alone (e.g. Figures 1 and 3), the inhibitory effect is unlikely to reflect functional antagonism through an independent signalling pathway. Rather, it appears that A_1 receptor stimulation may initiate events that interfere with the NHE-regulatory signalling mechanisms that lie distal to the α_{1A}-AR. Our recent work has shown that α_{1A}-AR-mediated stimulation of sarcolemmal NHE activity in rat ventricular myocytes requires the activation of both protein kinase C (PKC) and extracellular signal-regulated kinases 1 and 2 (Snabaitis *et al.*, 2000). PKC activation has been shown to be necessary also for thrombin-induced stimulation of sarcolemmal NHE activity (Yasutake *et al.*, 1996). In this context, it is interesting to note that stimulation of adenosine A_1 receptors in rat isolated hearts and ventricular myocytes attenuates the effects of dioctanoylglycerol, a direct activator of PKC, on contractility and Ca^{2+} handling (Narayan *et al.*, 1998). Furthermore, there is recent evidence that, in rat ventricular myocytes, activation of protein phosphatases contributes to the anti-β_1-adrenergic mechanisms of A_1 receptor stimulation (Narayan *et al.*, 2000). It is likely that the novel inhibitory effects of adenosine A_1 receptor stimulation reported here are mediated through changes in the activity of NHE-regulatory kinases and/or the phosphorylation status of their pertinent substrates, although this remains to be confirmed by further investigation.

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Expression and Activity of Protein Kinase D/Protein Kinase Cμ in Myocardium: Evidence for α_1-Adrenergic Receptor- and Protein Kinase C-Mediated Regulation

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R. S. Haworth, M. W. Goss, E. Rozengurt and M. Avkiran. Expression and Activity of Protein Kinase D/Protein Kinase Cμ in Myocardium: Evidence for α_1-Adrenergic Receptor- and Protein Kinase C-Mediated Regulation. *Journal of Molecular and Cellular Cardiology* (2000) 32, 1013–1023. Protein kinase D (PKD), which is also known as protein kinase C (PKC) μ, is a novel serine/threonine kinase that can be activated in parallel with or downstream of PKC in various cell types, but its expression and regulation in myocardium have not been characterized. In the present study, two proteins of 110 and 115 kDa were detected in rat ventricular myocardium using antibodies directed at the extreme N- or C-terminus of PKD. Both proteins were highly expressed in the fetal heart but showed a developmental decline in abundance. Fractionation studies showed that PKD was distributed between myocyte and non-myocyte fractions in the neonatal heart, but was found predominantly in the non-myocyte fraction in the adult heart. In cultured neonatal rat ventricular myocytes, an *in vitro* kinase assay revealed increased autophosphorylation of PKD (EC_{50} 2.8 nM) in response to phorbol-12-myristate-13-acetate (PMA). Exposure to norepinephrine also induced a dose-dependent increase in PKD autophosphorylation (EC_{50} 0.6 μM). Pretreatment with the α_1-adrenergic receptor (AR) antagonist prazosin blocked norepinephrine-induced PKD autophosphorylation, while the β_1-AR antagonist atenolol had no effect, indicating that activation of PKD by norepinephrine occurred via the α_1-AR. Involvement of the α_1-AR was confirmed by exposure of myocytes to the α_1-AR agonist phenylephrine, which induced a similar profile of PKD autophosphorylation to norepinephrine (EC_{50} 0.6 μM). The effects of both α_1-AR stimulation and PMA on PKD autophosphorylation were mediated by PKC, since these effects could be attenuated by pretreatment of myocytes with the PKC inhibitor bisindolylmaleimide. These data show that PKD is expressed in rat ventricular myocardium, where its expression is subject to developmental control, and that PKD activity in ventricular myocytes is regulated through α_1-AR- and PKC-mediated pathways. © 2000 Academic Press

Key Words: Protein kinase C; Protein kinase D; α_1-adrenergic receptors; Cardiac myocytes; Signal transduction.

[1] Abbreviations: G_qPCR, G_q protein-coupled receptor; PKC, protein kinase C; PKD, protein kinase D; PMA, phorbol-12-myristate-13-acetate; AR, adrenergic receptor; FCS, fetal calf serum.

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Introduction

G_q protein-coupled receptor (G_qPCR)[1] signaling pathways, which commonly involve activation of protein kinase C (PKC),[1] have been implicated in adaptive and maladaptive responses of myocardium to stress and injury (see recent review by Dorn and Brown, 1999[2]). Consistent with this, directly increasing myocardial PKC activity by molecular means is sufficient to induce hypertrophy[3] and improve resistance to simulated ischemia[4] in cultured neonatal rat ventricular myocytes, and to produce cardiomyopathy in transgenic mice.[5] Furthermore, PKC-mediated pathways have been implicated in the neurohumoral induction of myocyte hypertrophy[6,7] and regulation of other important physiological and pathophysiological processes in myocardium, such as sarcolemmal ion transport (e.g. by ion exchangers[8,9] and channels[10–12]), contractility[13,14] and protein synthesis.[15] Despite the apparent importance of PKC signaling in myocardium, however, the downstream targets of PKC in myocytes remain incompletely characterized.

Protein kinase D (PKD) is a recently identified serine/threonine kinase[16] which has distinct structural and enzymatic properties and is found in most tissues (see review by Rozengurt *et al.*[17]). PKD, which is also known as protein kinase Cμ,[18] has a catalytic domain that is distantly related to Ca^{2+}-regulated kinases and shows little similarity to the highly conserved regions of the kinase subdomains of the PKC family. Consistent with this, PKD does not phosphorylate a variety of substrates utilized by PKCs,[16,19] indicating that PKD is a protein kinase with distinct substrate specificity. The N-terminal region of PKD contains a tandem repeat of cysteine-rich motifs which bind phorbol esters with high affinity[16,19] and immunopurified PKD is stimulated *in vitro* by either diacylglycerol or biologically active phorbol esters in the presence of phosphatidylserine.[19] More recently, a second mechanism of PKD activation has been identified, which involves phosphorylation of PKD via a PKC-dependent pathway.[20] The finding that PKD can be activated in parallel with or downstream of PKC raises the possibility that some cellular responses that arise from PKC activation may be mediated by PKD.

In view of the potential importance of PKC/PKD signaling in the heart, in the present study we have (i) determined the expression of PKD in ventricular myocardium and in myocyte and non-myocyte fractions during development of the rat heart, and (ii) examined the regulation of PKD activity in primary cultures of neonatal rat ventricular myocytes, in particular by adrenergic receptors (ARs) and PKC-mediated pathways.

Methods

Animals and materials

Wistar rats (adult males, late term pregnant females, and 1–2-day-old neonates of mixed sex) were purchased from B & K Universal (Hull, UK). The investigation was performed in accordance with the Home Office *Guidance on the Operation of the Animals (Scientific Procedures) Act, 1986*, published by HMSO, London. [γ-^{32}P]-ATP (370 MBq/ml), ECL reagent, PVDF membrane and protein A sepharose were from Amersham Pharmacia Biotech (Little Chalfont, UK). Phorbol-12-myristate-13-acetate (PMA), phenylephrine and norepinephrine were from Sigma Aldrich (Poole, UK). Bisindolylmaleimide I was from Calbiochem (Nottingham, UK).

Preparation of cardiac myocytes

Ventricular myocytes were isolated from the hearts of neonatal and adult rats using collagenase-based enzymatic digestion techniques, as previously described.[8,21] Adult hearts were excised and perfused (37 °C) in the Langendorff mode for three sequential periods, as follows: (i) 5 min with Tyrode solution containing NaCl (137 mM), KCl (5.4 mM), $CaCl_2$ (1.8 mM), $MgCl_2$ (0.5 mM), *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES; 10 mM), glucose (10 mM), adjusted to pH 7.4 with NaOH, (ii) 5.5 min with nominally Ca^{2+}-free Tyrode solution containing NaCl (135 mM), KCl (5.4 mM), NaH_2PO_4 (0.33 mM), $MgCl_2$ (1.0 mM), HEPES (10 mM), glucose (10 mM), adjusted to pH 7.2 with NaOH, (iii) 10 min with nominally Ca^{2+}-free Tyrode solution containing collagenase (Worthington Type 1, 200 U/ml), and (iv) 5 min with storage buffer containing KOH (78 mM), KCl (30 mM), KH_2PO_4 (30 mM), $MgSO_4$ (3 mM), ethylene glycol-bis (β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid (EGTA; 0.5 mM), HEPES (10 mM), glutamic acid (50 mM), taurine (20 mM), glucose (10 mM), adjusted to pH 7.2 with KOH. All solutions were gassed with 100% O_2. After the perfusion procedure, the heart was removed from the cannula, and the atria and non-ventricular tissue carefully trimmed away. The tissue fragments were gently agitated in storage buffer to disperse ventricular cells. The cells

were subsequently filtered through a 40 μm mesh filter to remove undissociated tissue, pelleted by centrifugation (600 g for 7 min) and resuspended in plating medium (68% DMEM, 17% M199, 15% fetal calf serum (FCS), 100 units/ml penicillin and 100 μg/ml streptomycin). To separate myocytes from non-myocytes, the suspension was incubated in tissue culture dishes in a CO_2 incubator for 1 h. The unattached myocytes were removed and collected by centrifugation. The non-myocytes attached to the dish were washed twice with plating medium, and incubated for 4 days prior to harvesting.

Neonatal myocytes were prepared from 2-day-old animals as follows. The hearts from 24 animals were excised, the atria trimmed off and the ventricles cut into small pieces. Tissue was incubated with mixing for 20 min at 37 °C in a solution containing collagenase (112 U/ml), pancreatin (5 mg/ml), NaCl (117 mM), HEPES (20 mM), NaH_2PO_4 (0.87 mM), glucose (5.6 mM), KCl (5.4 mM) and $MgSO_4$ (1 mM), adjusted to pH 7.4 with NaOH. Intact tissue was allowed to settle, and the dispersed cells were removed and added to 2 ml FCS. A further four incubations with collagenase solution were performed. The cells were sedimented at 600 g for 6 min, pooled, filtered, sedimented and resuspended in plating medium. Again, myocytes were enriched by the differential plating method, with the non-myocyte fraction being cultured for 4 days prior to harvesting. For studies with cultured neonatal myocytes, the enriched myocyte preparation (90–95% myocytes, as determined by cell morphology) was cultured in 6-well plates for 2–3 days. Cells were transferred to serum-free medium 24 h prior to experiments.

Protein preparation

For whole ventricle studies, the hearts were rapidly excised and rinsed in ice-cold PBS. After the atria and connective tissue were trimmed away, the ventricles were mechanically homogenized in a lysis buffer containing 50 mM Tris/HCl (pH 7.5), 2 mM EGTA, 2 mM EDTA, 1 mM DTT, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 1 mM 4-(2′-aminoethyl)-benzenesulfonyl fluoride hydrochloride and 1% Triton X-100. Cell debris was removed by centrifugation at 10 000 g for 30 min. Cultured neonatal and adult non-myocytes, cultured neonatal myocytes and freshly isolated adult myocytes were washed three times with ice-cold PBS and lysed in 100 μl of the lysis buffer described above.

Western blots

After tissue/cell lysis, protein samples (15 μg) were subjected to SDS-PAGE on 7.5% acrylamide gels, and transferred to PVDF membrane using a Pharmacia LKB Multiphor II transfer apparatus. Membranes were probed for the presence of PKD using polyclonal antibodies against the N-terminus (sc-638; amino acids 6–25; Santa Cruz Biotechnology, Inc., Santa Cruz, California, USA) or the C-terminus (sc-935; amino acids 893–912; Santa Cruz Biotechnology, Inc.), or with a mouse monoclonal antibody against a central domain (P26720; amino acids 314–517; Transduction Laboratories, Lexington, USA). In some experiments, antibody sc-935 was preincubated with immunizing peptide sc-935P for 30 min on ice prior to Western blotting. Donkey anti-rabbit and sheep anti-mouse horseradish peroxidase-conjugated antibodies (Amersham) were used at a dilution of 1:2000 to label bound antibody, and the antibody complex was detected using the Amersham ECL system, as recommended by the manufacturer.

In vitro kinase assays

PKD was immunoprecipitated from tissue or cell lysates at 4 °C for 2 h with the PA-1 anti-peptide serum (1:100 dilution). PA-1 was raised against the synthetic peptide EEREMKALSERVSIL, which corresponds to amino acids 904–918 of PKD, as described previously.[16] Immune complexes were recovered by the addition of 30 μl protein A-sepharose (100 mg/ml), and pellets were washed three times with lysis buffer and three times with assay buffer [30 mM Tris/HCl (pH 7.5), 10 mM $MgCl_2$ and 1 mM DTT]. The final pellet was resuspended to a total volume of 40 μl with assay buffer. To initiate the phosphorylation reaction, 10 μl of phosphorylation mix (assay buffer containing 100 μM [γ-^{32}P]-ATP; 400–600 cpm/pmol) was added. The mixture was incubated at 30 °C for 5 min, and the reaction terminated by the addition of hot SDS-PAGE sample buffer. After boiling for 5 min, the samples were subjected to SDS-PAGE. Gels were dried, subjected to autoradiography and ^{32}P incorporation was quantified from autoradiographs (digitized with a Hewlett Packard ScanJet IIc flatbed scanner) using NIH Image software (version 1.59).

In one set of experiments, *in vitro* phosphorylation of the peptide syntide-2 (PLARTLSVAGLPGKK) was determined as an additional index of PKD activity.[16] For this study, syntide-2 (2.5 mg/ml) was added to the phosphorylation

mix. After incubation for 5 min, the reaction was terminated by adding 100 μl of ice-cold 75 mM H_3PO_4, and 100 μl of the reaction mix was spotted onto P-81 phosphocellulose paper. Free [γ-^{32}P]-ATP was removed by washing the P-81 paper four times for 5 min in 75 mM H_3PO_4. The radioactivity incorporated into syntide-2 was determined by Cerenkov counting.

Pharmacology protocols

Cells were maintained in 1 ml serum-free medium for 24 h prior to experiments with an adrenergic agonist (norepinephrine or phenylephrine) or PMA. The adrenergic agonist or PMA was added in 1 ml of serum-free medium 20 min prior to harvesting of cells. Where appropriate, bisindolylmaleimide or an adrenergic antagonist (prazosin or atenolol) was added 5 min prior to the adrenergic agonist or PMA, and was present until harvesting of cells.

Results

PKD is present in the rat heart, and is subject to developmental regulation

Western analysis of protein from ventricular tissue of fetal, neonatal and adult rat hearts revealed the presence of PKD in all samples (Fig. 1A). A distinct decline in the amount of immunoreactive protein was observed with increasing age. Interestingly, two immunoreactive bands (at 110 and 115 kDa) were detected by polyclonal antibodies raised against the extreme N-terminus (sc-638) or the extreme C-terminus (sc-935), as well as by a monoclonal antibody (P26720) directed against the central domain of PKD (Fig. 1A). Both bands were lost when sc-935 was preincubated with the immunizing peptide (Fig. 1A), confirming the specificity of the antibody. In contrast to our findings in rat ventricular myocardium, mouse PKD expressed in transiently-transfected COS-7 cells was detected as a single immunoreactive protein of 115 kDa (Fig. 1B).

Basal PKD activity in ventricular tissue from neonatal and adult rat hearts was assessed by an *in vitro* kinase assay following immunoprecipitation, using autophosphorylation as the indicator of activity.[19] The 110 and 115 kDa forms were both immunoprecipitated by the PA-1 antiserum, and

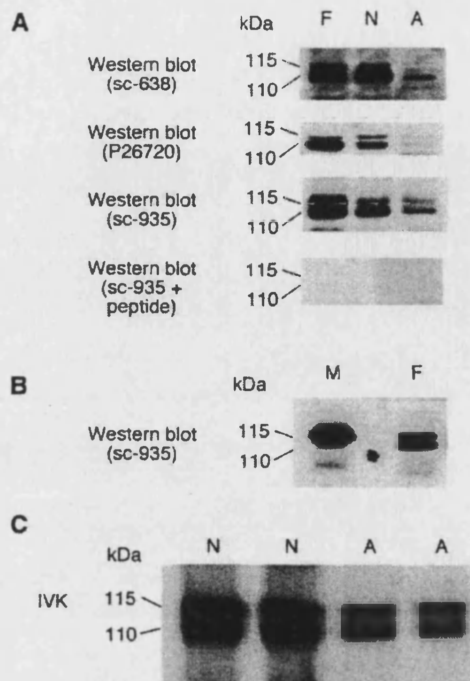

Figure 1 PKD expression and activity in the developing rat heart. A: Representative Western blots ($n = 3$) of protein from fetal (F), neonatal (N) and adult (A) rat hearts using the antibodies sc-638 (recognizes aa 6–25), P26720 (recognizes aa 314–517), and sc-935 (recognizes aa 893–912), and the latter either alone or following preincubation with the immunizing peptide sc-935P, as indicated. B: Representative Western blot ($n = 3$) of protein from COS-7 cells transfected with mouse PKD cDNA (M) and fetal rat heart (F), using sc-935 antibody. C: Representative autoradiogram ($n = 4$) of PKD autophosphorylation after immunoprecipitation from neonatal (N) and adult (A) rat hearts. Autophosphorylation was assessed by an *in vitro* kinase assay (IVK), as described in Methods.

both forms incorporated ^{32}P (Fig. 1C). Greater activity was observed in neonatal myocardium, consistent with the greater abundance of PKD protein in these samples.

To determine the presence of PKD in different cardiac cell types, fractionation of myocytes and non-myocytes was performed in both neonatal and adult rat ventricular tissue. Western blotting of equal quantities of protein with the sc-935 antibody revealed the presence of PKD in each fraction (Fig. 2A). PKD appeared to be more abundant in neonatal myocytes than in adult myocytes, since long exposures were required to detect PKD in adult myocytes (not shown). PKD was readily detectable in

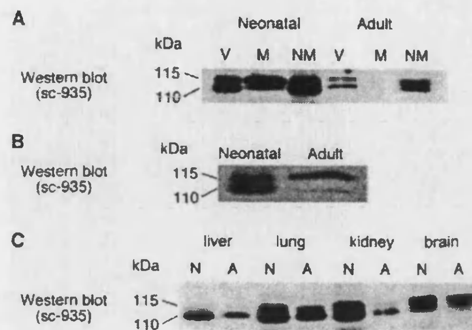

Figure 2 Tissue distribution of PKD in the rat. A: Representative Western blot ($n=2$) of equal amounts of protein (15 μg) from myocyte (M) and non-myocyte (NM) fractions and whole venticles (V) of neonatal and adult rat hearts, using sc-935 antibody. B: Representative Western blot ($n=4$) of protein from equal numbers of neonatal and adult myocytes (60 000 cells/lane), using sc-935 antibody. C: Representative Western blot ($n=2$) of equal amounts of protein (15 μg) from neonatal (N) and adult (A) tissues, using sc-935 antibody.

the non-myocyte fractions of both neonatal and adult samples. When protein samples from equal numbers of myocytes (60 000 cells/lane) were used, again PKD was more abundant in neonatal myocytes than in adult myocytes (Fig. 2B). Interestingly, in the adult cells, PKD was detected almost exclusively as an immunoreactive protein of 115 kDa (Fig. 2B).

To characterize the expression of PKD in different tissues of the rat, we also carried out a comparative Western analysis of protein from liver, lung, kidney and brain. As shown in Figure 2C, PKD was present in all four tissues, with a developmental decline in PKD protein expression, as in the heart.

PKD is activated by PMA in cultured neonatal myocytes

Activation of PKD by phorbol ester is well established in other cell types.[16,19,20] To determine whether PKD is activated by PMA in cardiac myocytes, we used a cultured neonatal myocyte preparation. Cells were incubated in serum-free medium for 24 h prior to treatment with PMA, since preliminary experiments showed that PKD was activated by serum (data not shown). *In vitro* kinase assays revealed a concentration-dependent increase in ^{32}P incorporation into PKD in response to PMA (Fig. 3A). Maximal PKD autophosphorylation was seen at 100 nM PMA, with an EC_{50} of 2.8 nM (Fig. 3B).

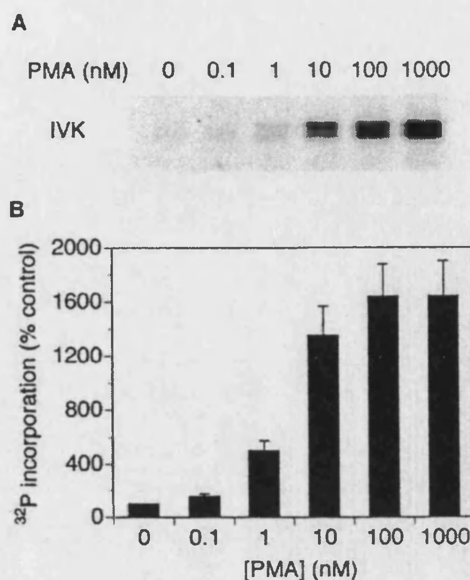

Figure 3 PMA activates PKD in cultured neonatal myocytes. A: Representative autoradiogram ($n=8$) showing PKD autophosphorylation following exposure of cells to various concentrations of PMA for 20 min prior to cell lysis and PKD immunoprecipitation. B: Concentration-dependent effects of PMA on ^{32}P incorporation into PKD. Autophosphorylation was assessed by an *in vitro* kinase assay (IVK), as described in Methods. Data are expressed as percentage of the control (vehicle) value ($n=8$).

PKD is activated by norepinephrine in cultured neonatal myocytes

In the light of the data obtained with PMA, we next determined whether a physiological stimulus could also activate PKD in neonatal myocytes. Exposure to norepinephrine for 20 min prior to cell lysis increased ^{32}P incorporation into PKD in a concentration-dependent manner (Fig. 4A), indicating activation of the kinase. The maximal increase in PKD autophosphorylation occurred with 100 μM norepinephrine, but measured only 3–4-fold over control (Fig. 4B), compared with approximately 16-fold in response to PMA (Fig. 3B). The EC_{50} for norepinephrine was 0.6 μM. The concentration-response profile of PKD autophosphorylation was mimicked by the concentration-response profile of syntide-2 phosphorylation, which was used as an additional index of PKD activity (Fig. 4B), confirming that PKD autophosphorylation status accurately reflects PKD activity in cultured neonatal myocytes.

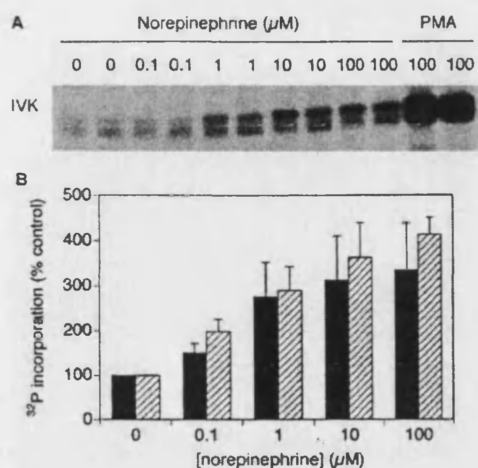

Figure 4 Norepinephrine activates PKD in cultured neonatal myocytes. A: Representative autoradiogram ($n=6$) showing PKD autophosphorylation following exposure of cells to various concentrations of norepinephrine or PMA for 20 min prior to cell lysis and PKD immunoprecipitation. B: Concentration-dependent effects of norepinephrine on ^{32}P incorporation into PKD (solid bars) and syntide-2 (hatched bars). Autophosphorylation and syntide-2 phosphorylation were both assessed by an *in vitro* kinase assay (IVK), as described in Methods. Data are expressed as percentage of the control (vehicle) value ($n=6$).

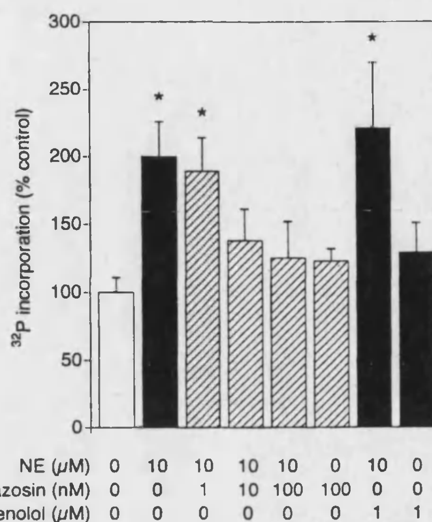

Figure 5 Norepinephrine-induced activation of PKD in cultured neonatal myocytes is inhibited by α_1-adrenergic receptor antagonist. Effects of norepinephrine on ^{32}P incorporation into PKD in the absence (solid bars) or presence of the α_1-AR antagonist prazosin (hatched bars) or the β_1-AR antagonist atenolol (stippled bars). Data are expressed as percentage of the control (vehicle) value ($n=4$–10). Norepinephrine was present for 20 min prior to cell lysis and PKD immunoprecipitation, and each antagonist was present for 5 min prior to and during norepinephrine exposure. * $P<0.05$ *v* control (open bar).

Norepinephrine activates PKD via α_1 adrenergic receptors in cultured neonatal myocytes

To determine whether norepinephrine-induced activation of PKD occurs via α_1- or β_1-ARs, we next tested the effects of the α_1-AR-selective antagonist prazosin and the β_1-AR-selective antagonist atenolol on this response. Prazosin (100 nM) or atenolol (1 μM) alone had little effect on PKD activity (Fig. 5). Atenolol also had no significant effect on PKD activation by norepinephrine, but prazosin inhibited this response in a concentration-dependent manner (Fig. 5). This finding suggests that norepinephrine activates PKD selectively via α_1-ARs in cultured neonatal myocytes.

Phenylephrine activates PKD in cultured neonatal myocytes

To confirm that α_1-AR stimulation is sufficient to activate PKD in cultured neonatal myocytes, we also tested the effects of the α_1-AR-selective agonist phenylephrine. Phenylephrine induced a pattern of PKD activation similar to that seen with norepinephrine, with an EC_{50} of 0.6 μM (Fig. 6). The maximal increase in PKD autophosphorylation was

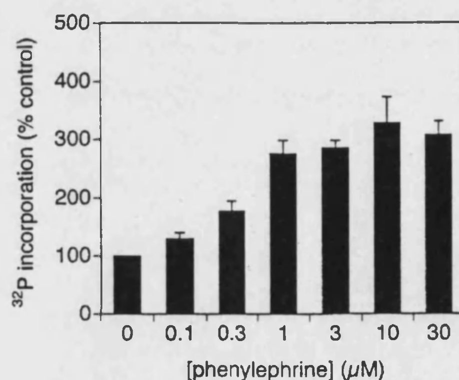

Figure 6 Phenylephrine activates PKD in cultured neonatal myocytes. Concentration-dependent effects of the α_1-AR agonist phenylephrine on ^{32}P incorporation into PKD. Data are expressed as percentage of the control (vehicle) value ($n=10$).

once again 3–4-fold, which is similar to that seen in response to norepinephrine. These findings confirm that PKD is activated via α_1-AR stimulation in cultured neonatal myocytes.

PKD activation in cultured neonatal myocytes is mediated by PKC

In vivo activation of PKD by phorbol ester in other cell types has been shown to involve direct phosphorylation of PKD by PKC.[20,22] To determine whether phorbol ester-induced activation of PKD in cardiac myocytes occurs via a PKC-dependent pathway, we determined the effects of the PKC inhibitor bisindolylmaleimide on the PMA response. As shown in Figure 7A, there was marked ^{32}P incorporation into PKD following exposure to PMA (lane 1). However, when cells were incubated with bisindolylmaleimide prior to and during exposure to PMA, PKD autophosphorylation was significantly reduced (lane 2). Importantly, exposure to bisindolylmaleimide *in vitro* (i.e. after PKD immunoprecipitation) had no effect on PKD autophosphorylation (lane 3), precluding a direct effect of bisindolylmaleimide on PKD activity.

To further characterize the role of PKC in the phorbol ester-induced response and to determine the involvement of PKC signaling in α_1-AR-mediated activation of PKD, we then quantified the effects of bisindolylmaleimide on the responses to phenylephrine and PMA. As shown in Figure 7B, exposure of control cells to bisindolylmaleimide led to a decrease in basal PKD activity, suggesting that maintenance of such activity requires active PKC. Phenylephrine once again induced a 3–4-fold increase in PKD activity, but this response was completely inhibited by pretreatment with 1 μM bisindolylmaleimide. As before, exposure of cells to PMA (100 nM) induced a much greater (16–17-fold) increase in PKD activity; this response was attenuated, in a concentration-dependent manner, by pretreatment of cells with bisindolylmaleimide. These observations confirm a key role for PKC in PKD activation in response to α_1-AR stimulation or exposure to PMA. In order to further define the role of PKC in the regulation of PKD activity in cultured neonatal myocytes, we also tested the effects of bisindolylmaleimide on the PKD response to the reintroduction of serum. Exposure to 10% FCS induced a 12-fold increase in PKD autophosphorylation, and this response was also attenuated, in a concentration-dependent manner, by bisindolylmaleimide. This indicates that the

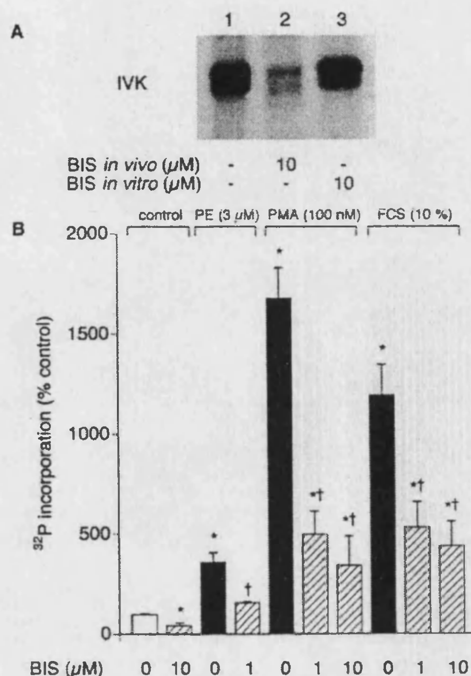

Figure 7 PKD activation in cultured neonatal myocytes is mediated by PKC. A: Representative autoradiogram ($n=2$) showing PKD autophosphorylation following exposure of cells to 100 nM PMA for 20 min, under control conditions (lane 1) or in the presence of the PKC inhibitor bisindolylmaleimide (BIS) prior to cell lysis (*in vivo*; lane 2) or during the kinase assay (*in vitro*; lane 3). B: Effects of phenylephrine (PE), PMA and FCS on ^{32}P incorporation into PKD in the absence (solid bars) or presence (hatched bars) of the PKC inhibitor bisindolylmaleimide (BIS). PE, PMA or FCS was present for 20 min prior to cell lysis and PKD immunoprecipitation, and BIS was present for 5 min prior to and during PE, PMA or FCS exposure. Autophosphorylation was assessed by an *in vitro* kinase assay (IVK), as described in Methods. Data are expressed as percentage of the control (vehicle) value ($n=4$). * $P<0.05$ *v* control (open bar), † $P<0.05$ *v* PE, PMA or FCS alone (solid bars).

factor(s) in serum which activate PKD induce this response by mechanism(s) that involve PKC.

PKD is not activated by phenylephrine or norepinephrine in cultured neonatal non-myocytes

To determine whether contaminating non-myocytes could make a significant contribution to the α_1-AR-mediated stimulation of PKD activity in our neonatal myocyte preparations, we additionally

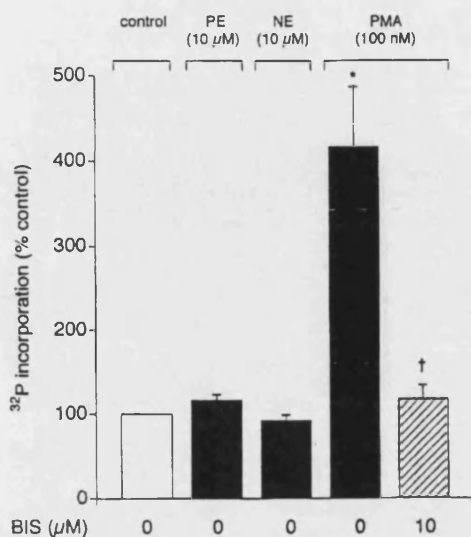

Figure 8 Phenylephrine and norepinephrine do not activate PKD in cultured neonatal non-myocytes. Effects of phenylephrine (PE), norepinephrine (NE) and PMA on ^{32}P incorporation into PKD in the absence (solid bars) or presence (hatched bars) of the PKC inhibitor bisindolylmaleimide (BIS). PE, NE or PMA was present for 20 min prior to cell lysis and PKD immunoprecipitation, and BIS was present for 5 min prior to and during PMA exposure. Autophosphorylation was assessed by an *in vitro* kinase assay, as described in Methods. Data are expressed as percentage of the control (vehicle) value ($n=3$). * $P<0.05$ *v* control (open bars), † $P<0.05$ *v* PMA alone.

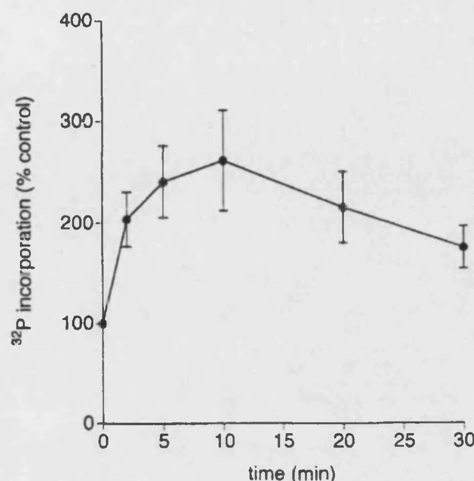

Figure 9 PMA activates PKD in freshly isolated adult myocytes. Time-dependent effects of PMA (100 nM) on ^{32}P incorporation into PKD. Autophosphorylation was assessed by an *in vitro* kinase assay, as described in Methods. Data are expressed as percentage of the control (vehicle) value ($n=4$).

studied the effects of norepinephrine and phenylephrine in cultured neonatal non-myocytes. As shown in Figure 8, neither phenylephrine nor norepinephrine produced a significant change in PKD activity in the non-myocyte preparations, at concentrations that produced maximum stimulation of PKD activity in neonatal myocytes (see Figs 4 and 6). In contrast, PMA produced significant PKC-mediated PKD activation in cultured non-myocytes (Fig. 8), as in cultured myocytes. This indicates that the α_1-AR-mediated increases in PKD activity in our neonatal myocyte preparations reflect such increases in myocytes themselves rather than in contaminating non-myocyte cells.

PKD is activated by PMA in freshly isolated adult myocytes

Since PKD was expressed in adult rat ventricular myocytes also, albeit at lower abundance than in neonatal ventricular myocytes, we also tested the effects of PMA on PKD activity in this cell type. Time-response experiments with PMA (100 nM) in freshly-isolated adult rat ventricular myocytes (500 000 cells/time point) revealed a modest 2.5-fold increase in ^{32}P incorporation into PKD, which peaked after 10 min of exposure to the phorbol ester (Fig. 9).

Discussion

The present study represents the first detailed characterization of myocardial PKD expression, activity and regulation. Our main conclusions are that, in the rat: (i) functional PKD is present in ventricular myocardium and its level of expression is subject to developmental regulation, (ii) PKD is abundant in both myocyte and non-myocyte fractions of neonatal ventricular myocardium, but PKD abundance in the myocyte fraction declines in adulthood, (iii) PKD activity in primary cultures of neonatal ventricular myocytes is increased by exposure to PMA, FCS and α_1-adrenergic agonists, and (iv) increased PKD activity in these cells in response to such stimuli occurs downstream of PKC activation.

An intriguing finding of our study was the consistent detection by Western blotting of two proteins

of 110 and 115 kDa, in ventricular myocardium and other tissues of neonatal and adult rats. These two proteins were immunoreactive to three independent antibodies that had been raised against distinct PKD/PKCμ domains in the rabbit (sc-638 and sc-935) or the mouse (P26720), and they were not detected when antibody (sc-935) was incubated with immunizing peptide prior to Western blotting. These observations strongly suggest that both proteins are PKD moieties, rather than related proteins. Furthermore, our observation that rabbit polyclonal antibodies directed against either the extreme N- or the extreme C-terminus of PKD (sc-638 and sc-935, respectively) both detected the same two moieties suggests that the 110 kDa band does not arise from proteolytic cleavage of the 115 kDa protein. A shift in PKD mobility upon activation, ascribed to PKD phosphorylation by PKC, has been reported previously in COS-7 cells (see Fig. 4C in Zugaza *et al.*, 1996[20]). However, the shift in PKD mobility induced by activation in that study was much more discrete than the difference in mobility between the two PKD moieties in our study, which suggests that these moieties may not arise as a consequence of such phosphorylation. In support of this, in our study, there was no significant change in the mobility of the two bands upon increased ^{32}P incorporation following activation, and treatment of protein samples with alkaline phosphatase prior to Western blotting had no effect on the mobility of either band (data not shown). Possible alternative explanations are that the 110 and 115 kDa proteins arise from: (i) two highly homologous genes, (ii) differential splicing of a single gene, or (iii) post-translational modification/protein maturation processes other than phosphorylation. Interestingly, unlike neonatal and adult ventricular tissue and neonatal ventricular myocytes, adult myocytes appear to express predominantly the 115 kDa protein, although the molecular basis of this divergence is currently unclear.

Consistent with previous observations regarding other signaling proteins (e.g. PKC isoforms,[23] components of the mitogen-activated protein kinase cascade[24]), our data suggest that PKD expression in the rat heart declines dramatically with maturation. The apparent difference in PKD abundance between neonatal and adult ventricular myocytes was retained even when protein samples from equal numbers of cells (as opposed to equal amounts of protein) were subjected to immunoblot analysis, indicating that the difference did not arise from an artefactual "dilution" of the target protein, due to changes in cardiac protein composition during post-natal maturation (such as hypertrophic growth of cardiac myocytes, with an associated increase in contractile protein content). The greater abundance of PKD in fetal and neonatal myocardium relative to the adult may suggest that PKD plays a role during development of the rat heart. Furthermore, we have observed a reduced immunoreactive PKD content in adulthood additionally in non-cardiac tissues (Fig. 2B), suggesting that expression of this kinase may be subject to developmental regulation in a tissue-independent manner. In this context, it is interesting to note that the expression of PKCμ, the PKD homologue, in mouse epidermis has also been reported to decline during the first week after birth.[25]

In primary cultures of neonatal rat ventricular myocytes, we have established that myocardial PKD can be activated by PMA in a dose-dependent manner, and that this activation is dependent upon activation of PKC. The latter conclusion is based on the effects of the PKC inhibitor bisindolylmaleimide; as shown before in other cell types, myocardial PKD activity *in vitro* was not directly inhibited by bisindolylmaleimide, which nevertheless inhibited PMA-induced activation of this kinase *in vivo* (Fig. 7). In the neonatal myocyte preparation, PKD was activated also by exposure to FCS, as well as the synthetic α_1-AR agonist phenylephrine and the endogenous catecholamine norepinephrine. PKD activation by norepinephrine occurred via the α_1-AR, since it was inhibited by the α_1-AR antagonist prazosin but not by the β_1-AR antagonist atenolol. Importantly, like the PMA-induced response, PKD activation by each of these stimuli was also sensitive to inhibition by bisindolylmaleimide, suggesting that PKC plays a central role in the *in vivo* regulation of myocardial PKD activity by diverse stimuli.

A potential problem in the interpretation of data obtained in cultured neonatal myocyte preparations is the unknown contribution from contaminating non-myocytes, mostly fibroblasts, to the detected signal. However, our studies in cultured neonatal *non-myocyte* cells revealed no increase in PKD activity in response to norepinephrine or phenylephrine, indicating that non-myocytes were unlikely to have contributed to the α_1-AR-mediated stimulation of PKD activity observed in the myocyte experiments. This is consistent with earlier reports that neonatal rat cardiac fibroblasts do not express α_1-AR mRNA,[26] and do not respond to α_1-adrenergic stimulation.[27] In contrast, a contribution from the non-myocyte fraction to the PMA-induced response cannot be discounted, since PMA produced a significant increase in PKD activity in cultured non-myocytes, as in the myocyte preparations.

In our studies in neonatal myocytes, the maximal

increase in PKD autophosphorylation was markedly greater following exposure to PMA (16–17-fold) than following α_1-AR stimulation by phenylephrine or norepinephrine (3–4-fold). This difference in PKD activation may reflect quantitative and/or qualitative differences in PKC activation in response to the distinct stimuli. In this regard, previous studies in neonatal rat ventricular myocytes have shown greater PKC activation by PMA than by norepinephrine.[28] Furthermore, other work in the same model has shown that phorbol ester treatment activates PKCα, PKCδ and PKCε, whereas stimuli that act via G_qPCRs, such as α_1-AR agonists, activate a limited subset of PKC isoforms.[29,30] The latter observation may be significant, since PKD appears to be differentially regulated by distinct PKC isoforms.[20]

To date, there is little information available regarding the physiological substrate(s) and function(s) of PKD in cardiac myocytes, or indeed in other cell types. An inhibitory role for PKD has been proposed in regulating glucagon-stimulated cAMP production in COS-7 cells[31] and antigen receptor complex-initiated signaling in B-cell lines.[32] Most recently, work from our laboratory has revealed that PKD may mediate a novel inhibitory pathway in the regulation of plasma membrane Na^+/H^+ exchanger activity, in both COS-7 and A-10 cells.[33] With regard to myocardial PKD, there has been only one previous study in the literature, also from our laboratory;[34] this investigated the potential role of PKD in the phenomenon of ischemic preconditioning, but failed to establish an involvement. Our novel finding in the present study that PKD activity in neonatal rat ventricular myocytes is subject to regulation by a variety of stimuli (including α_1-AR agonists) through a PKC-mediated pathway may suggest roles for PKD in modulating myocardial responses previously ascribed to PKC activation. In this context, primary cultures of neonatal rat ventricular myocytes have been used widely to study the regulation of myocyte growth and hypertrophy by humoral factors that signal via G_qPCRs (see reviews by Chien *et al.*, 1991[35] and Dorn and Brown, 1999[2]). Many studies have implicated PKC activation as an important proximal event in the induction of a hypertrophic phenotype in this preparation,[36,37] particularly following α_1-adrenergic stimulation.[6,7] In the light of the above and the current interest in the delineation of G_qPCR signaling pathways that mediate cardiac adaptive and maladaptive responses,[2] the functional significance of α_1-adrenoceptor- and PKC-mediated activation of PKD in myocardium warrants investigation.

Acknowledgements

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Protein kinase D inhibits plasma membrane Na^+/H^+ exchanger activity

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Haworth, Robert S., James Sinnett-Smith, Enrique Rozengurt, and Metin Avkiran. Protein kinase D inhibits plasma membrane Na^+/H^+ exchanger activity. *Am. J. Physiol.* 277 (*Cell Physiol.* 46): C1202–C1209, 1999.—The regulation of plasma membrane Na^+/H^+ exchanger (NHE) activity by protein kinase D (PKD), a novel protein kinase C- and phorbol ester-regulated kinase, was investigated. To determine the effect of PKD on NHE activity in vivo, intracellular pH (pH_i) measurements were made in COS-7 cells by microepifluorescence using the pH indicator cSNARF-1. Cells were transfected with empty vector (control), wild-type PKD, or its kinase-deficient mutant PKD-K618M, together with green fluorescent protein (GFP). NHE activity, as reflected by the rate of acid efflux (J_H), was determined in single GFP-positive cells following intracellular acidification. Overexpression of wild-type PKD had no significant effect on J_H (3.48 ± 0.25 vs. 3.78 ± 0.24 mM/min in control at pH_i 7.0). In contrast, overexpression of PKD-K618M increased J_H (5.31 ± 0.57 mM/min at pH_i 7.0; $P < 0.05$ vs. control). Transfection with these constructs produced similar effects also in A-10 cells, indicating that native PKD may have an inhibitory effect on NHE in both cell types, which is relieved by a dominant-negative action of PKD-K618M. Exposure of COS-7 cells to phorbol ester significantly increased J_H in control cells but failed to do so in cells overexpressing either wild-type PKD (due to inhibition by the overexpressed PKD) or PKD-K618M (because basal J_H was already near maximal). A fusion protein containing the cytosolic regulatory domain (amino acids 637–815) of NHE1 (the ubiquitous NHE isoform) was phosphorylated in vitro by wild-type PKD, but with low stoichiometry. These data suggest that PKD inhibits NHE activity, probably through an indirect mechanism, and represents a novel pathway in the regulation of the exchanger.

pH regulation; COS-7; A-10; green fluorescent protein; sodium/hydrogen exchanger type 1; protein kinase Cμ

IN RECENT YEARS, the type 1 isoform of the Na^+/H^+ exchanger (NHE1) has been the focus of considerable investigative effort as an important modulator of cellular function. Indeed, altered activity of this exchanger has been implicated in such diverse pathologies as cancer (17), epilepsy (5), hypertension (20), arterial restenosis (18), myocardial hypertrophy (40), and post-ischemic cardiac dysfunction (31). NHE1 is predicted to consist of two domains: an NH_2-terminal membrane-bound transport domain and a COOH-terminal cytoplasmic regulatory domain (25, 37). The exchanger is quiescent at physiological intracellular pH (pH_i) but is activated rapidly in response to intracellular acidosis via an allosteric modifier site in its transport domain (36). Several stimuli, including growth factors, hormones, hypertonic stress, and mechanical stretch, also increase NHE1 activity through various signaling pathways (25, 37). These include direct phosphorylation of the cytoplasmic regulatory domain (28), binding of calmodulin (2), and interaction with accessory proteins [e.g., CHP (23)]. Although NHE1-regulatory roles have been suggested for several kinase pathways (4, 22, 26, 33), the most extensively studied kinase-mediated mechanism of NHE1 activation involves protein kinase C (PKC). Thus activation of PKC, either directly by phorbol esters (8, 38) or indirectly via cell surface receptors (29, 38, 41), leads to increased exchanger activity in a variety of cell types, and putative PKC inhibitors inhibit such effects (38, 41). There is evidence that negative regulators of NHE1 activity also exist (21, 24), but less is known about inhibitory signaling pathways.

The recently identified protein kinase D [PKD; also known as PKCμ (16)] is a novel serine/threonine kinase (34) that has distinct structural and enzymatic properties and is found in most tissues [see review by Rozengurt et al. (27)]. The catalytic domain of PKD is distantly related to Ca^{2+}-regulated kinases and shows little similarity to the highly conserved regions of the kinase subdomains of the PKC family. Consistent with this, PKD does not phosphorylate a variety of substrates utilized by PKCs (34, 35), indicating that PKD is a protein kinase with distinct substrate specificity. The NH_2-terminal region of PKD contains a tandem repeat of cysteine-rich motifs that bind phorbol esters with high affinity (34, 35), and immunopurified PKD is stimulated in vitro by either diacylglycerol or biologically active phorbol esters in the presence of phosphatidylserine (35). More recently, a second mechanism of PKD activation has been identified, which involves phosphorylation of PKD via a PKC-dependent pathway (43).

In light of the evidence that PKD can be activated in parallel with or downstream of PKC, we determined whether PKD may be involved in the regulation of NHE1 activity. The results presented here suggest that PKD mediates a novel inhibitory pathway in the regulation of the exchanger and counteracts the stimulatory effects of the PKC pathway.

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EXPERIMENTAL PROCEDURES

Cell culture. Stock cultures of COS-7 (African Green monkey kidney) and A-10 (rat aortic smooth muscle) cells were maintained at 37°C in DMEM supplemented with 10% fetal bovine serum (FBS) in a humidified atmosphere containing 5% CO_2. For experimental purposes, cells were plated in 90-mm dishes, with or without acid-washed 15-mm diameter glass coverslips, at 6×10^5 cells/dish.

Transfection of cultured cells. The wild-type PKD cDNA fragment spanning bases −125 to 3179 was inserted into the mammalian expression vector pcDNA3, as described earlier (35). The kinase-deficient mutant PKD-K618M was also inserted into pcDNA3 (43). cDNA for green fluorescent protein (GFP), in the mammalian expression vector pCAGGS, was a kind gift from Drs L. Wightman and M. Marber (Department of Cardiology, St. Thomas' Hospital, London, UK).

Cultured cells (40–50% confluent) were transfected with the various plasmids using Lipofectin (Life Technologies), as recommended by the manufacturer. A total of 12 µg DNA was used per 90-mm dish, consisting of 6 µg pCAGGS-GFP and 6 µg pcDNA3, pcDNA3-PKD, or pcDNA3-PKD-K618M. Briefly, DNA was mixed with 1 ml Opti-MEM I (Life Technologies) and then mixed with Lipofectin (20 µl in 1 ml Opti-MEM I). After 20 min, the Lipofectin/DNA complex was diluted to 5 ml with Opti-MEM I and overlaid onto the cells. After overnight incubation, the procedure was completed by the addition of 5 ml of Opti-MEM I containing 20% FBS. Cells were used 24–48 h later.

Western blots. Cultured cells were washed three times with ice-cold PBS and lysed in 1 ml of lysis buffer, which contained 50 mM Tris·HCl (pH 7.5), 2 mM EGTA, 2 mM EDTA, 1 mM dithiothreitol (DTT), 10 µg/ml aprotinin, 10 µg/ml leupeptin, 1 mM 4-(2′-aminoethyl)-benzenesulfonyl fluoride hydrochloride, and 1% Triton X-100. Samples of cell lysate (15 µg protein) were then subjected to SDS-PAGE and transferred to polyvinylidene difluoride membrane (Amersham, Bucks, UK), using a Pharmacia LKB Multiphor II transfer apparatus. Membranes were probed with commercial antibody that recognizes both wild-type PKD and its kinase-deficient mutant (sc-935; Autogen Bioclear UK, Calne, UK). Donkey anti-rabbit horseradish peroxidase-conjugated antibody (Amersham) was used at a dilution of 1:2,000 to label bound antibody, and the antibody complex was detected using the Amersham enhanced chemiluminescence system, as recommended by the manufacturer.

NHE activity measurement. pH_i was measured in single cells using an adaptation of the microepifluorescence method that we have used previously in neonatal (9) and adult (9, 31, 41, 42) rat cardiac myocytes. Cultured cells grown on coverslips were mounted in a 150-µl volume imaging chamber (Warner RC-25F; Clark Electromedical Intruments, Reading, UK) and visualized on a Nikon Diaphot 300 inverted microscope, with a ×40 oil-immersion objective (numerical aperture 1.3). The cells were loaded with the fluorescent indicator carboxy-seminaphthorhodofluor-1 (cSNARF-1) by immersion in 5 µM of its acetoxymethyl ester for 20 min at room temperature, after which they were superfused with Tyrode solution (in mM: 137 NaCl, 5.4 KCl, 1.8 $CaCl_2$, 0.5 $MgCl_2$, 10 HEPES; pH 7.4 at 34°C) at a flow rate of 2–3 ml/min. To select successfully transfected cells for pH_i measurement, GFP fluorescence was initially visualized following excitation of cells at 486 nm. A single cell emitting green light was then selected and framed with adjustable shutters for cSNARF-1 fluorescence measurements. Fluorescence emission at 580 and 640 nm was detected during excitation at 540 nm, using a dual emission photometer system [model D-104; Photon Technology International (PTI), Surbiton, UK] with photon-counting photomultipliers (model 710; PTI). Emission intensity data were acquired at 2 Hz and stored on disk using PTI Felix software (version 1.1). The 580:640 emission intensity ratios were converted to pH_i values by reference to calibration curves constructed using nigericin, and cellular intrinsic buffering capacity (β_i) measurements were made during progressive washout of extracellular NH_4Cl, as described previously (9, 41). Because experiments were performed in bicarbonate-free medium, the rates of acid efflux (J_H) estimated during recovery from intracellular acidosis (induced by transient exposure to 20 mM NH_4Cl) could be used as indicators of plasma membrane NHE activity (9, 31, 41, 42). As described previously (9, 41), J_H was calculated at various pH_i during recovery from acidosis, from the equation $J_H = dpH_i/dt \cdot \beta_i$, in which dpH_i/dt is the rate of change of pH_i.

Generation of glutathione-S-transferase (GST)-NHE fusion protein. The regulatory domain of rabbit NHE1 was expressed as a fusion protein using the vector pGEX-3X (Pharmacia; see Ref. 32). DNA for the COOH-terminal 178 amino acids of NHE1 was amplified from rabbit heart cDNA by PCR using the primers 5′-GCG GAT CCT GCA GAA GAC CCG GCA GCG GCT-3′ and 5′-AAG AAT TCT ACT GCC CTT TGG GGA TGA-3′. The product generated was digested with *Eco*R I and *Bam*H I and ligated into pGEX-3X. Fusion protein expression in several strains of *Escherichia coli* was tested, as problems with degradation were encountered using DH5α (32). Fusion protein expressed in the protease-deficient strain BL21 (ompT−, lon−) was largely intact after purification using a glutathione-Sepharose 4B column, as recommended by the manufacturer (Pharmacia). Attempts to separate GST from the NHE1 fragment with Factor Xa led to degradation of the NHE1 portion, so intact fusion protein was used for in vitro phosphorylation assays. GST alone, expressed and purified in the same system, was used as control.

NHE1 phosphorylation assay. NHE1 phosphorylation by PKD was assessed following immunoprecipitation of the kinase as described previously (34, 35, 43). Cultured COS-7 cells were washed three times with ice-cold PBS and lysed in 1 ml of lysis buffer. PKD was immunoprecipitated from the cell lysate at 4°C for 3 h with the PA-1 anti-peptide serum [1:100 dilution (34)]. Immune complexes were recovered by the addition of 50 µl protein A-Sepharose (Pharmacia; 100 mg/ml), and pellets were washed three times with lysis buffer and three times with assay buffer [30 mM Tris·HCl (pH 7.5), 10 mM $MgCl_2$ and 1 mM DTT]. The final pellet was resuspended to a total volume of 40 µl with assay buffer. To initiate the phosphorylation reaction, 10 µl of phosphorylation mix [assay buffer containing GST-NHE fusion protein or GST (10 µg/ml) and 100 µM [γ-^{32}P]ATP; 400–600 counts·min^{-1}·pmol^{-1}] were added. The mixture was incubated at 30°C for 5 min, and the reaction was terminated by the addition of hot SDS-PAGE sample buffer. After boiling for 5 min, the samples were subjected to SDS-PAGE followed by autoradiography.

Statistical analysis. Data are expressed as means ± SE. Intragroup comparisons were by Student's paired *t*-test, whereas intergroup comparisons were by ANOVA followed by Bonferroni *t*-test. $P < 0.05$ was considered significant.

RESULTS

Characterization of NHE activity in COS-7 cells. To determine plasma membrane NHE activity selectively in transfected cells, we measured such activity in individual cells expressing the transfection marker GFP. The fluorescence characteristics of GFP and cSNARF-1 and the emission filters used precluded

signal interference between the two fluorophores. To confirm the absence of either such interference or a GFP-induced change in the pH_i sensitivity of cSNARF-1, we compared the pH_i calibration curves in nontransfected COS-7 cells and those transfected with GFP. As illustrated in Fig. 1, the calibration curves from these populations of cells were superimposed, indicating that expression of GFP does not affect the utility of cSNARF-1 as a pH_i indicator.

Because β_i is determined largely by intracellular protein composition and is a critical factor in calculating J_H (3), we tested whether overexpression of PKD affects β_i. To this end, β_i was determined at various pH_i in COS-7 cells transfected with pcDNA3 (control) wild-type PKD, or PKD-K618M, all concomitantly with GFP. Over the pH_i range 6.90–7.30 (mean 7.07 ± 0.01), there was no significant difference between the three groups in β_i (30.2 ± 1.5, 32.8 ± 2.0, and 31.8 ± 1.5 mM, respectively; n = 10 cells/group), indicating that overexpression of PKD has no effect on β_i in these cells.

To exclude an involvement of bicarbonate-dependent mechanisms in pH_i regulation, all microepifluorescence experiments were carried out in bicarbonate-free medium; under these conditions NHE represents the primary route of acid extrusion in other cell types (9, 17, 41). To verify that this is the case in COS-7 cells, we tested the effect of the NHE inhibitor HOE-642 (30) on pH_i recovery from acidosis. At 3 µM, HOE-642 completely inhibited pH_i recovery from acidosis after transient exposure to NH_4Cl (data not shown). This substantiates that, under the experimental conditions used, NHE activity represents the primary route of acid extrusion. Furthermore, it confirms that such activity arises from the NHE1 isoform, which is the only isoform that has been shown to be expressed in COS-7 cells (24), since at 3 µM HOE-642 is NHE1 selective (30).

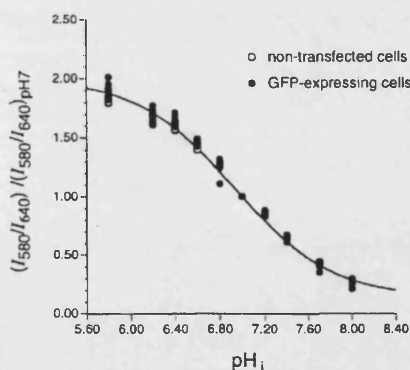

Fig. 1. Green fluorescent protein (GFP) does not interfere with cSNARF-1 fluorescence. COS-7 cells grown on coverslips were transfected with pCAGGS-GFP as described in EXPERIMENTAL PROCEDURES. Transfected cells were identified by GFP fluorescence, and cSNARF-1 emission intensity was measured at 580 (I_{580}) and 640 (I_{640}) nm in the presence of nigericin-containing calibration buffers of various pH. The normalized calibration curves, constructed as described by Yasutake et al. (41), for GFP-expressing cells (●) and nontransfected cells (○) were superimposed (10 cells/group).

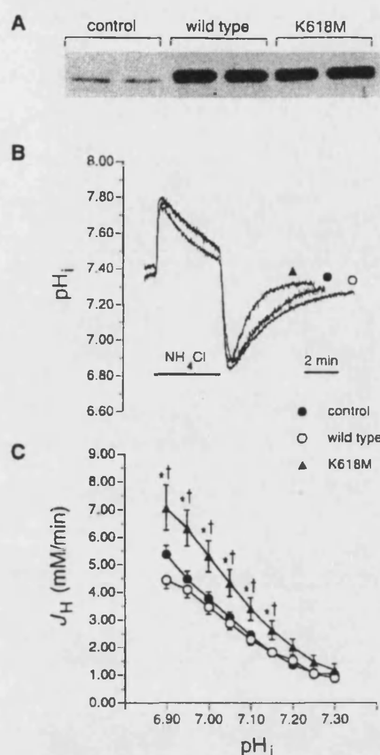

Fig. 2. Overexpression of protein kinase D (PKD)-K618M increases Na^+/H^+ exchanger (NHE) activity in COS-7 cells. *A*: Western blot of cell extracts. COS-7 cells were transfected with pCAGGS-GFP together with pcDNA3 (control), pcDNA3-PKD (wild type), or pcDNA3-PKD-K618M (K618M). Proteins from cell extracts were separated by SDS-PAGE, transferred to polyvinylidene difluoride (PVDF) membrane, and probed for presence of PKD using sc-935 antibody (1:500 dilution). Blot is representative of 3 experiments. *B*: representative pH_i recordings from single cells from each group. COS-7 cells grown on coverslips were transfected with pCAGGS-GFP together with pcDNA3 (control, ●), pcDNA3-PKD (wild type, ○), or pcDNA3-PKD-K618M (K618M, ▲). pH_i recordings were made from GFP-positive cells, and acid efflux (J_H) was estimated as an index of NHE activity, as described in EXPERIMENTAL PROCEDURES. *C*: J_H-vs.-pH_i curves for all groups (n = 36–37 cells/group). *$P < 0.05$ vs. control; †$P < 0.05$ vs. wild type, at same pH_i.

Overexpression of PKD-K618M increases NHE activity in COS-7 cells. To determine whether PKD had any effect on NHE activity in COS-7 cells, these were transfected with pcDNA3 (control), wild-type PKD, or the kinase-deficient mutant PKD-K618M before being exposed to intracellular acidosis. Figure 2*A* shows a representative Western blot of protein samples from COS-7 cells and illustrates the presence of native PKD in control cells as well as the equivalent overexpression of wild-type PKD and PKD-K618M following transfection. There was no significant difference between the groups in resting pH_i measured before exposure to NH_4Cl (control, 7.31 ± 0.01; wild-type PKD, 7.31 ± 0.01; PKD-K618M, 7.34 ± 0.01; n = 36–37 cells/group), and a similar extent of intracellular acidification was achieved in each group following NH_4Cl washout. Fig-

ure 2B shows representative pH_i recordings from a control cell and cells transfected with wild-type PKD or PKD-K618M. As illustrated, the rates of pH_i recovery from acidosis were similar in the cell transfected with wild-type PKD and the control cell transfected with empty vector; the salient feature in Fig. 2 is that recovery from intracellular acidosis was markedly faster in the cell transfected with PKD-K618M. Figure 2C shows quantitative data, in the form of J_H-vs.-pH_i curves, from several such experiments (n = 36–37 cells/group). In cells transfected with wild-type PKD, J_H was slightly lower than control at $pH_i < 7.10$, although this difference did not reach statistical significance. In marked contrast, in cells transfected with PKD-K618M, J_H was significantly greater than control over the pH_i range 6.90–7.15. This indicates that overexpression of the kinase-deficient PKD mutant increases NHE activity in COS-7 cells.

Overexpression of PKD-K618M increases NHE activity in A-10 cells. To determine whether NHE activity is regulated in a similar manner in cell types other than COS-7, A-10 cells were also transfected with pcDNA3 (control), wild-type PKD, or the kinase-deficient mutant PKD-K618M. Figure 3A shows a representative Western blot of protein samples from A-10 cells, demonstrating once again both the presence of native PKD and the equivalent overexpression of wild-type PKD and PKD-K618M following transfection. Over the pH_i range 6.60–7.20 (mean 6.96 ± 0.03), there was no significant difference between the three groups of A-10 cells in β_i (19.0 ± 1.2, 19.4 ± 1.8, and 15.9 ± 1.3 mM for control, wild-type PKD, and PKD-K618M groups, respectively; n = 8 cells/group), indicating that, as in COS-7 cells, overexpression of PKD had no effect on β_i. Nevertheless, mean β_i in the overall population was significantly lower in A-10 cells than in COS-7 cells (18.1 ± 0.8 vs. 31.5 ± 0.9 mM; $P < 0.05$).

There was again no significant difference between the groups in resting pH_i measured before exposure to NH_4Cl (control, 7.09 ± 0.03; wild-type PKD, 7.09 ± 0.02; PKD-K618M, 7.07 ± 0.02; n = 30–35 cells/group), which was ~0.2 pH unit lower than that observed in COS-7 cells. As in COS-7 cells, pH_i recovery from acidosis was inhibited by 3 μM HOE-642 (data not shown), confirming that, in A-10 cells also, such recovery arose from NHE1 activity. Figure 3B shows that J_H values were similar in control cells and cells transfected with wild-type PKD but were increased in cells transfected with PKD-K618M over the pH_i range 6.85–6.95 (n = 30–35 cells/group). These data indicate that, in common with our finding in COS-7 cells, overexpression of the kinase-deficient PKD mutant markedly increases NHE activity in A-10 cells. This common observation in two distinct cell types suggests that PKD may mediate a novel inhibitory pathway in the regulation of NHE, such that overexpression of its kinase-deficient mutant stimulates exchanger activity by attenuating the inhibitory action of native PKD.

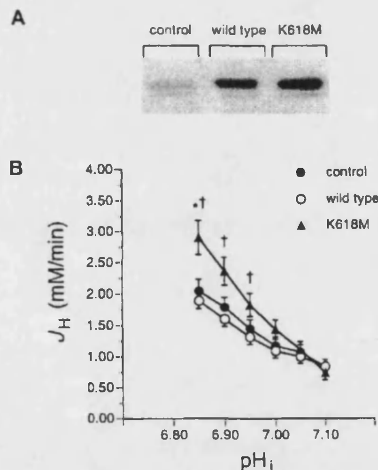

Fig. 3. Overexpression of PKD-K618M increases NHE activity in A-10 cells. *A*: Western blot of cell extracts. A-10 cells were transfected with pCAGGS-GFP together with pcDNA3 (control), pcDNA3-PKD (wild type), or pcDNA3-PKD-K618M (K618M). Proteins from cell extracts were separated by SDS-PAGE, transferred to PVDF membrane, and probed for presence of PKD using sc-935 antibody (1:500 dilution). Blot is representative of 3 experiments. *B*: J_H-vs.-pH_i curves for all groups. A-10 cells grown on coverslips were transfected with pCAGGS-GFP together with pcDNA3 (control, ●), pcDNA3-PKD (wild type, ○), or pcDNA3-PKD-K618M (K618M, ▲). pH_i recordings were made from GFP-positive cells, and J_H was estimated as an index of NHE activity, as described in EXPERIMENTAL PROCEDURES (n = 30–35 cells/group). $^*P < 0.05$ vs. control; $^\dagger P < 0.05$ vs. wild type, at same pH_i.

PKD inhibits PKC-induced stimulation of NHE activity in COS-7 cells. Direct activation of PKC is known to increase NHE1 activity in many cell types. Consistent with this, we have verified that in nontransfected COS-7 cells phorbol 12-myristate 13-acetate (PMA) increases plasma membrane NHE activity [as reflected by increases in J_H at pH_i 7.00 [$J_{H(7.0)}$], with an EC_{50} of 24 nM (Fig. 4A)]. The data illustrated in Figs. 2 and 3 and the regulatory association between PKC and PKD led us to hypothesize that cellular PKD activity may oppose PKC-mediated stimulation of the exchanger. To test this hypothesis, we determined the response to PMA of NHE activity in COS-7 cells transfected with pcDNA3 (control), wild-type PKD, or the kinase-deficient mutant PKD-K618M. As would be expected from the data shown in Fig. 4A, 100 nM PMA significantly increased $J_{H(7.0)}$ in control cells transfected with empty vector (Fig. 4B). In striking contrast, PMA failed to increase $J_{H(7.0)}$ in cells transfected with wild-type PKD, although basal $J_{H(7.0)}$ was similar to that observed in control cells (Fig. 4B). This is consistent with our hypothesis that PKD mediates a novel inhibitory pathway, such that overexpression of wild-type PKD counteracts the NHE-stimulatory effect of the PKC pathway.

Figure 4B additionally shows that PMA failed to increase $J_{H(7.0)}$ also in cells transfected with PKD-K618M. It should be noted, however, that basal $J_{H(7.0)}$ in these cells was greater than that in control cells, which is consistent with the findings reported above (Fig. 2C). Indeed, basal $J_{H(7.0)}$ in cells overexpressing PKD-K618M (7.34 ± 1.55 mM/min) was greater than the $J_{H(7.0)}$ obtained in response to PMA in control cells

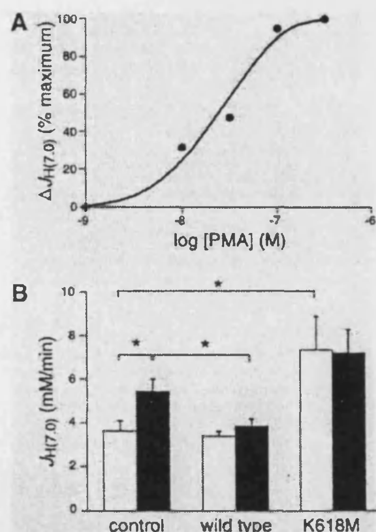

Fig. 4. PKD inhibits PKC-mediated stimulation of NHE activity in COS-7 cells. J_H at pH_i 7.00 [$J_{H(7.0)}$] was determined in single COS-7 cells following 2 consecutive exposures to intracellular acidosis, 1st under control conditions and 2nd in presence of phorbol 12-myristate 13-acetate (PMA) or vehicle. *A*: PMA dose-response curve in nontransfected cells, where $\Delta J_{H(7.0)}$ represents relative increase in $J_{H(7.0)}$ in response to PMA. *B*: $J_{H(7.0)}$ in absence (open bars) and presence (solid bars) of exposure to 100 nM PMA in COS-7 cells transfected with pCAGGS-GFP together with pcDNA3 (control), pcDNA3-PKD (wild type), or pcDNA3-PKD-K618M (K618M). $*P < 0.05$ (n = 8–12 cells/group).

(5.45 ± 0.53 mM/min). Thus it is likely that PMA did not increase NHE activity in cells transfected with the kinase-deficient PKD mutant, because NHE activity in these cells was already near maximal.

PKD phosphorylates NHE1 in vitro. To establish whether the observed inhibitory effect of PKD on NHE activity could arise from direct phosphorylation of the exchanger, we determined whether the regulatory region of NHE1, corresponding to the COOH-terminal 178 amino acids, was a substrate for PKD. COS-7 cells, transiently transfected with wild-type PKD, were treated with 200 nM PDB (to activate PKD) or vehicle for 10 min and lysed. PKD was immunoprecipitated from cell extracts with the PA-1 antibody, and the immune complexes were incubated with [γ-^{32}P]ATP in the absence or presence of the GST-NHE fusion protein (50 kDa). As shown in Fig. 5*A*, and in agreement with previous results (35, 43), PKD activity (as reflected by PKD autophosphorylation) was markedly increased by PDB stimulation of intact cells (*lanes 1* and *2*; ~10-fold increase). GST-NHE incubated with wild-type PKD showed some phosphorylation under basal conditions (*lane 5*), and this was markedly increased by PDB stimulation, concomitantly with PKD autophosphorylation (*lane 6*). These results indicate that PKD phosphorylates the COOH-terminal regulatory domain of NHE1 in vitro.

To confirm that phosphorylation of the NHE fusion protein requires active PKD, we also transfected COS-7 cells with the kinase-deficient mutant PKD-K618M and incubated PKD immunoprecipitates from these cells with GST-NHE in the presence of [γ-^{32}P]ATP. In contrast to the results obtained with wild-type PKD, immune complexes from cells transfected with PKD-K618M did not catalyze GST-NHE phosphorylation under basal conditions or after PDB stimulation (*lanes 7* and *8*). Indeed, consistent with earlier findings (43), PDB stimulation did not increase PKD activity in cells transfected with the kinase-deficient mutant (*lanes 3* and *4*). These results verify that the PDB-inducible NHE1 kinase activity observed in PA-1 immunoprecipitates was dependent on the kinase activity of PKD. Additional experiments confirmed that GST alone (30 kDa) was not significantly phosphorylated by activated PKD, suggesting that phosphorylation of the GST-NHE fusion protein occurred in the NHE domain (Fig. 5*B*).

To establish the potential functional importance of the observed phosphorylation by PKD of the NHE fusion protein, we determined the stoichiometry of this phosphorylation. Time course experiments showed that maximal ^{32}P incorporation into the NHE fusion protein occurred at 30 min and was maintained for up to 5 h (data not shown). Subsequent phosphorylation reactions were performed for 2 h, using known amounts of the GST-NHE fusion protein and the protocol described earlier. After autoradiography, the band corresponding to the fusion protein was excised from the gel and subjected to liquid scintillation counting. The stoichiometry of phosphorylation was calculated from the radioactive count, the specific activity of the [γ-^{32}P]ATP used, and the GST-NHE content of the reaction mix, as 0.068 ± 0.004 mol phosphate/mol fusion protein (n = 10).

DISCUSSION

The data presented above, which show the effects of changing the cellular PKD composition in intact cells on plasma membrane NHE activity, strongly suggest that PKD mediates a novel NHE-inhibitory pathway, through a mechanism that is dependent on its kinase activity. This conclusion is based to a significant extent on the ability of transfection with the kinase-deficient mutant PKD-K618M to increase plasma membrane NHE activity, in two distinct cell types. Kinase-deficient mutants of an extensive range of protein kinases [e.g., Raf (11), MEK (1), and Akt/PKB (19)] have been shown to exert dominant-negative effects in a variety of systems. Of more direct relevance to our work, recent studies have shown that kinase-deficient PKD/PKCμ mutants can also act in a dominant-negative fashion (14, 15). Thus, although an indirect effect of PKD-K618M cannot be completely discounted, the most likely mechanism underlying our findings is that native PKD exerts an inhibitory effect on NHE activity and that this inhibition is abolished by the dominant-negative action of the kinase-deficient PKD mutant.

Inhibition of NHE by a kinase that is downstream of PKC may at first appear paradoxical, since PKC activation is known to stimulate NHE1 activity in many cell types, as confirmed by our present findings in nontrans-

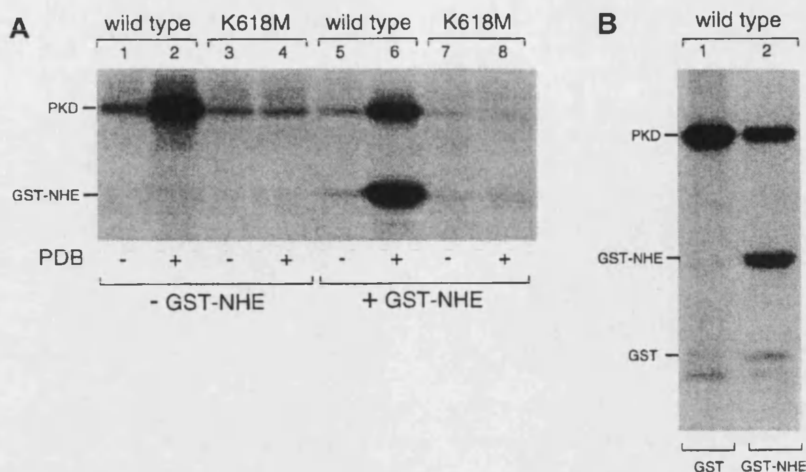

Fig. 5. PKD phosphorylates NHE1 regulatory domain in vitro. *A*: NHE phosphorylation assay. A fusion protein of the COOH-terminal 178 amino acids of NHE1 coupled to GST (GST-NHE) was prepared as described under EXPERIMENTAL PROCEDURES. COS-7 cells were transfected with pcDNA3-PKD (wild type) or pcDNA3-PKD-K618M (K618M). Cells from wild type (*lanes 1, 2, 5,* and *6*) and K618M (*lanes 3, 4, 7,* and *8*) groups were incubated for 10 min either in absence (−) or presence (+) of 200 nM PDB, and PKD was immunoprecipitated from cell lysates using antiserum PA-1 (1:100 dilution). After recovery of immune complexes with protein A-Sepharose, in vitro phosphorylation assays were performed in either the absence (*lanes 1–4*) or the presence (*lanes 5–8*) of 10 μg/ml GST-NHE. After SDS-PAGE, ^{32}P incorporation was visualized by autoradiography. Similar results were obtained in 3 experiments. *B*: GST phosphorylation assay. Cells from wild-type group were incubated for 10 min in presence of 100 nM PMA, and PKD was immunoprecipitated from cell lysates using antiserum PA-1 (1:100 dilution). After recovery of immune complexes with protein A-Sepharose, in vitro phosphorylation assays were performed in presence of 10 μg/ml GST (*lane 1*) or 10 μg/ml GST-NHE (*lane 2*). After SDS-PAGE, ^{32}P incorporation was visualized by autoradiography. Similar results were obtained in 3 experiments.

fected COS-7 cells exposed to PMA. However, our results may be readily reconciled with a hypothetical scheme whereby PKC stimulates NHE activity via a PKD-independent pathway, as well as activating PKD. Indeed, activation of PKD may curb PKC-mediated stimulation of the exchanger (which is supported by our observation that PMA cannot stimulate NHE activity in COS-7 cells that overexpress wild-type PKD), thereby allowing tighter control over NHE activity. In this regard, it would be informative to determine the time course of activation in response to PMA of the NHE-stimulatory pathway and to compare this with the time course of PKD activation. Unfortunately, such a comparison is not possible at present, since the identity of the NHE-stimulatory pathway that is downstream of PKC is unknown. In the context of the hypothesis proposed above, it is also notable that a parallel activation of both NHE-stimulatory and NHE-inhibitory pathways (mediated by ERK1/2 and p38 MAPK, respectively) has recently been reported in cultured rat vascular smooth muscle cells exposed to angiotensin II (21). Thus the PKD pathway may not be unique as an NHE-inhibitory pathway that is activated in parallel with an NHE-stimulatory pathway, in response to a given stimulus.

Despite our observation that transfection with PKD-K618M increases NHE activity in both COS-7 and A-10 cells, basal pH_i was not affected in either system by transfection with this kinase-deficient mutant. This is likely to arise from the absence of an increase in NHE activity at basal pH_i, since at $pH_i \geq 7.20$ and 7.00 in the COS-7 and A-10 systems, respectively, J_H was almost identical in cells transfected with PKD-K618M and those transfected with empty vector. Thus PKD-mediated inhibition of the exchanger may be of greater physiological impact under conditions of intracellular acidosis (which is commonly encountered in disease conditions such as ischemia) or during exposure to stimuli that increase cellular PKC activity (which, as noted above, are associated with stimulation of NHE activity).

Although considerable progress has been made in the understanding of the cellular regulation of PKD activity (13, 27, 35, 43, 44) since the original cloning and characterization of this enzyme (34), little is known about its cellular substrate(s). The present study indicates, for the first time, that PKD phosphorylates the regulatory COOH-terminal domain of NHE1 in vitro. However, the stoichiometry of this phosphorylation was <0.1 mol phosphate/mol fusion protein. For comparison, earlier studies have revealed stoichiometries of 0.5–1, 1, and 3 mol/mol, respectively, for the in vitro phosphorylation of similar NHE fusion proteins by ERK1/2 (39), p90rsk (21), and Ca^{2+}/calmodulin-dependent protein kinase II (6). Thus, relative to PKD, other putative regulators of NHE activity appear to possess greater efficiency as NHE1 kinases, suggesting that direct phosphorylation may not be the primary mechanism underlying PKD-mediated regulation of the exchanger. Nevertheless, such in vitro data may not

reflect accurately the efficiency of PKD as an NHE1 kinase in vivo and do not preclude a role for direct phosphorylation in PKD-mediated regulation of NHE activity in the intact cell. In this regard, recent evidence (33) suggests that a COOH-terminal-truncated NHE1 mutant, in which the region that we have shown to be phosphorylated by PKD in vitro has been deleted, exhibits increased basal activity in vivo. This is consistent with the existence of autoinhibitory domain(s) within the PKD-phosphorylatable region of the exchanger.

In our studies, we have used the expression of GFP as the marker for successful transfection, to enable the measurement of pH_i (and thereby NHE activity) selectively in cells transfected with a wild-type or mutant PKD construct. Although we have confirmed that GFP expression does not interfere with the measurement of pH_i in single cells, we cannot be certain that 100% of the cells that expressed GFP also expressed the cotransfected PKD construct. At present, it is impossible to determine the proportion of GFP-positive cells that also express the cotransfected PKD construct by conventional approaches such as immunocytochemistry, because available antibodies would not differentiate between native and transfected PKD protein. Nevertheless, in cotransfection experiments, it is generally accepted that cells that take up one expression vector are highly likely to also take up cotransfected vector(s), with numerous examples in the literature. Indeed, in a recent study, Ho et al. (10) have used cotransfection with GFP in a manner similar to ours, to monitor intracellular Ca^{2+} transients selectively in single cells transfected with various regulators of the ERK pathway. On this issue, it is important to stress that, even if the efficiency of cotransfection in our experiments was <100%, this would lead to an underestimation of the effects of wild-type PKD and PKD-K618M on plasma membrane NHE activity and would not invalidate our novel findings.

The majority of the work in the literature on the regulation of NHE activity has concentrated on the identification of stimuli that increase exchanger activity, such as a variety of growth factors/mitogens (28, 29) and neurohormonal and other mediators (7, 38, 41, 42), as well as the delineation of their signaling pathways (2, 4, 12, 22, 41). However, NHE-inhibitory pathways, such as those that involve $G\alpha_{12}$ (24), CHP (23), and p38 MAPK (21), also exist and are likely to be important in determining exchanger activity in various cell types. The findings of the present study strongly suggest that PKD mediates a novel NHE-inhibitory pathway, regardless of whether this inhibition occurs by direct phosphorylation of the exchanger or via intermediary proteins. A better understanding of NHE-inhibitory pathways may lead to the development of novel approaches to therapy, in diseases in which altered exchanger activity has been implicated as a causal mechanism.

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Cardiac Na^+–H^+ Exchanger During Postnatal Development in the Rat: Changes in mRNA Expression and Sarcolemmal Activity

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R. S. Haworth, M. Yasutake, G. Brooks and M. Avkiran. Cardiac Na^+–H^+ Exchanger During Postnatal Development in the Rat: Changes in mRNA Expression and Sarcolemmal Activity. *Journal of Molecular and Cellular Cardiology* (1997) 29, 321–332. We examined Na^+–H^+ exchanger isoform 1 (NHE-1) mRNA expression in ventricular myocardium and its correlation with sarcolemmal NHE activity in isolated ventricular myocytes, during postnatal development in the rat. The expression of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA did not change in ventricular myocardium between 2 and 42 days after birth. Therefore, at seven time points within that age range, GAPDH expression was used to normalize NHE-1 mRNA levels, as determined by reverse transcription polymerase chain reaction analysis. There was a progressive five-fold reduction in NHE-1 mRNA expression in ventricular myocardium from 2 days to 42 days of age. As an index of NHE activity, acid efflux rates (J_H) were determined in single neonatal (2–4-day-old) and adult (42-day-old) ventricular myocytes ($n=16$/group) loaded with the pH fluoroprobe carboxy-seminaphthorhodafluor-1. In HEPES-buffered medium, basal intracellular pH (pH_i) was similar at 7.28 ± 0.02 in neonatal and 7.31 ± 0.02 in adult myocytes, but intrinsic buffering power was lower in the former age group. The rate at which pH_i recovered from a similar acid load was significantly greater in neonatal than in adult myocytes (0.36 ± 0.07 *v* 0.16 ± 0.02 pH units/min at $pH_i = 6.8$). This was reflected by a significantly greater J_H (22 ± 4 *v* 9 ± 1 pmol/cm^2/s at $pH_i = 6.8$), indicating greater sarcolemmal NHE activity in neonatal myocytes. The concomitant reductions in tissue NHE-1 mRNA expression and sarcolemmal NHE activity suggest that myocardial NHE-1 is subject to regulation at the mRNA level during postnatal development. © 1997 Academic Press Limited

Key Words: Na^+–H^+ exchanger; Gene expression; Activity; Development; Rat.

Introduction

The regulation of intracellular pH (pH_i) is important in all cell types because numerous intracellular events are directed or modulated by changes in pH. In cardiac myocardium [in which pH_i is a key modulator of contractility (Orchard and Kentish, 1990)], a primary regulator of pH_i is the sarcolemmal Na^+–H^+ exchanger (NHE), which extrudes intracellular H^+ in exchange for extracellular Na^+ in an electroneutral manner (Karmazyn and Moffat, 1993). To date, four different isoforms of NHE have been described in the rat and other species (Sardet *et al.*, 1989; Tse *et al.*, 1991, 1992, 1993; Orlowski *et al.*, 1992), although the major isoform present in the heart is NHE-1 (Fliegel and Dyck, 1995; Orlowski *et al.*, 1992). In the absence of pharmacological modulation, the cardiac sarcolemmal NHE is quiescent at physiological pH_i, but is activated rapidly in response to intracellular acidosis (Wallert and Fröhlich, 1989, 1992).

There is evidence that a significant reduction

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in sarcolemmal NHE activity may occur during postnatal development of the rabbit myocardium (Meno *et al.*, 1989) and that this might be responsible, at least in part, for age-dependent differences in myocardial susceptibility to contractile dysfunction during respiratory or metabolic acidosis (Meno *et al.*, 1989; Nakanishi *et al.*, 1990). More recent studies in the same species have shown that cardiac NHE-1 mRNA expression decreases after birth (Chen *et al.*, 1995; Takewaki *et al.*, 1995), an observation that might provide a molecular basis for the earlier observation of reduced sarcolemmal NHE activity (Meno *et al.*, 1989). However, it is presently unknown whether the postnatal reduction observed in cardiac NHE-1 expression in the rabbit is a myocyte- or species-specific phenomenon. Furthermore, little is known regarding post-natal changes in NHE activity in *intact myocytes*, because the only published study was carried out in sarcolemmal vesicles (Meno *et al.*, 1989). In view of the multiple intracellular regulatory mechanisms (e.g. phosphorylation, calmodulin binding) that may be involved in determining the activity of the NHE-1 protein (Counillon and Pouysségur, 1995), it is possible that the activity measured in reconstituted sarcolemmal vesicles may not reflect accurately that present in intact cells.

The objectives of the present study were to determine: (1) whether a change in ventricular NHE-1 expression occurs during post-natal development in the rat; and (2) whether any such change in expression is reflected by a change in sarcolemmal NHE activity in intact myocytes.

Materials and Methods

Animals and materials

Adult (approximately 42-day-old) male and late-term pregnant Wistar rats were purchased from B & K Universal (Hull, UK). The investigation was performed in accordance with the Home Office *Guidance on the Operation of the Animals (Scientific Procedures) Act*, 1986, published by HMSO, London. Oligo dT cellulose was purchased from Becton Dickinson Labware (Bedford, MA, USA). Taq DNA polymerase, pancreatin, fetal calf serum and Dulbeeco's modified Eagles medium containing glutamax were from Gibco BRL Life Technologies Ltd. (Paisley, UK). All other chemicals were of the purest grade commercially available.

PolyA$^+$-enriched RNA preparation

To determine changes in myocardial NHE-1 expression during postnatal development, ventricular tissue was obtained from rats aged 2, 5, 10, 14, 21, 28, and 42 days. Tissue samples were frozen in liquid nitrogen immediately after excision, ground to a powder using a pestle and mortar, precooled with liquid nitrogen, and stored in liquid nitrogen until required. PolyA$^+$ RNA was prepared from 300–500 mg of powdered tissue by mechanical homogenization in a buffer containing NaCl (200 mM), Tris(hydroxymethyl)aminomethane (Tris, 200 mM, pH 7.5), magnesium chloride (150 μM), sodium dodecyl sulfate (SDS; 2%) and proteinase K (200 μg/ml). In the case of ventricular tissue from very young rats, hearts from more than one animal were pooled (ten hearts at 2 days, six hearts at 5 days and three hearts at 10 days). The lysate was forced through a 21-gauge needle until a homogeneous viscosity was obtained. Samples were incubated at 45°C for 12 h with gentle agitation. Oligo dT cellulose was hydrated for 60 min in binding buffer containing NaCl (0.5 M), Tris (10 mM, pH 7.5), washed once with binding buffer, and added to the lysate. NaCl was added to a final concentration of 0.5 M, followed by incubation at room temperature with gentle agitation for 60 min. The bound polyA$^+$ RNA was washed six times with binding buffer, and eluted with Tris (10 mM, pH 7.5). PolyA$^+$ RNA concentration was determined by measuring absorption at 260 nm.

Dot blots

Nylon membrane (Hybond-N, Amersham International) was pre-soaked first in RNase free water for 5 min and then 20 × saline–sodium citrate (SSC; 20 × SSC contains NaCl (3 M) and trisodium citrate (0.3 M; pH 7.0)) for 1 h. Samples of polyA$^+$ RNA (0.5 μg) were denatured (68°C for 15 min) and applied directly to the membrane, using vacuum filtration in conjunction with a dot blot manifold (Schleicher and Schuell; Keene, NH, USA). The RNA was cross-linked to the membrane using a Stratagene UV Stratalinker 2400 (200 μJ × 100 s). The membrane was prehybridized in formamide (50%), 5 × SSC, 5 × Denhardt's solution, SDS (0.1%) and denatured herring sperm DNA (100 μg/ml) for 5 h at 42°C. The dot blots were first exposed to an oligo(dT)$_{15}$ probe, stripped by boiling in 1 × SSC for 15 min, and subsequently exposed to a glyceraldehyde-3-phosphate dehydrogenase (GAPDH)

probe. The oligo$(dT)_{15}$ probe was prepared by labelling the oligonucleotide (Promega; 0.5 μg) using the Promega DNA 5′-End Labelling System, as recommended by the manufacturer. The GAPDH probe was prepared using the Multiprime DNA Labelling Kit (Amersham International, Amersham, UK), as recommended by the manufacturer, with a 1-kb rat GAPDH fragment (100 ng) used as template. Following column purification and heat denaturation, the probes (specific activity 2×10^7 ct/min/μg) were added directly to the prehybridization solution, and incubation continued at 42°C overnight. Dot blots were washed with 2 × SSC/0.1% SDS for 15 min and 1 × SSC/0.1% SDS for 30 min, both at 42°C. For GAPDH, a final wash with 0.1 × SSC/0.1% SDS for 30 min at 65°C was also performed. The membrane was exposed to film (Kodak X-OMAT-AR) for different time periods to ensure that the linear range of the film was not exceeded. The signals obtained at 6 h (which were within the linear range) were quantified using an LKB Ultrascan XL Laser Densitometer.

Reverse transcription polymerase chain reaction (RT-PCR)

Samples of mRNA (1 μg) were reverse-transcribed using the Invitrogen cDNA Cycle Kit, as recommended by the manufacturer. Following ethanol precipitation, the cDNA samples were dissolved in RNase-free water (20 μl). An aliquot (1 μl) was diluted to 50 μl prior to PCR. At this dilution, the products of both NHE-1 and GAPDH amplification were within the linear phase of the reaction (data not shown). PCR was performed using 1 μl of diluted cDNA in a total volume of 50 μl containing HEPES (50 mM, pH 7.9), $MgCl_2$ (1.5 mM), dNTPs (100 μM each), 100 pmol each of the forward and reverse primers, and 2.5 U of Taq DNA polymerase. The reactions also contained 1 μCi of [α^{32}P] dCTP (3000 Ci/mmol). The following primers were used: NHE-1 forward primer 5′-ACC CTG CTC TTC TGC CTC-3′, NHE-1 reverse primer 5′-TGC GGA TCT CCT CCT CCT T-3′, GAPDH forward primer 5′-CCT TCA TTG ACC TCA AC-3′, GAPDH reverse primer 5′-AGT TGT CAT GGA TGA CC-3′. The primers were designed to span one intron for each gene, thus reducing the possibility of signals due to genomic DNA contamination. Amplification was for 30 cycles of 94°C for 45 s, 62°C for 45 s and 72°C for 90 s, using a Techne PHC-3 Thermal Cycler. Duplicate aliquots (10 μl) were resolved on 9% polyacrylamide gels. Gels were exposed to Kodak X-OMAT-AR film to identify amplified DNA. Radiolabelled bands were excised and quantitated by liquid scintillation counting.

Northern blots

Samples of mRNA (5 μg) were subjected to electrophoresis using a 1% agarose/6% formaldehyde gel and transferred to a nylon membrane (Hybond-N; Amersham International, Amersham, UK). The membrane was prehybridized in formamide (50%), 5 × SSC, 5 × Denhardt's solution, SDS (0.1%) and denatured herring sperm DNA (100 μg/ml) for 4 h at 42°C. The membrane was subsequently hybridized to a gel-purified 1.3-kb *PstI* fragment from the rat NHE-1 cDNA (100 ng), radiolabelled to a specific activity of 3×10^7 ct/min/μg using the Amersham Multiprime DNA Labelling Kit, by adding the probe directly to the prehybridization solution. The membrane was washed in 2 × SSC/0.1% SDS for 15 min at room temperature, followed by 0.1 × SSC/0.1% SDS for 30 min at 60°C, prior to autoradiography using Kodak X-Omat AR film. The blot was stripped by boiling in 0.1 × SSC for 30 min, and re-probed using the GAPDH probe described earlier.

Preparation of cardiac myocytes

For estimation of cell volume and surface area, and measurement of intracellular pH and NHE activity, ventricular myocytes were isolated from the hearts of neonatal and adult rats using collagenase-based enzymatic digestion techniques.

Adult hearts were excised and perfused (37°C) in the Langendorff mode for three sequential periods, as follows: (1) 5 min with Tyrode solution containing NaCl (137 mM), KCl (5.4 mM), $CaCl_2$ (1.8 mM), $MgCl_2$ (0.5 mM), N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid (HEPES; 10 mM), glucose (10 mM), adjusted to pH 7.4 with NaOH; (2) 5.5 min with nominally Ca^{2+}-free Tyrode solution containing NaCl (135 mM), KCl (5.4 mM), NaH_2PO_4 (0.33 mM), $MgCl_2$ (1.0 mM) HEPES (10 mM), glucose (10 mM), adjusted to pH 7.2 with NaOH; (3) 10 min with nominally Ca^{2+}-free Tyrode solution containing collagenase (Worthington Type 1, 200 U/ml); and (4) 5 min with storage buffer containing KOH (78 mM), KCl (30 mM), KH_2PO_4 (30 mM), $MgSO_4$ (3 mM), ethylene glycol-bis(β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid (EGTA; 0.5 mM), HEPES (10 mM), glutamic acid (50 mM), taurine (20 mM), glucose (10 mM),

adjusted to pH 7.2 with KOH. All solutions were gassed with 100% O_2. After the perfusion procedure, the heart was removed from the cannula, and the atria and non-ventricular tissue carefully removed. The tissue fragments were gently agitated in storage buffer to disperse ventricular cells. The cells were suspended in storage buffer at 25°C for 60 min prior to loading with fluorescent dye (see later) and were used within 6 h of preparation.

Neonatal myocytes were prepared from 2–4-day-old animals as follows. The hearts from ten animals were excised, the atria trimmed off and the ventricles cut into small pieces. Tissue was incubated with continuous stirring for 20 min at 37°C in a solution containing collagenase (112 U/ml), pancreatin (5 mg/ml), NaCl (117 mM), HEPES (20 mM), NaH_2PO_4 (0.87 mM), glucose (5.6 mM), KCl (5.4 mM) and $MgSO_4$ (1 mM), adjusted to pH 7.4 with NaOH. Intact tissue was allowed to settle, and the dispersed cells were removed and added to fetal calf serum (FCS; 10% v/v final concentration) for 10–20 min. The cells were then sedimented at 200 × g for 2 min and resuspended in Dulbecco's modified Eagles medium (DMEM) containing glutamax at 25°C for 60 min, prior to loading with fluorescent dye. Cells were used within 6 h of preparation.

Estimation of cell volume and surface area

Cell dimensions were measured on a calibrated monitor, using a CCD camera in conjunction with the 40 × objective lens. The volumes of neonatal myocytes were estimated from cell diameter measurements, assuming a spherical shape (Rook *et al.*, 1988). The volumes of adult myocytes were calculated from the length and width of each cell, using the formula volume = width × length × 0.00759 pl/μm^2 (Satoh *et al.*, 1996). To obtain an estimate of cell surface area, capacitance measurements were made using heat-polished patch electrodes, made from thin-walled glass capillaries (1.5 mm o.d., 1.2 mm i.d.; World Precision Instruments, New Haven, CT, USA) using a two-stage horizontal microelectrode puller (DMZ-Universal Puller; Munich, Germany). Electrode resistance ranged from 2–8 MΩ. The electrode solution contained aspartic acid (110 mM), KCl (20 mM), EGTA (10 mM), HEPES (10 mM), ATP (5 mM) and $MgCl_2$ (5.92 mM), adjusted to pH 7.2 with KOH. A hydraulic micromanipulator (model MO-102, Narishige Scientific Instruments, Tokyo, Japan) was used to position the electrode near the centre of the cell. A seal was established between the electrode tip and the membrane by applying slight negative pressure. After compensation for stray capacitance, the patch membrane was ruptured. Recordings of membrane current were made using a patch clamp amplifier, with a holding potential of −80 mV. Voltage clamp pulses (to −85 mV) were generated using pClamp software (Axon Instruments, Foster City, CA, USA), and membrane capacitance calculated, as previously described (Bénitah *et al.*, 1993). In estimating cell surface area, a specific capacitance of 1 pF/100 μm^2 was assumed (Satoh *et al.*, 1996).

Measurement of intracellular pH

Intracellular pH (pH_i) was monitored in single ventricular myocytes using the pH-sensitive fluorescent dye carboxy-seminapththorhodafluor-1 (C.SNARF-1), as previously described (Yasutake *et al.*, 1996). Aliquots of cells were loaded with C.SNARF-1 by incubating them in a 5 μM solution of the acetoxymethyl ester (Calbiochem, Nottingham, UK) for 10 min at room temperature. Cells loaded with C.SNARF-1 then were allowed to settle on a glass coverslip at the bottom of a 100 μl chamber, mounted on the stage of an inverted microscope (Nikon Diaphot) which comprised a 40 × magnification oil immersion objective with a numerical aperture of 1.3. After adherence to the coverslip, cells were superfused (3.5 ml/min) with Tyrode solution at 34°C. Cells were excited with light at 540 nm and the resulting fluorescence emission intensity from a selected area of a single myocyte measured simultaneously at 580 nm (I_{580}) and 640 nm (I_{640}), using a dual emission photometer system (Model D104C; Photon Technology International Inc., London, UK) which comprised an adjustable aperture and two multialkali photomultiplier tubes (Type R928; Hamamatsu Photonics UK Ltd., Enfield, UK). Background fluorescence was measured using an identical aperture and subtracted from the signal. After I–V conversion, the acquired signals were digitized at 1.7 Hz and stored on digital acquisition tape and computer hard disk, the latter using pClamp software (Axon Instruments, Foster City, CA, USA).

The emission intensity ratio (I_{580}/I_{640}) was calculated and converted to a pH_i scale, using *in situ* calibration data obtained by exposing cells loaded with C.SNARF-1 to nigericin-containing calibration solutions of pH 5.8–8.0. The calibration solutions consisted of KCl (140 mM), $MgCl_2$ (1 mM), and nigericin (10 μM), and were buffered with 10 mM of

one of the following buffers: 2-[N-morpholino]ethanesulphonic acid (MES) at pH 5.8, 6.2 and 6.4, piperazine-N,N′-bis[2-ethanesulfonic acid] (PIPES) at pH 6.6, 6.8 and 7.0, and HEPES at pH 7.2, 7.4, 7.7 and 8.0. All calibration solutions were adjusted to the correct pH with NaOH. The data acquired from calibration experiments were normalized by dividing all I_{580}/I_{640} ratios by the ratio obtained at a pH_i of 7.0; thus, the normalized I_{580}/I_{640} ratio at pH_i 7.0 was always 1 (Gupta *et al.*, 1994). A calibration curve was obtained by a non-linear least-squares fit of the normalized data to the equation given below, with the curve constrained to pass through the point having the coordinates $I_{580}/I_{640}/(I_{580}/I_{640})_{pH7} = 1.0$, pH = 7.0.

$$\frac{I_{580}/I_{640}}{(I_{580}/I_{640})_{pH7}} = 1 + a \left[\frac{10(pH - pK)}{1 + 10(pH - pK)} - \frac{10(7 - pK)}{1 + 10^{(7-}pK)} \right]$$

The best-fit values for pK and a were 7.12 ± 0.03 and -1.384 ± 0.03, respectively. No significant difference was observed in the respective calibration curves for adult and neonatal myocytes.

Determination of intrinsic buffering power

Intracellular intrinsic buffering power (β_i) was estimated by stepwise removal of extracellular NH_4Cl (Lagadic-Gossmann *et al.*, 1992). Cells were exposed to Tyrode solution containing 20 mM NH_4Cl for 1–2 min, followed by a stepwise reduction of extracellular NH_4^+ to 10, 5, 2.5, 1 and 0 mM. At each step, calculated changes in $[NH_4^+]_i$ and measured changes in pH_i were used to estimate β_i, from the equation $\beta_i = \Delta[NH_4^+]_i/\Delta pH_i$. These experiments were carried out in the presence of HOE-694 (30 μM) in order to prevent acid extrusion via the Na^+–H^+ exchanger and $BaCl_2$ (5 mM) to reduce NH_4^+ efflux through potassium channels.

Calculation of sarcolemmal acid efflux

Because all experiments were carried out in the nominal absence of bicarbonate, the rate of acid efflux (J_H) was used as a direct index of sarcolemmal Na^+/H^+ exchanger activity (Lagadic-Gossmann *et al.*, 1992). Intracellular acidosis was induced by the wash-out (for 6 min) of NH_4Cl (20 mM), after its transient (2–3 min) application. At the end of every experiment, the cell under study was exposed to nigericin-containing calibration solution at pH 7.0, thus enabling normalisation of the I_{580}/I_{640} ratios. Equation 2 and the fitted values for pK and a were then used to calculate pH_i from the normalized I_{580}/I_{640} values. The pH_i trace during the recovery phase was fitted to a single exponential function (Boyarsky *et al.*, 1988). At pH_i intervals of 0.05 during this phase, dpH_i/dt (rate recovery of pH_i) was calculated as the differential derivative of the exponential fit. To take account of the differences in the size of neonatal versus adult myocytes, "true" J_H was calculated, in units of $pmol/cm^2/s$, using the equation $J_H = \beta_i \times dpH_i/dt \times v/s$, where v/s represents the estimated cell volume:surface area ratio in the appropriate age group (Bevensee and Boron, 1995).

Statistical analysis

Gaussian-distributed variables were expressed as mean ± S.E.M. Multiple comparisons were by analysis of variance, followed by Dunnetts test for comparison with the 2-day-old group. To assess changes between two groups, an unpaired t-test was used. A P value of <0.05 was considered significant.

Results

Ventricular GAPDH mRNA expression

The level of expression of GAPDH mRNA relative to the total ventricular mRNA pool was determined by dot blot analysis of samples from animals aged 2, 5, 10, 14, 21, 28, and 42 days. The results are presented in Figure 1. Data were obtained from three separate experiments performed at different times and, in each experiment, individual GAPDH: oligo $(dT)_{15}$ ratios were expressed as a percentage of the ratio obtained with the 2-day-old sample in that experiment. No significant change in GAPDH mRNA expression was seen over the age range investigated, thereby validating its use as an internal standard in subsequent studies using RT-PCR.

Ventricular NHE-1 mRNA expression

Typical RT-PCR results are shown in Figure 2, in the form of an autoradiogram. NHE-1 (upper panel) and GAPDH (lower panel) were detected using simultaneous PCR analysis of samples from the same reverse-transcription reaction. The lengths of the fragments obtained matched those predicted

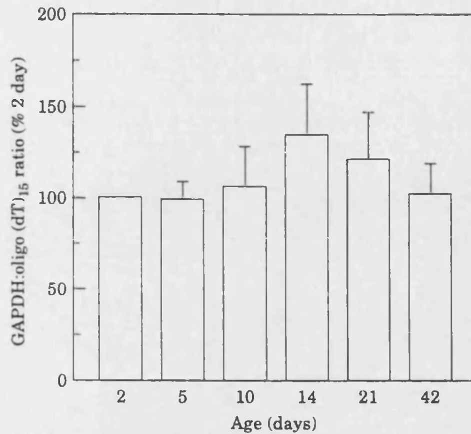

Figure 1 Postnatal changes in GAPDH mRNA expression, determined by dot blot analysis. mRNA samples were probed using a GAPDH cDNA fragment and oligo $(dT)_{15}$, and the signals obtained quantitated by scanning densitometry. The results are presented as mean ± S.E.M. of the GAPDH:oligo $(dT)_{15}$ signal ratios in the various age groups, normalized to the ratio seen at 2 days (n = 3).

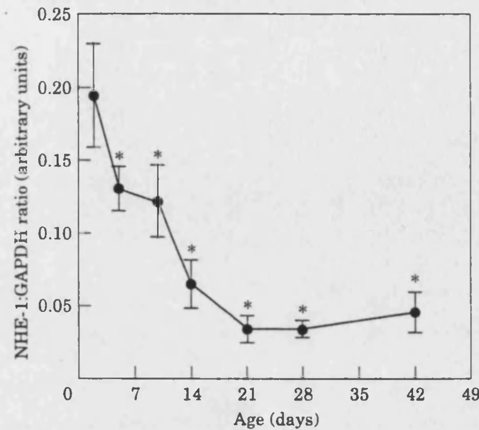

Figure 3 Postnatal changes in NHE-1 mRNA expression determined by RT-PCR analysis. Data are presented as mean ± S.E.M. of the NHE-1:GAPDH ratios in the various age groups (n = 3–5). *P<0.05 v 2-day-old.

from the published sequences for rat NHE-1 (Orlowski *et al.*, 1992) and GAPDH (Fort *et al.*, 1985). The mean NHE-1:GAPDH ratios, obtained from 3–5 experiments, are shown in Figure 3. As can be seen, there was a progressive five-fold reduction in NHE-1 mRNA expression during the first 21 days after birth, which then remained constant for the rest of the period studied. These findings obtained with RT-PCR were supported by additional analysis of NHE-1 mRNA expression in 2-day-old and 42-day-old samples using Northern blotting (Fig. 4) and a dot blot assay (data not shown).

Cell volume and surface area

The size characteristics of neonatal (2–4-day-old and adult (42-day-old) myocytes are presented in

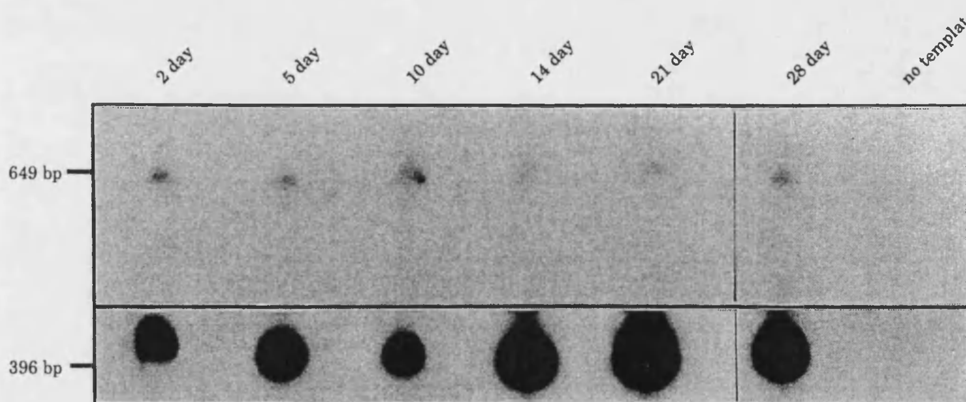

Figure 2 Representative autoradiogram illustrating the detection of NHE-1 and GAPDH mRNA, using RT-PCR. mRNA samples isolated from whole ventricles of rats of different ages were subjected to reverse transcription and polymerase chain reaction. For PCR, specific primers were used to amplify NHE-1 (upper panel) or GAPDH (lower panel), as described in Materials and Methods.

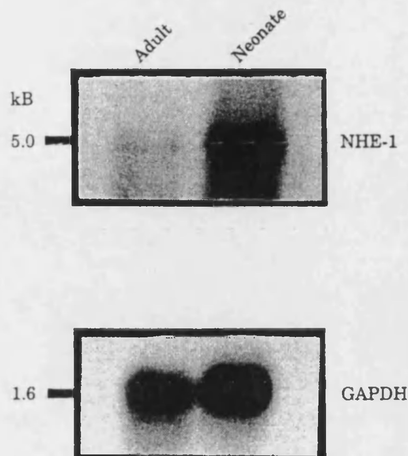

Figure 4 Northern blot of adult (42-day-old) and neonatal (2-day-old) ventricular mRNA probed for NHE-1 (upper panel) and GAPDH (lower panel). The lengths of the detected mRNA species are as indicated in the Figure.

Table 1 Size characteristics of neonatal (2–4-day-old) and adult (42-day-old) ventricular myocytes

	Neonate ($n=21$)	Adult ($n=18$)
Diameter (μm)	27.5 ± 0.4	NA
Width (μm)	NA	20 ± 2
Length (μm)	NA	108 ± 3
Volume (pl)	1.39 ± 0.07	34 ± 3*
Capacitance (pF)	9.7 ± 0.4	197 ± 12*
Surface area (μm^2)	970 ± 40	19700 ± 1200*

NA: not applicable. *$P<0.05$ *v* neonate.

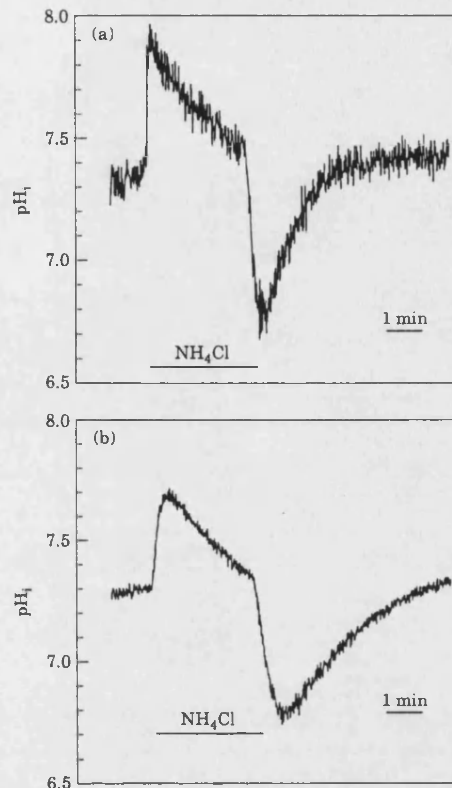

Figure 5 Representative recordings of pH_i recovery in individual neonatal [2-day-old; (a)] and adult [42-day-old; (b)] ventricular myocytes following an acid load. Intracellular acidosis was induced by transient exposure to NH_4Cl (20 mM).

Table 1. As expected, both surface area and cell volume were significantly greater in adult *v* neonatal myocytes. The estimated volumes of neonatal and adult myocytes were comparable to previously reported values, based on morphometric analysis (Anversa *et al.*, 1980) and a volume rendering algorithm used in conjunction with confocal microscopy (Satoh *et al.*, 1996). Cell volume:surface area ratios (v/s) based on the data shown in Table 1 were subsequently used to calculate the rates of acid efflux (see Materials and Methods) in the two age groups.

pH_i regulation

Because NHE-1 has been shown to be essentially quiescent at physiological values of pH_i, an acute acid load was induced experimentally in neonatal (2–4-day-old) and adult (42-day-old) myocytes loaded with C.SNARF-1 by transient exposure to NH_4Cl (Fröhlich and Wallert, 1995). Recordings from typical experiments with both a neonatal (upper panel) and an adult (lower panel) myocyte are shown in Figure 5, which illustrates that in both age groups pH_i was able to recover from acute acidosis. To demonstrate that, under the nominally bicarbonate-free conditions employed, the recovery of pH_i from acidosis was due to NHE activity, some cells were exposed to a Na^{+}-free Tyrode solution (in which NaCl was replaced iso-osmotically with choline chloride) during the first 3 min of NH_4Cl wash-out. This was then followed by superfusion with Na^{+}-containing Tyrode solution. The results of typical experiments with both a neonatal (upper panel) and an adult (lower panel) myocyte are shown in Figure 6. In the absence of extracellular

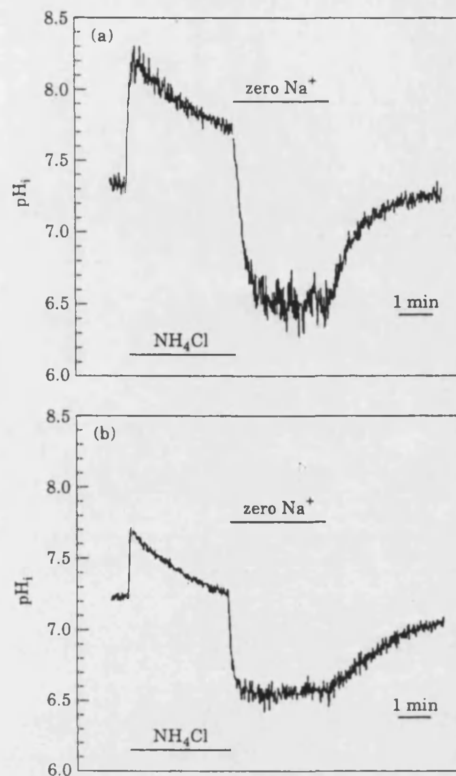

Figure 6 Representative illustration of the Na^+-dependency of pH_i recovery from acidosis in both neonatal [2-day-old; (a)] and adult [42-day-old; (b)] ventricular myocytes. Intracellular acidosis was induced by transient exposure to NH_4Cl (20 mM). There was no recovery of pH_i in either age group until extracellular Na^+ was re-introduced.

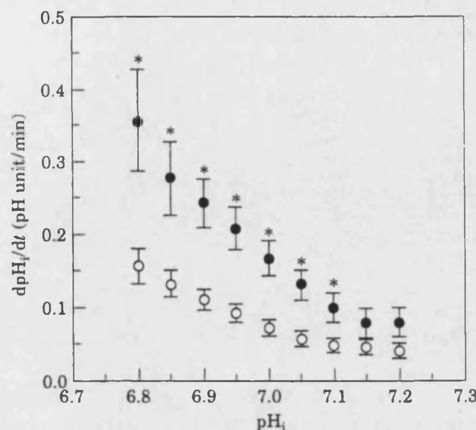

Figure 7 Dependency of the rate of pH_i recovery (dpH_i/dt) on pH_i in neonatal (2–4-day-old) (●) and adult (42-day-old) (○) ventricular myocytes. Data are expressed as mean ± S.E.M. ($n=16$). *$P<0.05$ v adult.

Na^+, there was no recovery of pH_i from acidosis in either neonatal or adult cells. pH_i recovery was initiated only upon the re-introduction of extracellular Na^+, confirming that such recovery occurred via NHE in both age-groups.

Basal pH_i was not significantly different between the two age groups (7.28 ± 0.02 ($n=34$) and 7.31 ± 0.02 ($n=23$), in neonatal and adult myocytes, respectively). Although a similar degree of alkalosis was observed in the two age groups following exposure to NH_4Cl (maximum pH_i of 7.89 ± 0.03 and 7.82 ± 0.02, in neonatal and adult myocytes, respectively), the rate at which pH_i recovered from such alkalosis during the 3 min exposure period was different, with average pH_i changes of 0.27 ± 0.02 and 0.39 ± 0.02 units in neonatal and adult myocytes, respectively ($P<0.05$). Presumably, as a consequence of this, the maximum acidification attained following NH_4Cl wash-out tended to be greater in adult myocytes (minimum $pH_i = 6.65 \pm 0.04$) than in neonatal cells (minimum $pH_i = 6.73 \pm 0.03$).

The rates of change of pH_i (dpH_i/dt) following NH_4Cl wash-out, as a function of pH_i, are shown in Figure 7. Little difference was observed between the two age groups at physiological pH_i (pH>7.1). However, at lower pH_i, dpH_i/dt was significantly greater in neonatal myocytes.

Intrinsic buffering power

The dependence of intrinsic buffering power (β_i) on pH_i in neonatal (2–4-day-old) and adult myocytes is shown in Figure 8. Linear regression analysis revealed very similar correlation coefficients of 0.63 and 0.62 for neonatal and adult myocytes, respectively. The gradients of the best-fit lines were −23 and −35 mM/pH unit for neonatal and adult cells, respectively, indicating a 33% lower β_i in the younger age group.

Sarcolemmal NHE activity

Figure 9 shows the pH_i v J_H relationships in neonatal (2–4-day-old) and adult myocytes. As shown, J_H was significantly greater in neonatal cells at pH_i

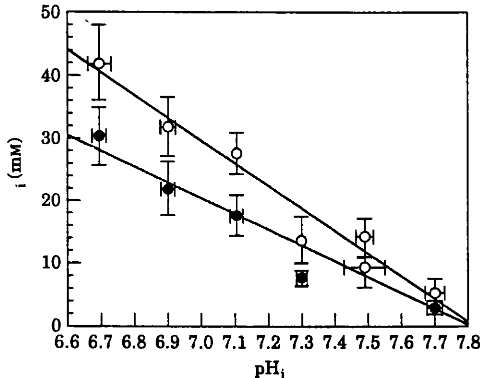

Figure 8 Dependency of the intrinsic buffering power (β_i) on pH_i in neonatal (2–4-day-old (●) and adult (42-day-old) (○) ventricular myocytes. Data from 15–20 cells in each age group were pooled into 0.2 pH unit bins ($n=8$–31), and are expressed as the mean ± S.E.M.

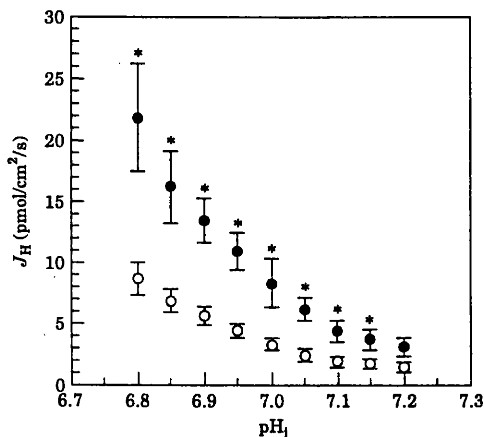

Figure 9 Dependency of the rate of acid efflux (J_H) on pH_i in neonatal (2–4-day-old) (●) and adult (42-day-old) (○) ventricular myocytes. Data are presented as the mean ± S.E.M. ($n=16$). *$P<0.05$ v adult.

6.80–7.15, indicating 120–140% greater NHE activity in this age group within this pH_i range.

Additional experiments were carried out to confirm that this marked difference in NHE activity arose from intrinsic age-dependent differences, rather than differences in the cell isolation protocols used at the two ages. In these experiments, adult myocytes were isolated as described earlier but an aliquot of cells was exposed to FCS (10% v/v) for 20 min, which is equivalent to the maximum duration for which neonatal myocytes were exposed to FCS. These cells were then resuspended in DMEM containing glutamax at 25°C for 60 min prior to loading with fluorescent dye, as with the neonatal cells. These studies revealed that there was no significant difference in J_H between cells ($n=7$ or 8/group) exposed to FCS/DMEM with glutamax (J_H range of 5.1 ± 2.0–0.9 ± 0.1 pmol/cm²/s over the pH_i range 6.75–7.05) and contemporary controls (J_H range of 5.9 ± 1.0–9 ± 0.1 pmol/cm²/s over the pH_i range 6.75–7.05). This suggests that exposure to FCS/DMEM with glutamax is unlikely to account fully for the greater NHE activity observed in neonatal myocytes.

Discussion

In the present study, we have determined the steady-state mRNA levels of the Na^+–H^+ exchanger (NHE-1 isoform) in the developing rat ventricular myocardium, and correlated these with the relative activity of the sarcolemmal exchanger in intact neonatal v adult ventricular myocytes. Our findings with respect to postnatal changes NHE-1 mRNA expression are qualitatively similar to data obtained recently from studies in the rabbit (Chen *et al.*, 1995; Takewaki *et al.*, 1995), which also utilized GAPDH as an internal control. Indeed, GAPDH has been used frequently as a control housekeeping gene in studies of myocardial gene expression in a variety of settings, on the assumption that its expression remains constant. However, recently, Knoll *et al.* (1994) have provided data that challenge the dogma of invariable GAPDH expression in the myocardium. In view of this, we initially investigated whether the steady-state GAPDH mRNA level (relative to the total mRNA pool) in rat ventricular myocardium changed significantly in our experimental setting, namely during postnatal development. Our observation of a constant level of GAPDH expression during the first 42 days after birth confirms that, at least in the rat, the use of GAPDH as an internal housekeeping gene in the study of postnatal changes in myocardial gene expression is a valid approach.

Although qualitatively similar, the extent of the decline in NHE-1 mRNA expression in rat myocardium in the present study (five-fold reduction between 2 days of age and adulthood) was notably greater than that previously reported in rabbit ventricular myocardium (1.6-fold reduction between 2–5 days of age and adulthood (Chen *et al.*, 1995). Although the reason for this is unclear, it may be related to species-differences in the extent of maturity at birth and/or the rate of postnatal development. In this regard, it is interesting to note

that for the sarcolemmal Na^+–Ca^{2+} exchanger also, the relative profiles of steady-state mRNA levels in the developing rat *v* rabbit myocardium apppear to exhibit such a quantitative difference [eight-fold reduction between 1 day of age and adult in the rat *v* 2.8-fold reduction over the same period in the rabbit (Boerth *et al.*, 1994)]. It remains to be determined whether the postnatal reduction in the steady-state NHE-1 mRNA level observed in rat myocardium in the present study is due to a reduction in the rate of transcription and/or in mRNA stability. In this regard, direct evidence of the factors involved in transcriptional regulation of the NHE-1 gene has only recently begun to emerge (Kolyada *et al.*, 1994, 1995; Dyck *et al.*, 1995), although there is still little known regarding the role of changes in the rate of mRNA degradation in regulating NHE-1 expression (Fliegel and Dyck, 1995).

Data based solely on whole tissue mRNA expression (Chen *et al.*, 1995; Takewaki *et al.*, 1995) is difficult to interpret in relation to the mechanisms that may be involved in the regulation of the sarcolemmal Na^+–H^+ exchanger, because: (1) a heterogeneous population of cells (not just myocytes, but also cardiac fibroblasts, vascular smooth muscle cells and endothelial cells) may contribute to the message; and (2) changes in NHE-1 mRNA expression may not necessarily reflect changes in NHE-1 protein expression. With regard to the latter point, it has not been possible to detect NHE-1 protein by the conventional methods of immunoprecipitation and Western blotting in rat or rabbit ventricular myocardium (authors' unpublished observations), most likely due to the very low level of expression of the protein in cardiac tissue (Fliegel and Dyck, 1995). In the absence of data on NHE-1 protein expression, the qualitative similarity of the developmental changes observed in NHE-1 mRNA expression in rabbit ventricular tissue to the previously reported changes in Na^+–H^+ exchanger activity in sarcolemmal vesicles from the same species (Meno *et al.*, 1989) prompted Chen *et al.* (1995) to hypothesize that the NHE-1 mRNA level may determine cardiac Na^+–H^+ exchanger activity. Although this is a feasible hypothesis (particularly in the light of the results of the present study—see below), it should be noted that age-dependent differences in Na^+–H^+ exchanger activity in isolated sarcolemmal vesicles may not reflect accurately developmental changes in sarcolemmal Na^+–H^+ exchanger activity in intact myocytes, because: (1) vesicles from different age groups may not represent similar sarcolemmal membrane fractions or have comparable properties of orientation and permeability (Artman *et al.*, 1995); and (2) in intact cells, there are a variety of post-translational regulatory mechanisms (e.g. phosphorylation, calmodulin binding) that are involved in determining Na^+–H^+ exchanger activity (Counillon and Pouysségur, 1995), which themselves may be subject to developmental regulation and which may be impaired or lost in reconstituted vesicles.

The present study is the first to determine Na^+–H^+ exchanger activity, in terms of the H^+-efflux rate via the exchanger per unit area of cell membrane, in intact myocytes from neonatal and adult ventricular tissue. Our observation of a significant decline in such activity, occurring concomitantly with the decline in tissue NHE-1 mRNA expression, suggests that: (1) the changes in tissue mRNA levels may reflect the changes in myocyte mRNA levels; and (2) transcriptional/post-transcriptional regulatory mechanisms may indeed play a dominant role in determining sarcolemmal Na^+–H^+ exchanger activity. These findings are analogous to those of Artman and colleagues with respect to the sarcolemmal Na^+–Ca^{2+} exchanger (Boerth *et al.*, 1994; Artman *et al.*, 1995). It should be noted, however, that between the neonatal period and adulthood, the decline in sarcolemmal Na^+–H^+ exchanger activity (approximately 2–3-fold) was less than that in tissue NHE-1 mRNA expression (approximately five-fold). This suggests that changes in cells other than myocytes may have contributed to the changes in the message, and/or that sarcolemmal Na^+–H^+ exchanger activity may be additionally subject to translational/post translational regulation during post-natal development.

During our determination of sarcolemmal Na^+–H^+ exchanger activity, we observed a significant difference in the responses of neonatal *v* adult myocytes to the NH_4Cl pulse, such that adult myocytes exhibited a greater rate of recovery from alkalosis during the period of exposure to NH_4Cl. The primary mechanism of recovery from intracellular alkalosis in mammalian myocytes is the Cl^-–HCO_3^- exchanger (Vaughan-Jones, 1986). However, because our experiments were carried out in HCO_3^- -free medium, it is unlikely that a difference in the activity of the Cl^--HCO_3^- exchanger contributed significantly to the age-dependent difference in the rate of recovery from alkalosis. This difference is more likely to have arisen from a difference in membrane permeability to NH_4^+, the influx (probably through K^+ channels) of which is thought to mediate pH_i recovery during exposure to NH_4Cl (Roos and Boron, 1981; Wu *et al.*, 1994). We also observed a greater intrinsic

buffering power in adult myocytes relative to their neonatal counterparts. Because intrinsic intracellular buffers [i.e. buffers that cannot traverse the cell membrane (Bevensee and Boron, 1995)] are believed to comprise H^+-titratable groups on cytoplasmic proteins (Bountra *et al.*, 1990), the age-dependent difference in the intrinsic buffering power is likely to reflect a change in the protein composition of the myocyte during postnatal development.

In conclusion, we have demonstrated, for the first time, a concomitant postnatal decline in the steady-state NHE-1 mRNA level in ventricular myocardium and the sarcolemmal activity of the Na^+–H^+ exchanger in isolated intact ventricular myocytes in the same species, namely the rat. These findings, taken together with previous observations in rabbit ventricular myocardium (Chen *et al.*, 1995; Takewaki *et al.*, 1995) and rabbit sarcolemmal vesicles (Meno *et al.*, 1989), support an important role for transcriptional/post-transcriptional mechanisms in the regulation of the sarcolemmal Na^+–H^+ exchanger. Furthermore, they are consistent with the hypothesis (Meno *et al.*, 1989; Nakanishi *et al.*, 1990) that a greater sarcolemmal Na^+–H^+ exchanger activity may be responsible for the reduced susceptibility of neonatal myocardium to dysfunction during respiratory or metabolic acidosis.

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Effects of moderate hypothermia on sarcolemmal Na^+/H^+ exchanger activity and its inhibition by cariporide in cardiac ventricular myocytes

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1 Specific inhibitors of the sarcolemmal Na^+/H^+ exchanger (NHE) such as cariporide are being evaluated for cardioprotective therapy during cardiac surgery. We determined the effects of moderate hypothermia (25°C), as occurs during cardiac surgery, on (1) sarcolemmal NHE activity and (2) the NHE-inhibitory potency of cariporide, in isolated adult rat ventricular myocytes.

2 As the index of NHE activity, trans-sarcolemmal acid efflux rate (J_H) was determined by microepifluorescence in single cells (n=8 to 11 per group), during recovery from intracellular acidosis in bicarbonate-free conditions.

3 Initially, myocytes were subjected to two consecutive acid pulses; these both occurred at 37°C in the normothermic control group but the second pulse was at 25°C in the moderate hypothermia group. J_H values obtained after the first pulse were superimposed in both groups, indicating comparable cell populations. However, after the second pulse, J_H values in the moderate hypothermia group were approximately 50% of those in the normothermic control group over the pH_i range 6.80–7.10.

4 Similar results were obtained in cells subjected to a single acid pulse at 37 or 25°C, with J_H values in the latter group measuring approximately 60% of those in the former over the pH_i range 6.80–7.10.

5 Cariporide (0.01, 0.03, 0.1, 0.3, 1.0 or 3.0 μM), present during recovery from a single acid pulse, reduced J_H in a concentration-dependent manner, with IC_{50} values of 150 and 130 nM at 37 and 25°C, respectively.

6 We conclude that moderate hypothermia produces (1) a significant, but partial, inhibition of sarcolemmal NHE activity, and (2) no significant effect on the NHE-inhibitory potency of cariporide.

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Keywords: Na^+/H^+ exchange; cariporide; hypothermia; temperature; myocyte

Abbreviations: β_i, intrinsic buffering power; cSNARF-1, carboxy-seminaphthorhodafluor-1; GUARDIAN, Guard During Ischaemia Against Necrosis trial; HOE-642, 4-isopropyl-3-methylsulphonylbenzoyl-guanidine methanesulphonate; J_H, rate of acid efflux; NHE, Na^+/H^+ exchange; pH_i, intracellular pH

Introduction

There is substantial pre-clinical evidence that recently-developed, specific Na^+/H^+ exchanger (NHE) inhibitors protect the myocardium during ischaemia and reperfusion (see reviews by Avkiran (1999b) and Karmazyn *et al.* (1999)). Indeed, the degree of protection afforded by NHE inhibition appears to be at least as good as that afforded by ischaemic preconditioning (Avkiran, 1999a; Gumina *et al.*, 1999; Shipolini *et al.*, 1997b). In the vast majority of pre-clinical studies with NHE inhibitors, hearts have been subjected to ischaemia and reperfusion under normothermic conditions, in an attempt to mimic the situation that occurs during spontaneous coronary occlusion and subsequent revascularization in patients with coronary artery disease. Nevertheless, a few studies (Kim *et al.*, 1998a, b; Myers & Karmazyn, 1996; Shipolini *et al.*, 1997a; Yamauchi *et al.*, 1997) have employed global hypothermic ischaemia, as encountered during cardiac surgery and transplantation, and have used NHE inhibitors in combination with established surgical cardioprotection techniques (such as hyperkalaemic cardioplegic arrest), with encouraging findings. For example, our laboratory was the first to show that the specific NHE inhibitor cariporide (HOE-642; 4-isopropyl-3-methylsulphonylbenzoyl-guanidine methanesulphonate), used as an adjunct or additive to crystalloid cardioplegia, provides additional cardioprotective benefit under conditions of both moderate hypothermia (28°C), as encountered during routine cardiac surgery, and severe hypothermia (7.5°C), as used for cardiac preservation for transplantation (Shipolini *et al.*, 1997a). Interestingly, data from the GUARDIAN trial (Théroux *et al.*, 2000) indicate that a subgroup of high-risk patients subjected to iatrogenic myocardial ischaemia during coronary artery bypass graft surgery uniquely benefited from treatment with cariporide.

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Its common occurrence under hypothermic conditions is a potentially important factor that distinguishes iatrogenic myocardial ischaemia during cardiac surgery from myocardial ischaemia that manifests during spontaneous coronary events. Despite the experimental work that has been carried out to determine the cardioprotective efficacy of NHE inhibitors under conditions of normothermic and hypothermic ischaemia, however, the effects of reduced temperature *per se* on sarcolemmal NHE activity and on the potency of NHE inhibitors have not been fully characterized. Therefore, the objectives of the work described here were to determine, in adult rat ventricular myocytes, the effects of moderate hypothermia (25°C) on (1) sarcolemmal NHE activity and (2) the NHE-inhibitory potency of cariporide.

Methods

This investigation was performed in accordance with the Home Office 'Guidance on the Operation of the Animals (Scientific Procedures) Act 1986', published by Her Majesty's Stationery Office, London.

Isolation of ventricular myocytes

Adult male Wistar rats (200–250 g) were anaesthetized with sodium pentobarbitone (60 mg kg^{-1} i.p.) and injected with heparin (50 u i.v.), and their hearts were excised for the isolation of ventricular myocytes by enzymatic digestion, as described previously (Yasutake *et al.*, 1996).

Measurement of pH_i

Intracellular pH (pH_i) was monitored in single myocytes loaded with the pH-sensitive fluoroprobe carboxy-seminaphthorhodafluor-1 (cSNARF-1), using an established microepifluorescence technique (Avkiran & Yokoyama., 2000; Gunasegaram *et al.*, 1999; Haworth *et al.*, 1997; 1999; Snabaitis *et al.*, 2000; Yasutake *et al.*, 1996; Yokoyama *et al.*, 1998; 2000). Calibration of the cSNARF-1 signal was carried out *in situ* at both 37°C (13 cells) and 25°C (10 cells) using nigericin-containing calibration solutions, as described in detail previously (Yasutake *et al.*, 1996). Also as described previously (Yasutake *et al.*, 1996), at the end of each experiment, myocytes were exposed to the pH 7.0 calibration solution and the normalized fluorescence emission ratios were converted to pH_i values by reference to a calibration curve that was obtained by a nonlinear least-squares fit of data to the equation given below.

$$\frac{I_{580}/I_{640}}{(I_{580}/I_{640})_{pH7}} = 1 + a\left[\frac{10^{(pH-pK)}}{1+10^{(pH-pK)}} - \frac{10^{(7-pK)}}{1+10^{(7-pK)}}\right]$$

Estimation of intracellular intrinsic buffering power

Intracellular intrinsic buffering power (β_i) was estimated by stepwise removal of extracellular NH_4Cl at both 37°C (10 cells) and 25°C (11 cells), as described in detail previously (Yasutake *et al.*, 1996). At each step, calculated changes in $[NH_4^+]_i$ and measured changes in pH_i were used to estimate β_i, from the equation $\beta_i = [NH_4^+]_i/pH_i$.

Determination of sarcolemmal NHE activity

The myocytes were maintained in bicarbonate-free Tyrode's solution throughout each experiment, thus enabling the rate of acid efflux (J_H), which was calculated from the equation $J_H = \beta_i \cdot dpH_i/dt$ (where dpH_i/dt is the rate of recovery of pH_i), to be used as an indicator of sarcolemmal NHE activity (Yasutake *et al.*, 1996). J_H values were determined either at pH_i intervals of 0.05 throughout recovery from intracellular acidosis (when studying the effects of temperature) or during the first 60 s after the induction of intracellular acidosis (when studying the effects of cariporide).

Experimental protocols

In initial studies, myocytes (n=9 or 10 per group) were subjected to intracellular acidosis by transient exposure to 20 mM NH_4Cl and its subsequent washout for 8 min (first acid pulse), which was repeated 10–12 min later (second acid pulse) (Avkiran & Yokoyama., 2000; Gunasegaram *et al.*, 1999; Snabaitis *et al.*, 2000; Yasutake *et al.*, 1996; Yokoyama *et al.*, 1998). In both normothermic control and moderate hypothermia groups, the first acid pulse occurred at 37°C and was induced by a 4 min exposure to NH_4Cl. In the normothermic control group, cells were maintained at 37°C throughout the experiment and the second acid pulse was induced under identical conditions to the first. In contrast, in the moderate hypothermia group, cells were switched to superfusion at 25°C from 10 min before the second acid pulse and were maintained at this temperature thereafter; in this group, the second acid pulse was induced by a 6 min exposure to NH_4Cl. In subsequent experiments, cells (n=8 to 11 per group) were subjected to a single acid pulse at either 37 or 25°C by transient (4 min at 37°C, 6 min at 25°C) exposure to 20 mM NH_4Cl and its subsequent washout for 8 min; when used, cariporide (0.01, 0.03, 0.1, 0.3, 1.0 or 3.0 μM) was present throughout NH_4Cl washout.

Data analysis

Data are expressed as mean ± s.e.mean. Experiments within each protocol were randomized. For inter-group comparisons of data, either analysis of variance (for multi-group comparisons) or the unpaired t-test (for comparisons between normothermic control and moderate hypothermia groups) was used. $P<0.05$ was considered significant. Dose-response curves and IC_{50} values were obtained by nonlinear regression analysis, using GraphPad Prism software.

Results

Effects of moderate hypothermia on cSNARF-1 calibration

The *in situ* calibration curves obtained at 37 and 25°C are shown in Figure 1. As illustrated, moderate hypothermia altered the pH_i-dependence of the fluorescence emission ratio

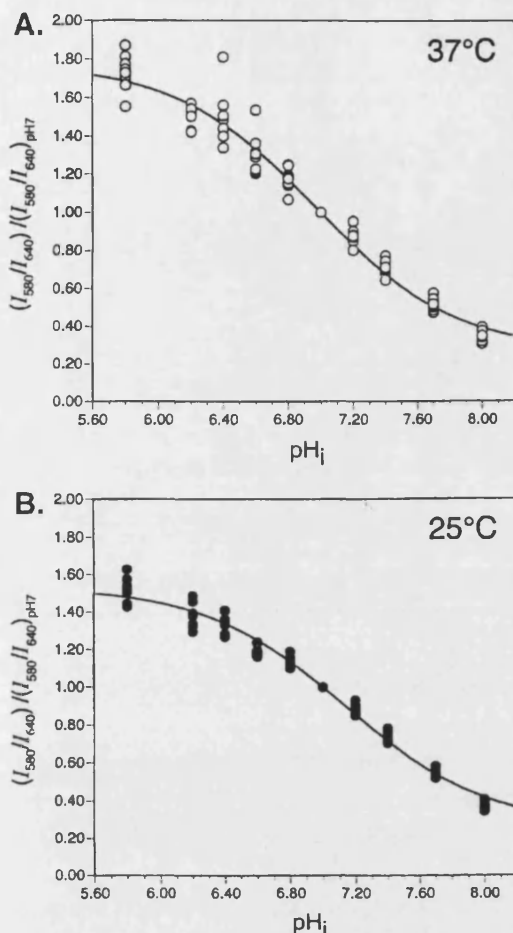


Figure 1 Calibration curves constructed using normalized I_{580}/I_{640} ratio data obtained during exposure of adult rat ventricular myocytes to nigericin-containing calibration solutions at (A) 37°C (n = 13 cells) or (B) 25°C (n = 10 cells). See text for details.

of cSNARF-1, with estimated pK values for the fluoroprobe of approximately 7.00 at 37°C and 7.15 at 25°C. A comparable temperature-dependent pK change has been reported previously for another pH-sensitive fluoroprobe, 2 7-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein (Graber *et al.*, 1992). In subsequent experiments, fluorescence emission ratios were converted to pH_i values by reference to the calibration curve obtained at the appropriate temperature (Figure 1).

Effects of moderate hypothermia on β_i

As shown in Figure 2, moderate hypothermia produced a small change in the pH_i-dependence of β_i, such that β_i tended to be greater at 25°C than at 37°C, particularly under non-acidic conditions. For the calculation of J_H in subsequent experiments, β_i was estimated by reference to the β_i-versus-

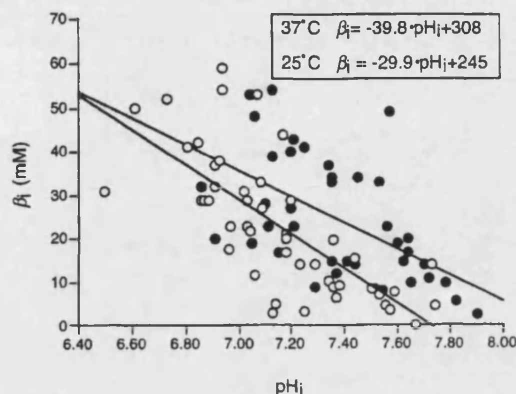


Figure 2 The relationship between intracellular pH (pH_i) and intrinsic buffering power (β_i) in adult rat ventricular myocytes at 37°C (n = 10 cells, open symbols) and 25°C (n = 11 cells, solid symbols). Inset shows the equations obtained by linear regression analysis of the data obtained at each temperature.

pH_i relationship obtained at the appropriate temperature (Figure 2).

Effects of moderate hypothermia on sarcolemmal NHE activity

In the first set of experiments to determine the effect of moderate hypothermia on sarcolemmal NHE activity, two groups of cells were subjected to consecutive acid pulses, after each of which NHE activity was determined. The first pulse was at 37°C in both groups (to confirm comparable cell populations) while the second pulse was at either 37°C (normothermic control group, n = 10) or 25°C (moderate hypothermia group, n = 9). Basal pH_i values at 37°C, obtained prior to the first acid pulse, were comparable in the normothermic control and moderate hypothermia groups, measuring 7.31 ± 0.02 and 7.28 ± 0.04, respectively (NS). Cells in the normothermic control and moderate hypothermia groups acidified to a similar extent during the first acid pulse at 37°C, with minimum pH_i values of 6.62 ± 0.03 and 6.64 ± 0.07, respectively; during the second acid pulse, however, the minimum pH_i was 6.66 ± 0.03 in the normothermic control group but tended to be higher at 6.76 ± 0.06 in the moderate hypothermia group. Figure 3 shows the J_H-versus-pH_i curves obtained after both acid pulses in the two study groups. NHE activity during recovery from the first acid pulse at 37°C was similar in both groups, with comparable J_H values obtained throughout the pH_i range 6.80–7.10 (Figure 3A). For example, J_H at pH_i 6.90 was 3.79 ± 0.75 mM min^{-1} in the normothermic control group and 3.78 ± 0.43 mM min^{-1} in the moderate hypothermia group (NS). In contrast, after the second acid pulse (which was carried out at 37°C in the normothermic control group but at 25°C in the moderate hypothermia group), J_H in the moderate hypothermia group measured approximately 50% of that seen in the normothermic control group throughout the pH_i range 6.80–7.10 (Figure 3B). This difference between the groups arose predominantly from an increase in NHE activity between the two acid pulses in the normothermic

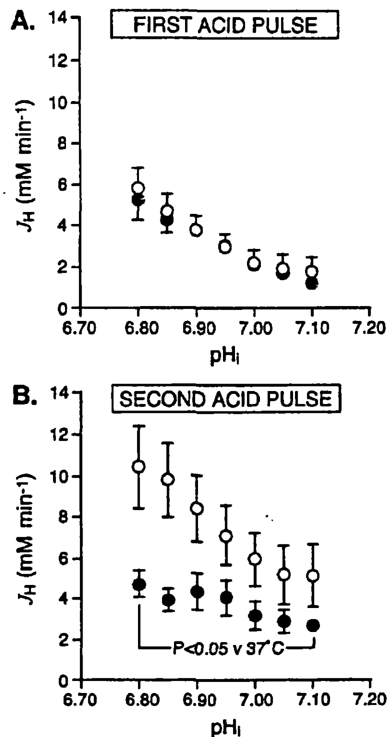


Figure 3 Effect of moderate hypothermia on sarcolemmal NHE activity in adult rat ventricular myocytes subjected to two consecutive acid pulses. Figure shows the J_H-versus-pH$_i$ curves obtained during (A) the first acid pulse and (B) the second acid pulse. In the normothermic control group ($n=10$ cells, open symbols) both acid pulses occurred at 37°C, whereas in the moderate hypothermia group ($n=9$ cells, solid symbols) cells were switched from 37 to 25°C from 10 min before the second acid pulse. The curves were constructed by determining J_H values at pH$_i$ intervals of 0.05 in each cell, throughout recovery from both acid pulses.

control group but not in the moderate hypothermia group. It appears therefore that consecutive acid pulses produce an increase in sarcolemmal NHE activity in adult rat ventricular myocytes under normothermic conditions and that this increase is inhibited by exposure of the cells to moderate hypothermia.

To determine the effect of moderate hypothermia on NHE activity in the absence of any changes arising from repeated episodes of intracellular acidosis, we next examined NHE activity in two groups of cells ($n=8$ or 9 per group) subjected to a single acid pulse, at either 37 or 25°C. In these experiments, the basal pH$_i$ value obtained prior to the acid pulse was 7.32 ± 0.07 in the normothermic control group ($n=9$) but was significantly higher at 7.46 ± 0.03 in the moderate hypothermia group ($n=8$). The minimum pH$_i$ achieved during the acid pulse was also significantly higher in the moderate hypothermia group (6.83 ± 0.03) relative to the normothermic control group (6.67 ± 0.04). Figure 4 shows the J_H-versus-pH$_i$ curves obtained in both study groups; as can be seen, the curve was shifted to the left under conditions of moderate hypothermia, reflecting significantly lower NHE activity

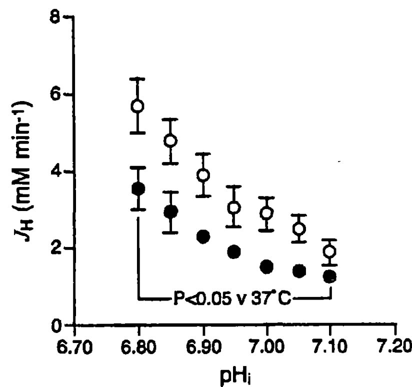


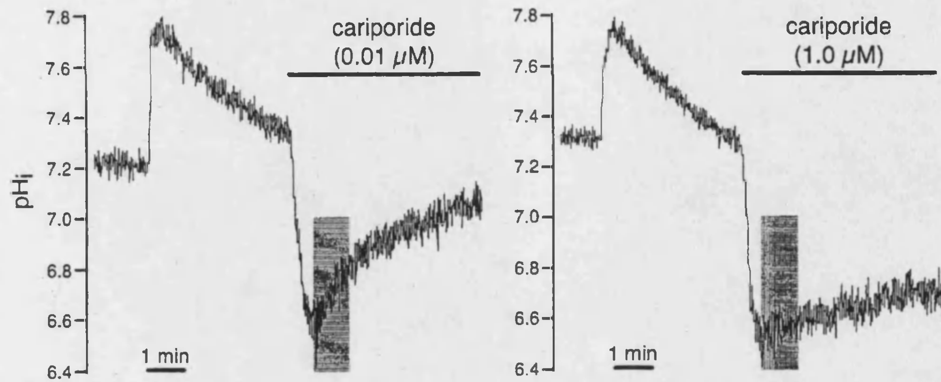
Figure 4 Effect of moderate hypothermia on sarcolemmal NHE activity in adult rat ventricular myocytes subjected to a single acid pulse. Figure shows the J_H-versus-pH$_i$ curves for the normothermic control group ($n=9$ cells, open symbols), in which the acid pulse occurred at 37°C, and the moderate hypothermia group ($n=8$ cells, solid symbols), in which the acid pulse occurred at 25°C. The curves were constructed by determining J_H values at pH$_i$ intervals of 0.05 in each cell, throughout recovery from the acid pulse.

throughout the pH$_i$ range 6.80–7.10. For example, J_H at pH$_i$ 6.90 was 3.91 ± 0.55 mM min^{-1} in the normothermic control group but measured only 59% of that, at 2.32 ± 0.24 mM min^{-1}, in the moderate hypothermia group ($P<0.05$).

Effects of moderate hypothermia on the NHE-inhibitory potency of cariporide

In this protocol, we sought to determine whether the NHE-inhibitory potency of cariporide is altered under conditions of moderate hypothermia. Twelve groups of cells ($n=8$ to 11 per group) were again subjected to a single acid pulse at either 37 or 25°C, with cariporide (0.01–3.0 μM) present in the superfusion solution throughout the recovery phase. Since cariporide inhibits recovery from intracellular acidosis, J_H was determined only at the nadir of the acid pulse, as illustrated in Figure 5. The basal pH$_i$ value obtained prior to the acid pulse was 7.25 ± 0.01 in the cells studied at 37°C ($n=52$) and again was significantly higher at 7.45 ± 0.02 in the cells studied at 25°C ($n=53$). The minimum pH$_i$ value, obtained upon NH_4Cl washout, was 6.64 ± 0.02 in the cells studied at 37°C ($n=52$) and was also significantly higher at 6.72 ± 0.02 in the cells studied at 25°C ($n=53$). At each temperature, however, there was no significant difference in either the basal pH$_i$ or the minimum pH$_i$ between the six groups that received the different concentrations of cariporide (Table 1).

Figure 6A shows the J_H values obtained in the various study groups at 37 and 25°C. As can be seen, in the presence of each concentration of cariporide, the J_H obtained at 25°C was approximately 50–60% of that obtained at 37°C, which is consistent with our observations above. At both temperatures, cariporide produced a concentration-dependent reduction in J_H (Figure 6A). Notably, even in the presence of 3.0 μM cariporide, J_H was not completely abolished, most likely due to residual Na^+/HCO_3^- cotransport activity (Wu



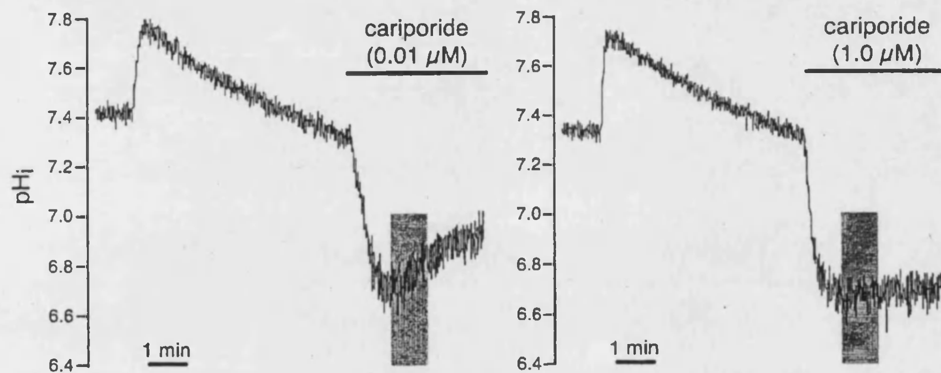


Figure 5 Effect of cariporide on recovery from intracellular acidosis in adult rat ventricular myocytes. Figure shows representative pH_i recordings obtained in cells exposed to a low, non-inhibitory concentration (0.01 μM) or a high, inhibitory concentration (1.0 μM) of cariporide during acid pulses carried out under conditions of (A) normothermia (37°C) or (B) moderate hypothermia (25°C). Shaded areas indicate the period during which J_H was estimated from the rate of recovery of pH_i.

Table 1 Basal and minimum pH_i values in cells exposed to cariporide at 37 or 25°C.

Cariporide		*37°C*			*25°C*	
(μM)	n	*basal* pH_i	*min* pH_i	n	*basal* pH_i	*min* pH_i
0.01	9	7.23±0.03	6.62±0.03	9	7.44±0.03	6.78±0.03
0.03	8	7.23±0.03	6.66±0.06	9	7.51±0.04	6.75±0.05
0.1	9	7.25±0.04	6.59±0.03	8	7.41±0.04	6.68±0.06
0.3	8	7.29±0.04	6.70±0.04	8	7.47±0.06	6.74±0.04
1.0	9	7.31±0.03	6.67±0.03	11	7.45±0.04	6.72±0.03
3.0	9	7.22±00.3	6.58±0.02	8	7.43±0.03	6.66±0.03

For each group, *n* indicates the number of cells studied. In each cell, basal and minimum pH_i values were noted immediately before exposure to and immediately after washout of NH_4Cl, respectively.

et al., 1994). Figure 6B illustrates the dose-response curves for NHE inhibition by cariporide, obtained after the correction of J_H values for residual Na^+/HCO_3^- cotransport activity, at both 37 and 25°C. As can be seen, moderate hypothermia did not have a significant effect on the NHE-inhibitory potency of cariporide, with IC_{50} values of approximately 150 nM at 37°C and 130 nM at 25°C.

Discussion

Although several studies have attempted to determine the effects of moderate hypothermia (20–30°C) on plasma membrane NHE activity, their findings have been somewhat contradictory, probably due to the variety of cell types that have been used and the manner in which NHE activity has

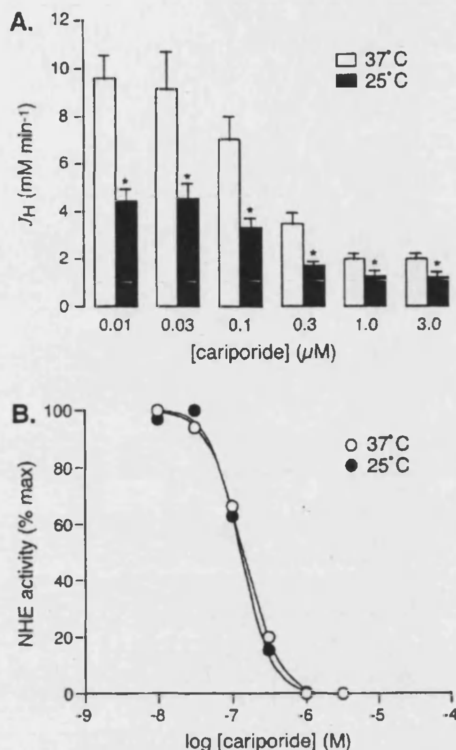


Figure 6 Effect of moderate hypothermia on the NHE-inhibitory potency of cariporide in adult rat ventricular myocytes. Figure shows (A) absolute J_H values and (B) relative NHE activity, in cells subjected to a single acid pulse under conditions of normothermia (37°C, open bars and symbols) or moderate hypothermia (25°C, solid bars and symbols), in the presence of 0.01, 0.03, 0.1, 0.3, 1.0 or 3.0 μM cariporide (n=8 to 11 cells per group). *P<0.05 versus 37°C.

been assessed. In guinea-pig erythrocytes, lowering the temperature from 37 to 20°C has been shown to produce an increase in Na^+ influx that is sensitive to inhibition by the NHE inhibitor amiloride, which is indicative of a hypothermia-induced increase in NHE activity (Zhou & Willis, 1989). More recent work has shown that lowering the temperature from 37 to 27°C induces rapid swelling of rat glial cells in a manner that is inhibited by the amiloride analogue ethylisopropylamiloride, again suggesting increased NHE activity under conditions of moderate hypothermia (Plesnila *et al.*, 2000). In contrast, the rate of swelling of rat lymphocytes following exposure to sodium propionate has been shown to be considerably slower at 22 and 27°C than at 37°C (and to be inhibited at each temperature by the NHE inhibitor FR168888), suggesting a hypothermia-induced decrease in NHE activity (Yamauchi *et al.*, 1997). In the above studies (Plesnila *et al.*, 2000; Yamauchi *et al.*, 1997), NHE activity was surmized from the inhibitory effects of NHE inhibitors on the observed increase in cellular volume and direct measurements of NHE activity (i.e. the rate of NHE-mediated Na^+ influx or H^+ efflux) at known values of pH_i, which is the principal regulator of NHE activity (Wakabayashi *et al.*, 1997), are scarce. In this context, Graber *et al.* (1992) have measured the rate of recovery of pH_i after the induction of an intracellular acid load in opossum kidney cells and shown this to be slower at 25°C than at 37°C. A similar observation has been reported in sheep cardiac Purkinje fibres, upon lowering of the ambient temperature from 37 to 22°C (Ellis & Macleod, 1985). Although these findings may indicate a reduction in NHE activity in the presence of moderate hypothermia, it is notable that, in both studies, the rate of recovery of pH_i was measured at a different level of intracellular acidosis under conditions of normothermia versus moderate hypothermia.

The present study is the first detailed characterization of the effects of moderate hypothermia on sarcolemmal NHE activity in adult mammalian ventricular myocytes, and demonstrates a significant inhibition of such activity upon lowering of the ambient temperature from 37 to 25°C. Notably, this inhibition is not absolute, such that at 25°C sarcolemmal NHE activity is retained at approximately 50–60% of that observed at 37°C. A recent preliminary report indicates that moderate hypothermia (27°C) may produce a similar degree of inhibition of sarcolemmal NHE activity in guinea-pig ventricular myocytes also (Ch'en & Vaughan-Jones, 2000). Interestingly, other evidence in the literature suggests that pathophysiologically significant sarcolemmal NHE activity may be retained even under conditions of severe hypothermia (<20°C). Thus, Na^+ has been shown to accumulate intracellularly during 6 h storage of embryonic chick cardiac myocytes at 10°C (Knerr & Lieberman, 1993) and 12 h storage of adult rat hearts at 4°C (Askenasy *et al.*, 1996) in a manner that was significantly attenuated by the NHE inhibitor ethylisopropylamiloride.

In our experiments that employed two consecutive acid pulses, there was a marked increase in sarcolemmal NHE activity after the second acid pulse relative to the first, when both pulses occurred at 37°C (Figure 3). In contrast, no such increase in NHE activity was observed when the second pulse was at 25°C (Figure 3). In our previous studies that used similar 2-pulse protocols at 34°C (Avkiran & Yokoyama, 2000; Gunasegaram *et al.*, 1999; Snabaitis *et al.*, 2000; Yasutake *et al.*, 1996; Yokoyama *et al.*, 1998), there was only a small (<30%) increase in NHE activity after the second acid pulse (in the absence of any other intervention) and this increase was not statistically significant. It appears therefore that repeated episodes of intracellular acidosis can lead to increased sarcolemmal NHE activity, through a mechanism that is very sensitive to the ambient temperature. It would be of interest to determine the role of altered activity of NHE-regulatory signalling pathways (such as the protein kinase C and extracellular signal regulated kinase pathways (Moor & Fliegel, 1999; Snabaitis *et al.*, 2000)) in such stimulation of sarcolemmal NHE activity by repeated episodes of acidosis. Regardless of the precise mechanisms underlying this interesting phenomenon, however, it is important to note that a similar reduction in sarcolemmal NHE activity by moderate hypothermia was evident also when cells were exposed to a single acid pulse (Figure 4). Therefore, it is likely that this negative effect of moderate hypothermia arose principally from the inhibition of sarcolemmal NHE activity *per se* rather than the attenuation of its stimulation by repeated episodes of intracellular acidosis.

The present work has also revealed that cariporide inhibits sarcolemmal NHE activity with comparable potency at 25

and 37°C, with an IC_{50} of 130–150 nM under each condition. Such an IC_{50} value is approximately 15 fold greater than that we have previously estimated for this drug in rat ventricular myocytes (Shipolini *et al.*, 1997b). However, in our earlier study (Shipolini *et al.*, 1997b), intracellular acidosis to activate the exchanger was induced in the absence of extracellular Na^+, which was reintroduced concomitantly with cariporide. In contrast, in the present study, intracellular acidosis was induced by the washout of NH_4Cl with Tyrode's solution, which contains Na^+ at a concentration of 137 mM. Extracellular Na^+ is known to antagonize competitively the binding of benzoylguanidine-based inhibitors such as cariporide to NHE (Baumgarth *et al.*, 1998), which is likely to underlie the different IC_{50} values obtained in our studies. Indeed, in guinea-pig ventricular myocytes, the IC_{50} for HOE-694 (another benzoylguanidine-based NHE inhibitor that is closely related to cariporide structurally) has been estimated to be approximately 16 fold greater in the presence of an extracellular Na^+ concentration of 150 mM, relative to the value obtained in the virtual absence of extracellular Na^+ (Loh *et al.*, 1996).

An interesting observation in the present study was the difference in basal pH_i under conditions of normothermia versus moderate hypothermia, such that this was 0.15–0.20 pH unit greater at 25°C than at 37°C. To our knowledge, this is the first report of this phenomenon in isolated ventricular myocytes, although similar effects of moderate hypothermia have been reported previously in sheep Purkinje fibres (pH_i increase of 0.21 (Ellis & Macleod, 1985) or 0.31 (Bright & Ellis, 1994) on reducing temperature from 35 to 21–22°C), isolated rat hearts (pH_i increase of 0.16 on reducing temperature from 36 to 20°C (Gruwel *et al.*, 1998)) and sheep myocardium *in vivo* (pH_i increase of 0.19 on reducing temperature from 37 to 26°C (Swain *et al.*, 1991)). Although the precise mechanism(s) underlying this increase in steady-state pH_i have not been determined, hypothermia-induced changes in the pK of intracellular buffers, such as the imidazole moiety of histidine, are likely to play an important role (Roos & Boron, 1981). In this context, it is notable that the pK of imidazole has been estimated to be 6.75 at 37°C but to increase to 7.30 at 25°C (Durand *et al.*, 1998). On the basis that a low level of sarcolemmal NHE activity appears to be retained under steady-state conditions in ventricular myocytes (Leem *et al.*, 1999), our data suggest that the inhibition of such activity may also contribute to the increase in basal pH_i during exposure to moderate hypothermia.

Previous data in sheep Purkinje fibres suggest that, under conditions of moderate hypothermia, the higher steady-state pH_i is associated with an attenuated level of intracellular acidification in response to the NH_4Cl pulse (Ellis & Macleod, 1985). Since pH_i is a critical determinant of NHE activity (Wakabayashi *et al.*, 1997), we attempted to compensate for this and obtain comparable levels of intracellular acidosis in the normothermic control and moderate hypothermia groups, by extending the duration of the NH_4Cl pulse from 4 min at 37°C to 6 min at 25°C. This approach was only partially successful, however, in that the minimum pH_i achieved at 25°C remained approximately 0.10 pH unit higher than that at 37°C. This difference is unlikely to contribute to the lower NHE activity observed at 25°C (Figures 3 and 4), since J_H values were compared at identical pH_i values in the two groups. Nevertheless, in the cariporide study, where J_H was determined at the nadir of the acid pulse, a higher minimum pH_i value may have contributed to the lower NHE activity at 25°C. Indeed, in the presence of a non-inhibitory concentration of cariporide (0.01 μM), J_H at 25°C was only 45% of that at 37°C (Figure 6A). In contrast, when the comparison was made at identical values of pH_i in a similar protocol in the absence of cariporide (Figure 4), hypothermia-induced inhibition of sarcolemmal NHE activity was somewhat attenuated, with J_H values at 25°C measuring approximately 60% of those at 37°C.

The temperature-independence of the NHE-inhibitory potency of cariporide, at least within the temperature range that we have studied, suggests that this agent is likely to retain its cardioprotective efficacy under moderately hypothermic conditions. This is indeed borne out by our earlier work in isolated rat hearts (Shipolini *et al.*, 1997a), which revealed that the use of cariporide as an additive to crystalloid cardioplegia improved the recovery of contractile function and reduced the leakage of creatine kinase following 120 min of global ischaemia at 28°C. This property is potentially important in relation to the application of cariporide for surgical myocardial protection and distinguishes this agent from other ion transport inhibitors, such as L-type calcium channel blockers. In this context, unlike cariporide (Shipolini *et al.*, 1997a), verapamil (Hearse *et al.*, 1984) and nifedipine (Fukunami & Hearse, 1985) have been shown to provide no significant cardioprotective benefit in isolated rat hearts when used as an additive to hyperkalaemic cardioplegia under conditions of moderate hypothermia, although both were effective at temperatures >30°C.

In conclusion, our work in isolated adult rat ventricular myocytes has shown that moderate hypothermia (25°C) produces a significant, but only partial, inhibition of sarcolemmal NHE activity. Furthermore, the NHE-inhibitory potency of cariporide is not affected by such a reduction in temperature. These findings may help provide a mechanistic basis for the previously demonstrated ability of cariporide to protect ischaemic myocardium under conditions of moderate hypothermia and suggest that this effect is likely to arise from the inhibition of retained NHE activity.

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Regulation of sarcolemmal Na^+/H^+ exchange by hydrogen peroxide in adult rat ventricular myocytes

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Abstract

Objective: To characterise the effects of exogenous H_2O_2 on sarcolemmal Na^+/H^+ exchanger (NHE) activity and determine the roles of extracellular signal-regulated kinase (ERK), p38 mitogen-activated protein kinase (p38 MAPK) and protein kinase C (PKC) in observed effects. **Methods:** Sarcolemmal H^+ efflux rate (J_H) was determined by microepifluorescence at a pH_i of 6.70 in adult rat ventricular myocytes, after two consecutive acid pulses in HCO_3^--free medium; before the second pulse, cells (n=7–10/group) were exposed to H_2O_2 or vehicle and the change in J_H (ΔJ_H) was used to quantify the change in NHE activity. ERK and p38 MAPK activities were determined by immunoblotting with phosphospecific antibodies. **Results:** Relative to control, ΔJ_H was increased by a 10-min exposure to 100, but not 1 or 10 μM H_2O_2 (1000 μM was not tolerated); 3 or 6 min exposure to 100 μM H_2O_2 was without effect. ERK and p38 MAPK activities were both increased by 100 μM H_2O_2 (peak at 6 min); the ERK kinase inhibitor PD98059 (10 μM), but not the p38 MAPK inhibitor SB203580 (1 μM), inhibited the H_2O_2-induced increase in ΔJ_H. H_2O_2-induced ERK activation was inhibited not only by PD98059 (10 μM), but also by the non-selective tyrosine kinase inhibitor genistein (3–100 μM), the EGF receptor kinase inhibitor AG1478 (3–300 nM) and the Src family kinase inhibitor PP2 (0.1–10 μM). The PKC inhibitors GF109203X (0.3–10 μM) and chelerythrine (1–30 μM) were without effect on ERK activation, although the former abolished the H_2O_2-induced increase in ΔJ_H. **Conclusions:** Our data demonstrate that, in adult rat ventricular myocytes, (i) hydrogen peroxide stimulates sarcolemmal NHE activity, (ii) this response requires activation of ERK and PKC, but not p38 MAPK, (iii) ERK activation occurs through tyrosine kinase-mediated, but PKC-independent, mechanisms © 2002 Elsevier Science B.V. All rights reserved.

Keywords: Free radicals; Myocytes; Na/H-exchanger; Signal transduction; Protein kinases

1. Introduction

The sarcolemmal Na^+/H^+ exchanger (NHE) is encoded by the NHE-1 gene [1] and contributes to the integrated control of intracellular pH (pH_i) in cardiac myocytes [2]. Basal activity of the sarcolemmal NHE is low under physiological conditions [2] but increased exchanger activity and resultant increases in intracellular Na^+ and/or pH_i may mediate the inotropic responses of myocardium to neurohormonal stimuli [3–6] and myocardial stretch [7]. With respect to cardiac pathophysiology, sarcolemmal NHE activity may play a permissive role in the hypertrophic response of cardiac myocytes to neurohormonal [8] and mechanical [9] stimuli in vitro and in the adverse ventricular remodelling that occurs following myocardial infarction in vivo [10–12]. Perhaps the strongest experimental evidence for a pathophysiological role for the sarcolemmal NHE, however, is that which implicates the exchanger in the development of myocardial injury and dysfunction during ischaemia and reperfusion (see reviews by Avkiran [13] and Karmazyn et al. [14]). Importantly, recent studies suggest that NHE inhibition by the NHE-1-selective inhibitor cariporide may provide cardioprotective benefit in certain clinical settings also, such as in high-risk patients who are subjected to elective myocardial ischaemia and reperfusion during coronary artery bypass graft surgery [15].

There is strong evidence that oxidative stress also

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contributes to the development of myocardial injury and dysfunction during ischaemia and reperfusion [16,17]. Intriguingly, data from Karmazyn's laboratory suggest that the adverse myocardial effects of oxidative stress (induced by exposure to H_2O_2), when applied either alone [18] or in combination with ischaemia and reperfusion [19], are attenuated by NHE inhibition. Although sarcolemmal NHE activity was not determined in either study, these findings raise the possibility that an increase in such activity may mediate some of the detrimental consequences of exposure to H_2O_2, or that NHE inhibitors (or the consequences of NHE inhibition) may enhance myocardial tolerance to such oxidative stress. Recent data from neonatal rat ventricular myocytes are consistent with the former possibility [20]. However, the effects of oxidative stress induced by H_2O_2 on sarcolemmal NHE activity in *adult* myocardium and the signalling mechanism(s) underlying any such effects are unknown. Notably, qualitative and quantitative changes are known to occur during post-natal development in the myocardial expression of NHE-regulatory signalling molecules, such as ERK [21] and protein kinase C (PKC) [22–24]. Furthermore, the basal expression and activity of NHE differs significantly between neonatal and adult rat ventricular myocytes [25]. Thus, the regulation of NHE activity by H_2O_2 may differ between adult and neonatal myocardium.

The present study used adult rat ventricular myocytes to characterise the time- and dose-dependent effects of H_2O_2 on the activities of the sarcolemmal NHE and the putative NHE-regulatory signalling pathways mediated by ERK and p38 MAPK. Subsequently, any causal link between the observed changes was probed through the use of specific inhibitors of ERK kinase (MEK) and p38 MAPK. Finally, we explored the proximal signalling mechanisms that are responsible for H_2O_2-induced ERK activation.

2. Materials and methods

The present investigation was performed in accordance with the *Home Office Guidance on the Operation of the Animals (Scientific Procedures) Act 1986*, published by HMSO, London.

2.1. Isolation of ventricular myocytes

Ventricular myocytes were isolated from the hearts of adult male Wistar rats (200–250 g) using a collagenase-based enzymatic digestion technique that provides a yield of >80% rod-shaped myocytes, as described previously [26].

2.2. Determination of sarcolemmal NHE activity

Sarcolemmal NHE activity was determined in quiescent single myocytes loaded with the pH-sensitive fluoroprobe cSNARF-1, using a microepifluorescence technique [26–29]. Cells were maintained in HCO_3^--free medium (34°C) throughout each experiment, thus enabling the rate of acid efflux (J_H) to be used as the indicator of sarcolemmal NHE activity. J_H was determined at a pH_i of 6.70 during recovery from intracellular acidosis before and after exposure to H_2O_2, and the change in J_H (ΔJ_H) used to quantify H_2O_2-induced changes in NHE activity.

2.3. Determination of cellular MAPK, p90 ribosomal S6 kinase ($p90^{rsk}$) and Src tyrosine kinase activities

Protein samples (40 μg) from whole cell lysates were separated by SDS–PAGE on 9% polyacrylamide gels. MAPK activities were determined through the detection of dual phosphorylation of ERK1/2 and p38 MAPK by immunoblot analysis using phosphospecific antibodies (New England BioLabs, MA), as described previously [27,28]. Activities of $p90^{rsk}$ and Src tyrosine kinase were determined by the detection of Ser^{381} or Tyr^{416} phosphorylation, respectively, using phosphospecific antibodies (New England BioLabs) [27,28]. To confirm equal protein loading, non-phosphospecific antibodies for ERK2, p38, $p60^{Src}$ (all from Santa Cruz Biotechnology, CA) and $p90^{rsk}$ (Transduction Laboratories, KY) were used. Specific protein bands were detected by enhanced chemiluminescence and autoradiography, and phosphorylation status was quantified by using a LKB 2222 Bromma Ultroscan XL laser densitometer.

2.4. Determination of cellular MAPK-activated protein kinase-2 (MAPKAPK-2) activity

The inhibitory effect of SB203580 on the cellular activity of p38 MAPK was determined by immunoprecipitating MAPKAPK-2 (which is phosphorylated and activated by p38 MAPK) from cell lysates and subjecting it to an in vitro kinase assay (Upstate Biotechnology, NY). In this assay, activity of MAPKAPK-2 is determined by incubation of the immune complex with the specific MAPKAPK-2 substrate peptide KKLNRTLSVA in a reaction mixture containing [γ-^{32}P]:ATP and quantifying the resulting incorporation of ^{32}P into the peptide by scintillation counting [30].

2.5. Experimental protocols

For determination of H_2O_2-induced changes in NHE activity, myocytes were subjected to intracellular acidosis by transient exposure to 20 mM NH_4Cl (first acid pulse), which was repeated 17–27 min later (second acid pulse). Dose-dependent effects of H_2O_2 were assessed by exposing cells to vehicle or 1, 10, 100 or 1000 μM H_2O_2 (Sigma–Aldrich, Poole, UK) from 10 min before the second acid pulse (see Fig. 1A). Time-dependent effects

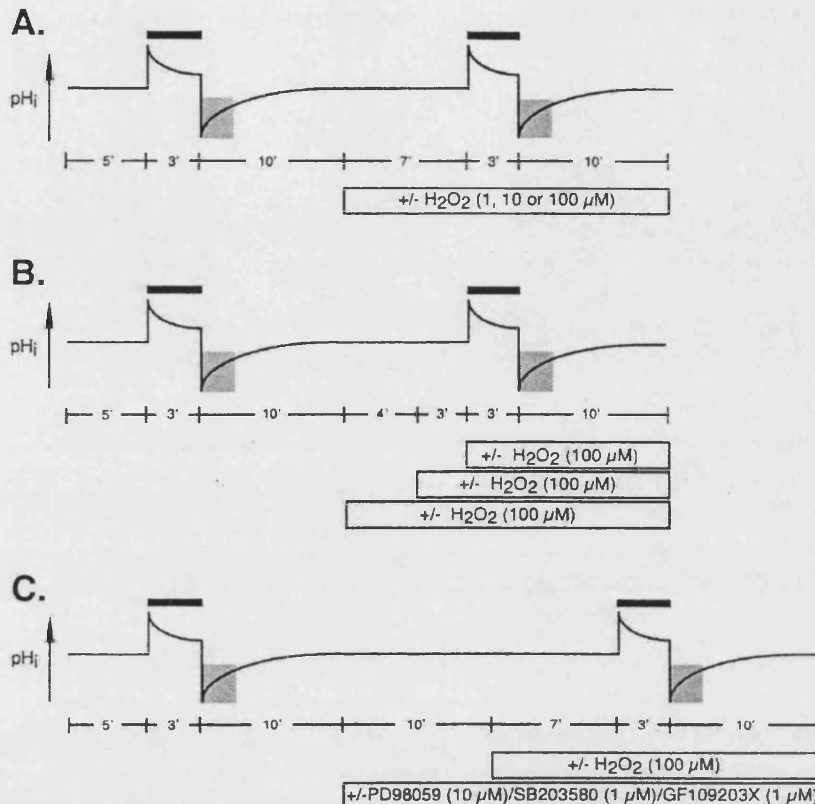

Fig. 1. Experimental protocols used to determine the effects of H_2O_2 on sarcolemmal Na^+/H^+ exchanger activity. Protocols are shown for the determination of (A) dose-dependent, (B) time-dependent and (C) protein kinase-mediated effects of H_2O_2 on sarcolemmal Na^+/H^+ exchanger activity in adult rat ventricular myocytes. Solid bars represent the periods of exposure to 20 mM NH_4Cl, whose washout produced intracellular acidosis; J_H was determined during recovery from such acidosis (shaded areas).

were assessed by exposing cells to vehicle or 100 μM H_2O_2 from 3, 6 or 10 min before the second acid pulse (see Fig. 1B). When studying the effects of the MEK inhibitor PD98059 (10 μM), the p38 MAPK inhibitor SB203580 (1 μM) or the PKC inhibitor GF109203X (1 μM) (all from Calbiochem–Novabiochem, Nottingham, UK), the kinase inhibitor was present from 10 min before the start of exposure to 100 μM H_2O_2 (see Fig. 1C). The concentrations of PD98059 and GF109203X were selected on the basis of our earlier work on receptor-mediated NHE regulation in the same preparation [27,28] and the concentration of SB203580 was selected since it fully inhibits p38 MAPK activity while not affecting other kinase pathways in neonatal myocytes [31].

For the determination of H_2O_2 effects on kinase activity ($n=4$ experiments in each protocol, with each experiment using cells from a separate heart), myocytes in suspension were exposed to H_2O_2, using identical concentrations and exposures to those described above; in time-response studies, duration of H_2O_2 exposure was additionally extended to 20 and 30 min. When studying the effects of PD98059 (10 μM) or SB203580 (1 μM) on H_2O_2-induced kinase activation, this was present from 10 min before the start of exposure to 100 μM H_2O_2 (10 min), as above. When studying the effects of PKC inhibitors (GF109203X and chelerythrine), the non-selective tyrosine kinase inhibitor genistein, the Src family kinase inhibitor PP2 and the epidermal growth factor (EGF) receptor kinase inhibitor AG1478 (all from Calbiochem-Novabiochem) on H_2O_2-induced kinase activation, various concentrations of each inhibitor were present from 30 min before the start of exposure to 100 μM H_2O_2 (10 min).

Stock solutions of all kinase inhibitors were dissolved in DMSO, which was also included in superfusion solutions (at its final concentration of 0.05%) in control and H_2O_2-only experiments, for the appropriate periods.

2.6. Statistical analysis

Data are mean±S.E.M. For the microepifluorescence

experiments, cells from one heart were used on each day and these were allocated in a randomised manner to the experimental groups of the protocol under study; eight to 10 separate hearts were required to complete each protocol (see figure legends), within which each experimental group comprised seven to 10 myocytes. The biochemical experiments were repeated four times, each time using myocytes from a separate heart. For inter-group comparison of ΔJ_H or protein kinase phosphorylation, data were subjected to ANOVA; further analysis was by Dunnett's test, to compare each treatment group with the control group. $P<0.05$ was considered significant.

3. Results

3.1. *Effects of H_2O_2 on sarcolemmal NHE activity*

3.1.1. *Dose–response studies*

In control cells that were exposed to vehicle, there was little change in J_H during the second acid pulse relative to the first, as reflected by a ΔJ_H of around zero (Fig. 2A). Relative to the control group, a 10-min exposure to 100 μM H_2O_2 produced a significant increase in ΔJ_H; in contrast, ΔJ_H was not changed significantly by a similar exposure to 1 or 10 μM H_2O_2 (Fig. 2A). Following a 10-min exposure to 1000 μM H_2O_2, cells became hypercontracted and detached from the cover slip during the second acid pulse; thus, ΔJ_H could not be determined in this group. These data indicate that H_2O_2 stimulates sarcolemmal NHE activity in a dose-dependent manner, such that >10 μM H_2O_2 is required to achieve a significant effect.

3.1.2. *Time–response studies*

These experiments were performed to delineate the temporal profile of H_2O_2-induced stimulation of NHE activity. Consistent with the above results, a 10-min exposure to 100 μM H_2O_2 again produced a significant increase in ΔJ_H (Fig. 2B). However, when exposure time was limited to 3 or 6 min, there was no significant change in ΔJ_H in response to 100 μM H_2O_2 (Fig. 2B). These data indicate that H_2O_2-induced stimulation of sarcolemmal NHE activity requires an exposure time of >6 min.

3.2. *Effect of H_2O_2 on cellular ERK and p38 MAPK activity*

We next determined the dose- and time-dependent effects of H_2O_2 on ERK and p38 MAPK activity, to test whether either pathway is activated in a manner that is consistent with a potential NHE-regulatory role. Exposure of cells to 1–1000 μM H_2O_2 for 10 min produced dose-dependent activation of both ERK1/2 and p38 MAPK, with significant increases in activity achieved with 100 μM H_2O_2 (Fig. 3A). These data also showed that in adult rat

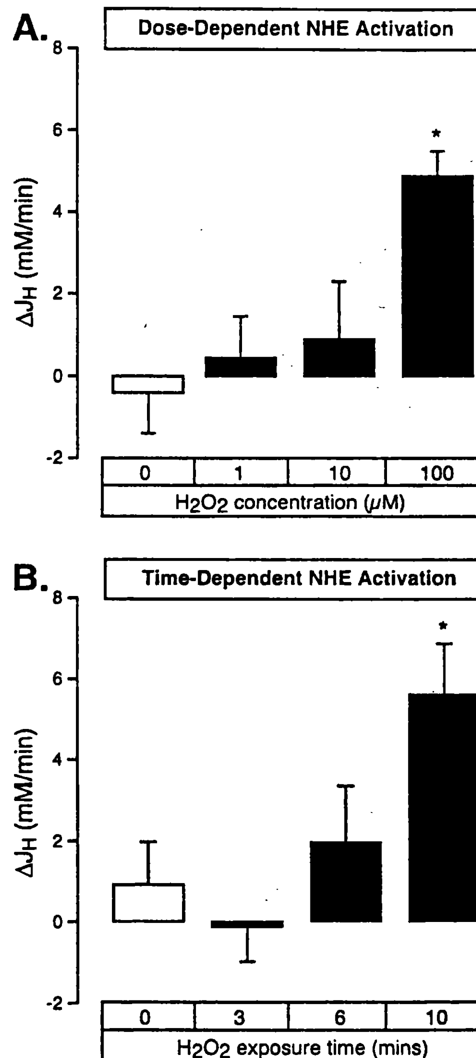

Fig. 2. Dose- and time-dependent effects of H_2O_2 on sarcolemmal Na^+/H^+ exchanger activity. The change in J_H (ΔJ_H) during the second acid pulse relative to the first is shown in (A) cells exposed to vehicle or 1, 10 or 100 μM H_2O_2 from 10 min before the second acid pulse, and (B) cells exposed to vehicle or 100 μM H_2O_2 from 3, 6 or 10 min before the second acid pulse. *$P<0.05$ versus control (seven to 10 cells per group, obtained from 10 hearts).

ventricular myocytes, ERK2 (cf. ERK1) was the predominant isozyme detected by the ERK1/2 phosphospecific antibody with readily detectable basal phosphorylation in the untreated control cells. Therefore, subsequent quantitation of the effects of H_2O_2 on ERK activity was based on the change in the phosphorylation status of ERK2. The temporal profiles indicated that peak ERK and p38 MAPK activation occurred after 6 min of exposure to 100 μM H_2O_2, and that such activation was sustained for

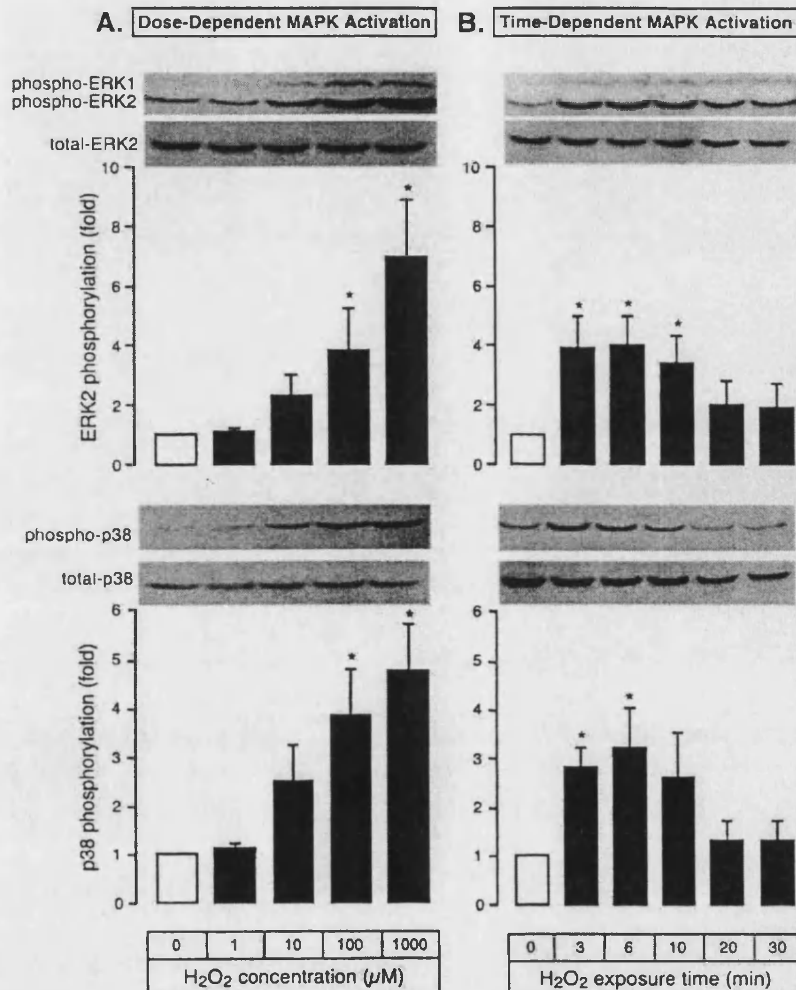

Fig. 3. Dose- and time-dependent effects of H_2O_2 on ERK and p38-MAPK activity. Cells were exposed to (A) vehicle or 1, 10, 100 and 1000 μM H_2O_2 for 10 min, and (B) vehicle or 100 μM H_2O_2 for 3, 6, 10, 20 or 30 min. Autoradiograms show representative Western blots with phosphospecific (top panel) and non-phosphospecific (bottom panel) for ERK and p38-MAPK antibodies. *$P<0.05$ versus control (four experiments, with cells from four hearts).

approximately 10 min (Fig. 3B). The concordance between the dose- and time-dependence characteristics of H_2O_2-induced activation of ERK and p38 MAPK and H_2O_2-induced stimulation of sarcolemmal NHE activity suggests that either kinase may play a role in the latter response.

3.3. Role of ERK and p38 MAPK in H_2O_2-induced stimulation of sarcolemmal NHE activity

This protocol was designed to test whether activation of the ERK and/or the p38 MAPK pathway was necessary for H_2O_2-induced stimulation of sarcolemmal NHE activity. In the absence of any pre-treatment, a 10-min exposure to 100 μM H_2O_2 again produced a significant increase in ΔJ_H (Fig. 4A). This effect was abolished by pre-treatment of cells with the MEK inhibitor PD98059 but was unaffected by pre-treatment with the p38 MAPK inhibitor SB203580 (Fig. 4A). The lack of effect of SB203580 was not due to inadequate inhibition of p38 MAPK activity, since H_2O_2-induced activation of MAPKAPK-2 was abolished by the SB203580 pre-treatment protocol (Fig. 4B). These data indicate that H_2O_2-induced stimulation of sarcolemmal NHE activity requires activation of the ERK but not the p38 MAPK pathway.

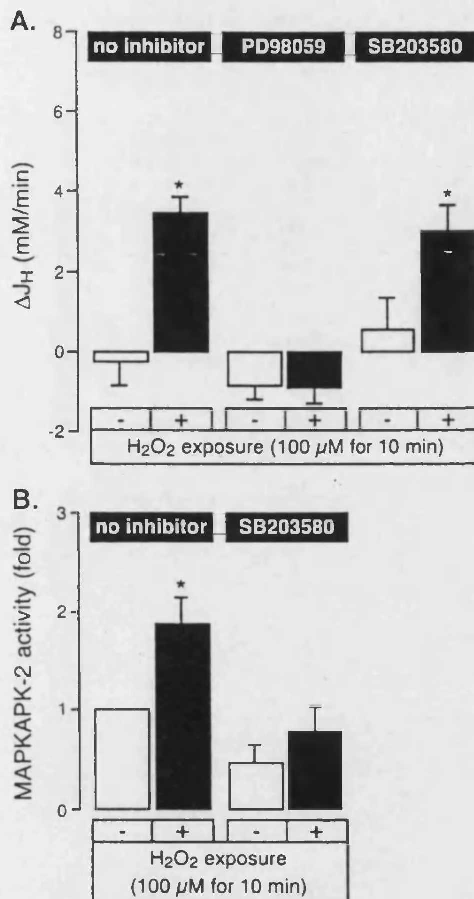

Fig. 4. Effects of MAPK inhibition on H_2O_2-induced increases in sarcolemmal Na^+/H^+ exchanger and cellular MAPKAPK-2 activities. In (A), cells were subjected to two consecutive acid pulses. The change in J_H (ΔJ_H) during the second acid pulse relative to the first is shown in cells exposed to vehicle or 100 μM H_2O_2 from 10 min before the second acid pulse, in the absence or presence of pre-treatment with the MEK inhibitor PD98059 (10 μM) or the p38 MAPK inhibitor SB203580 (1 μM). *$P<0.05$ versus control (seven to 10 cells per group, obtained from 10 hearts). In (B), cells were exposed to vehicle or 100 μM H_2O_2 for 10 min in the absence or presence of pre-treatment with the p38 MAPK inhibitor SB203580 (1 μM). *$P<0.05$ versus control (four experiments, with cells from four hearts).

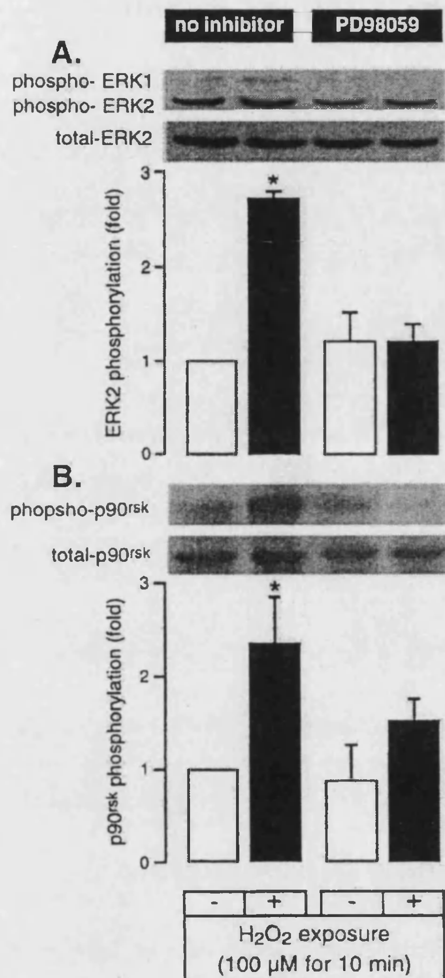

Fig. 5. Effects of MEK inhibition on H_2O_2-induced increases in ERK and $p90^{rsk}$ activity. Cells were exposed to vehicle or 100 μM H_2O_2 for 10 min in the absence or presence of pre-treatment with the MEK inhibitor PD98059 (10 μM). Autoradiograms show representative Western blots with phosphospecific (top panel) and non-phosphospecific (bottom panel) antibodies for (A) ERK and (B) $p90^{rsk}$. *$P<0.05$ versus control (four experiments, with cells from four hearts).

To explore whether the inhibitory effect of PD98059 on NHE stimulation occurred through the predicted mechanism of MEK inhibition, we next determined its effect on ERK activity. As shown in Fig. 5, a 10-min exposure to 100 μM H_2O_2 activated not only ERK (thus confirming our earlier observation) but also its downstream substrate $p90^{rsk}$, which is a putative NHE-1 kinase [32,33]. PD98059 abolished H_2O_2-induced increases in ERK and $p90^{rsk}$ activity, whilst having no effect on basal activity (Fig. 5). These data support an effector role for ERK and/or $p90^{rsk}$ in H_2O_2-induced stimulation of sarcolemmal NHE activity.

3.4. Role of PKC in H_2O_2-induced stimulation of sarcolemmal NHE activity

Our previous work has shown that, in addition to ERK/$p90^{rsk}$ activation, PKC activation is also necessary for the stimulation of sarcolemmal NHE activity through α_{1A}-adrenergic [28] and angiotensin II AT_1 [27] receptors.

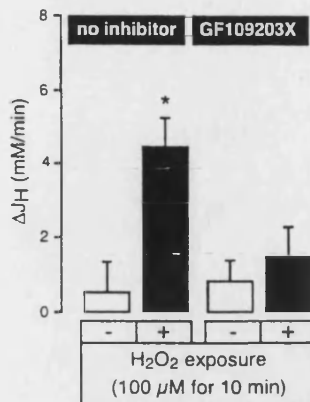

Fig. 6. Effects of PKC inhibition on H_2O_2-induced increases in sarcolemmal Na^+/H^+ exchanger activity. The change in J_H (ΔJ_H) during the second acid pulse relative to the first is shown in cells exposed to vehicle or 100 μM H_2O_2 from 10 min before the second acid pulse in the absence or presence of pre-treatment with the PKC inhibitor GF109203X (1 μM). $*P<0.05$ versus control (seven to 10 cells per group, obtained from eight hearts).

Here, we tested whether this holds true also for H_2O_2-induced stimulation of sarcolemmal NHE activity. As shown in Fig. 6, pre-treatment with the PKC inhibitor GF109203X (used at a concentration of 1 μM, which we have shown previously to inhibit phorbol ester-induced effects in an identical preparation [28]) completely abolished the H_2O_2-induced increase in ΔJ_H. This suggests that, in addition to ERK/p90^{rsk} activation, PKC activation is also necessary for the stimulation of sarcolemmal NHE activity by H_2O_2.

3.5. Proximal regulators of ERK activity: role of PKC

In cultured neonatal rat ventricular myocytes, H_2O_2-induced ERK activation has been suggested to occur through a PKC-mediated mechanism [20,34]. However, when we examined the effects of GF109203X (Fig. 7A) and the structurally distinct PKC inhibitor chelerythrine (Fig. 7B), we found no inhibition of the H_2O_2-induced increase in ERK activity over a broad concentration range. Thus, it appears that H_2O_2-induced ERK activation in freshly isolated adult rat ventricular myocytes occurs through PKC-*independent* mechanisms.

3.6. Proximal regulators of ERK activity: role of tyrosine kinases

In the light of the above, we examined whether H_2O_2-induced ERK activation might be mediated through tyrosine kinase pathways. To address this, we initially determined the effects of the non-selective tyrosine kinase inhibitor genistein, which produced a dose-dependent inhibition of the H_2O_2-induced increase in ERK activity

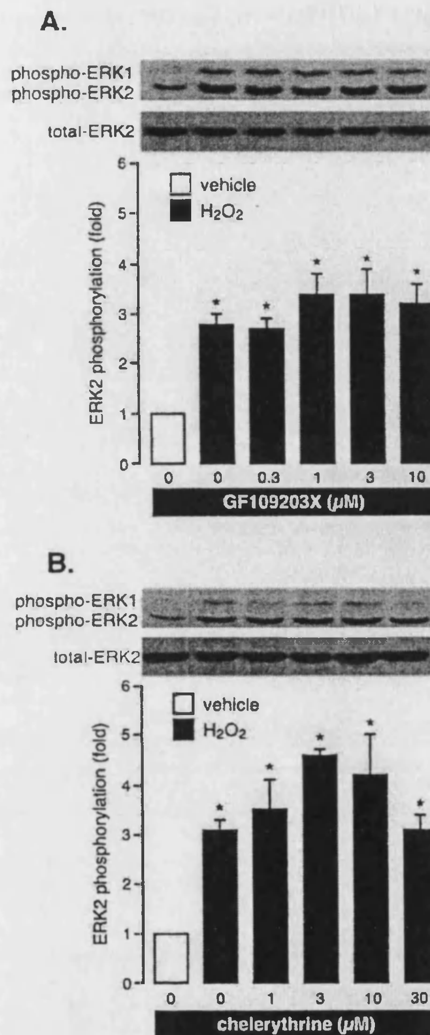

Fig. 7. Effects of PKC inhibition on H_2O_2-induced increases in ERK activity. Cells were exposed to vehicle or 100 μM H_2O_2 for 10 min in the absence or presence of pre-treatment with the PKC inhibitor (A) GF109203X (0.3–10 μM) or (B) chelerythrine (1–30 μM). Autoradiograms show representative Western blots with phosphospecific (top panel) and non-phosphospecific (bottom panel) antibodies for ERK. $*P<0.05$ versus control (four experiments, with cells from four hearts).

(Fig. 8A). Daidzein, a structural analog of genistein without tyrosine kinase inhibitory activity, had no effect on the H_2O_2-induced response. Thus, relative to control, H_2O_2 increased ERK phosphorylation by 4.1±0.5-fold in the absence of daidzein and by 4.0±0.3-, 3.3±0.4-, 3.1±0.8- and 4.8±1.2-fold in the presence of 3,10, 30 and 100 μM daidzein, respectively (four experiments, with cells from four hearts). These findings indicate that H_2O_2-

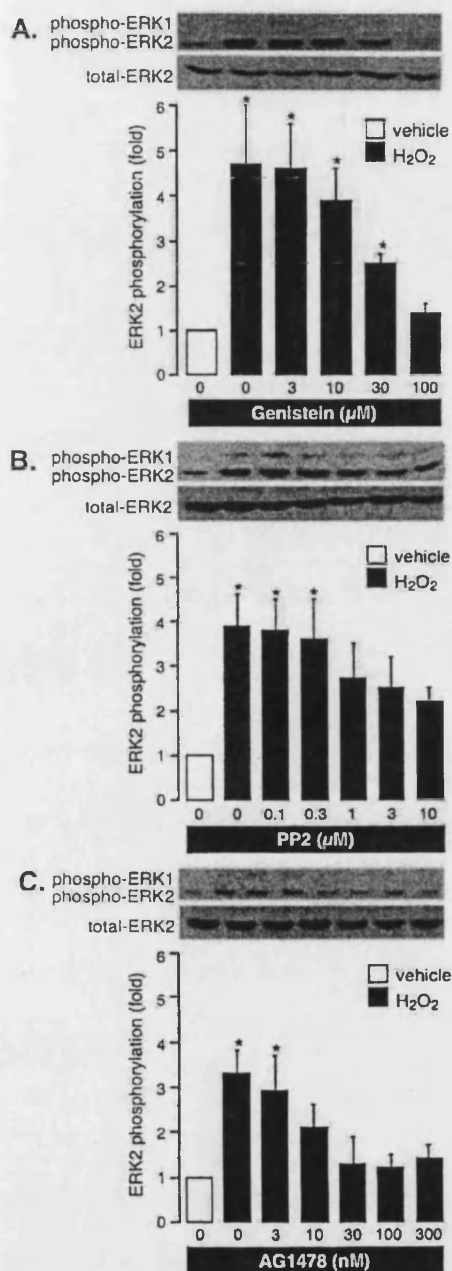

Fig. 8. Effects of tyrosine kinase inhibition on H_2O_2-induced increases in ERK activity. Cells were exposed to vehicle or 100 μM H_2O_2 for 10 min in the absence or presence of pre-treatment with (A) the non-selective tyrosine kinase inhibitor genistein (3–100 μM), (B) the Src-family kinase inhibitor PP2 (0.1–10 μM) or (C) the EGF receptor kinase inhibitor AG1478 (3–300 nM). Autoradiograms show representative Western blots with phosphospecific (top panel) and non-phosphospecific (bottom panel) antibodies for ERK. $^*P<0.05$ versus control (four experiments, with cells from four hearts).

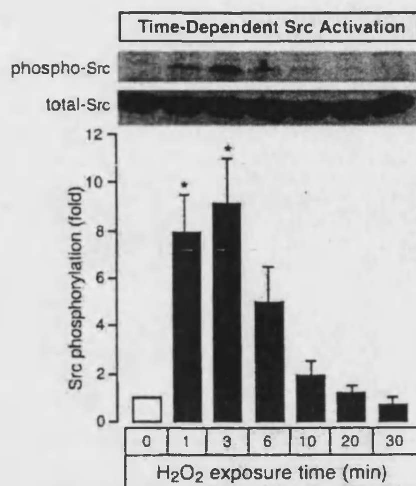

Fig. 9. Time-dependent effects of H_2O_2 on Src tyrosine kinase activity. Cells were exposed to vehicle or 100 μM H_2O_2 for 1, 3, 6, 10, 20 or 30 min. Autoradiograms show representative Western blots with phosphospecific (top panel) and non-phosphospecific (bottom panel) antibodies for Src tyrosine kinase. $^*P<0.05$ versus control (four experiments, with cells from four hearts).

induced ERK activation in the adult myocyte occurs through tyrosine kinase-mediated mechanisms.

To explore the role of non-receptor tyrosine kinases of the Src family, which have been shown to play an important role in H_2O_2 signalling in neonatal myocytes [35], we next determined the effects of the Src-selective inhibitor PP2. This agent, like genistein, produced a dose-dependent inhibition of the H_2O_2-induced increase in ERK activity (Fig. 8B), indicating a key role for Src family tyrosine kinases in this response. Indeed, Src tyrosine kinase was found to be activated rapidly upon exposure to H_2O_2 (Fig. 9), which supports a proximal role for Src-family tyrosine kinases in the H_2O_2-induced increase in ERK activity. In other cell types, c-Src (a member of the Src family) can phosphorylate the EGF receptor and thereby facilitate its full activation [36], and activation of this receptor tyrosine kinase has been implicated in ERK activation by H_2O_2 [37]. We therefore also investigated the effects of the EGF receptor kinase inhibitor AG1478 in our adult myocyte system. The data revealed that AG1478 inhibited H_2O_2-induced ERK activation in a dose-dependent manner (Fig. 8C), suggesting that the signalling mechanisms underlying this response are likely to involve activation of the EGF receptor.

4. Discussion

The present study has shown that exposure to H_2O_2 stimulates sarcolemmal NHE activity in freshly isolated

adult rat ventricular myocytes. Our data extend earlier findings of accelerated recovery from intracellular acidosis in response to H_2O_2 in cultured neonatal rat ventricular myocytes [20], and provide additional evidence that H_2O_2-induced stimulation of NHE activity requires activation of not only the ERK (but not the p38) pathway of the MAPK cascade, but also PKC. Intriguingly, unlike in neonatal myocytes [20], in our adult myocyte preparation PKC and ERK activation are not proximal and distal components, respectively, of a contiguous signalling pathway. Instead, H_2O_2-induced ERK activation in the adult myocyte appears to occur through the activation of tyrosine kinases of both receptor and non-receptor families.

Fig. 10 is based on the data obtained in the present study with rationally selected concentrations of specific kinase inhibitors (shown in italics) and the information that is available in the literature, and illustrates the signalling processes that are likely to mediate H_2O_2-induced stimulation of sarcolemmal NHE activity in the adult myocyte. According to this model, H_2O_2-induced stimulation of sarcolemmal NHE activity requires the parallel activation of PKC and ERK, in an analogous manner to α_{1A}-adrenoceptor-mediated stimulation of the exchanger [28]. In response to H_2O_2, activation of both PKC and ERK may be facilitated through the activation of non-receptor tyrosine kinases, including members of the Src family. Exposure to H_2O_2 has been shown to activate a variety of PKC isozymes, including PKCδ and PKCε (the predominant PKC isozymes in adult rat myocardium [24]), in transfected COS-7 cells, through tyrosine phosphorylation by unidentified kinases [38]. ERK is also activated by H_2O_2, as shown by previous work in neonatal myocytes [20,34,35] and the present study in adult myocytes. Although it has been suggested that this activation may occur through a PKC-mediated pathway in cultured neonatal myocytes [20,34], contradictory data have also been reported [35]. Our present data with GF109203X and chelerythrine oppose a PKC-mediated mechanism for H_2O_2-induced ERK activation in freshly isolated adult myocytes. Instead, our data suggest that ERK activation in these cells occurs through tyrosine kinase-mediated mechanisms that involve both Src family enzymes (which are inhibited selectively by PP2) and the EGF receptor (which is inhibited selectively by AG1478). EGF receptor activation has been suggested previously to mediate ERK activation in response to H_2O_2 in vascular smooth muscle cells [37]; furthermore, recent evidence suggests that c-Src can facilitate EGF receptor activation by direct phosphorylation of the receptor [36]. Such complementary interaction between Src family kinases and the EGF receptor would be consistent with our observations that genistein (a non-selective tyrosine kinase inhibitor), PP2 and AG1478 each inhibited H_2O_2-induced ERK activation in adult ventricular myocytes. In this context, it is noteworthy that, in adult guinea pig hearts, perfusion with H_2O_2 activates Src, as well as ERK and $p90^{rsk}$ [39]. Furthermore, in neonatal myocytes, overexpression of C-terminal Src kinase (a negative regulator of Src family tyrosine kinases) by transfection has been shown to inhibit H_2O_2-induced ERK activation [35]. On the basis of the above, it is likely that H_2O_2 activates PKC through tyrosine phosphorylation (by an as yet unidentified kinase) and ERK through Src-dependent EGF receptor activation and subsequent stimulation of the classical Ras-Raf-MEK pathway.

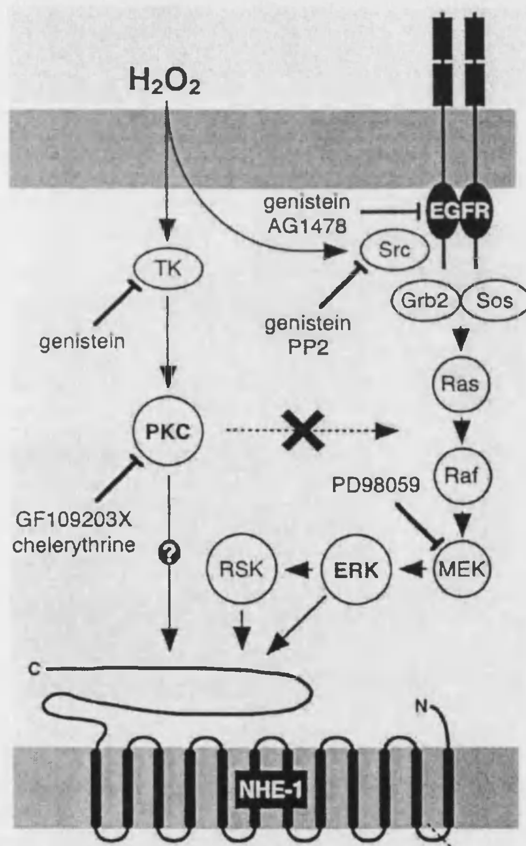

Fig. 10. Potential integration of signalling pathways in H_2O_2-induced stimulation of sarcolemmal Na^+/H^+ exchanger activity in adult rat ventricular myocytes. The kinase inhibitors used in the present study are shown in italics, with their target enzymes indicated (see Section 4 for details).

A potential limitation of the present study is the reliance on pharmacological kinase inhibitors to dissect the intracellular signalling pathways that mediate H_2O_2-induced stimulation of sarcolemmal NHE activity. In previous studies in a variety of cell types, these inhibitors have been used widely as pharmacological tools and have contributed significantly to the delineation of the physiological roles of pertinent pathways. Furthermore, extensive characterisa-

tion of inhibitors such as PD98059 [40], SB203580 [41], GF109203X [42], chelerythrine [43], AG1478 [44], and PP2 [45] indicates that, over the carefully selected concentration ranges used in the present study, they inhibit their target kinases but not a variety of other common kinases. Indeed, recent reports of non-specific effects of some of these kinase inhibitors [31,46] have observed such effects at concentrations that are considerably higher than those which produced significant effects in the present study.

An issue that is pertinent to the scheme proposed in Fig. 10 is how parallel activation of ERK and PKC may result in the stimulation of sarcolemmal NHE activity. Activation of the ERK pathway can lead to the phosphorylation of serine residues in the regulatory C-terminal domain of the NHE-1 protein, either directly by ERK itself [47] or indirectly through $p90^{rsk}$ [32], in a manner that may alter the interaction of this domain with accessory protein(s) that regulate exchanger activity. PKC, in contrast, does not directly phosphorylate the regulatory domain of NHE-1 [48] and, as we have suggested previously [28], may promote the stimulation of NHE activity by producing a concomitant change in the phosphorylation status of pertinent accessory protein(s).

There are likely to be cell type-dependent differences in the manner in which $p90^{rsk}$ activity is regulated in response to H_2O_2. The ability of the MEK inhibitor PD98059 to inhibit H_2O_2-induced $p90^{rsk}$ activation in the present study suggests that such activation in adult rat ventricular myocytes occurs predominantly through ERK. In contrast, Abe et al. [49] have shown that, in cultured fibroblasts and Jurkat cells, H_2O_2-induced $p90^{rsk}$ activation occurs through an ERK-independent pathway that is mediated via Fyn and Ras.

In conclusion, the present study has shown that H_2O_2 stimulates sarcolemmal NHE activity of adult rat ventricular myocytes, through the concomitant (but independent) activation of PKC- and ERK-mediated signalling pathways. In these cells, H_2O_2-induced stimulation of ERK occurs through Src family kinase- and EGF receptor kinase-mediated mechanisms. H_2O_2-induced stimulation of sarcolemmal NHE activity is likely to have functional consequences, particularly in the setting of myocardial ischaemia and reperfusion.

Acknowledgements

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Regulation of Cardiac Sarcolemmal Na^+/H^+ Exchanger Activity by Endogenous Ligands

Relevance to Ischemia[a]

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ABSTRACT: The cardiac sarcolemmal Na^+/H^+ exchanger (NHE) extrudes one H^+ in exchange for one Na^+ entering the myocyte, utilizing for its driving force the inwardly directed Na^+ gradient that is maintained by the Na^+/K^+ ATPase. The exchanger is quiescent at physiological values of intracellular pH but becomes activated in response to intracellular acidosis. Recent evidence suggests that a variety of extracellular signals (e.g., adrenergic agonists, thrombin, and endothelin) also modulate sarcolemmal NHE activity by altering its sensitivity to intracellular H^+. Since sarcolemmal NHE activity is believed to be an important determinant of the extent of myocardial injury during ischemia and reperfusion, regulation of exchanger activity by endogenous ligands associated with ischemia is likely to be of pathophysiological importance.

The plasma membrane Na^+/H^+ exchanger (NHE) is a ubiquitous electroneutral exchanger that extrudes one H^+ in exchange for one Na^+, utilizing as its driving force the inwardly directed Na^+ gradient maintained by Na^+/K^+ ATPase.[1,2] The exchanger is thought to mediate a number of physiological functions in various cell types, including the regulation of intracellular pH and cell volume (by virtue of the ability of the exchanger to transport H^+ and Na^+, respectively) and the control of cell growth and proliferation (by mediating the actions of a number of mitogens and growth factors).[3] Abnormalities in NHE activity have also been implicated in pathophysiological processes, such as renal acid-base disorders.[3]

With respect to the cardiovascular system, increased NHE activity has been linked with hypertension,[4] platelet activation,[5] and the proliferative response of arterial smooth muscle cells to injury.[6] Additionally, recent evidence suggests that upregulation of NHE expression and/or activity may be associated with cardiac hypertrophy in both *in vivo*[7] and *in vitro*[8,9] models. However, as discussed in depth elsewhere in this volume, the strongest evidence for an important role for NHE in

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cardiac pathophysiology is that implicating the sarcolemmal exchanger in the unfavorable sequelae of ischemia and reperfusion, such as arrhythmias, contractile dysfunction, and infarction (for reviews see Avkiran[10] and Fröhlich and Karmazyn[11]). Indeed, novel pharmacological inhibitors of the exchanger, such as HOE694[12] and cariporide (HOE642),[13] have been developed for potential use in the therapy of ischemic heart disease.[14] Notably, the clinical efficacy of cariporide is currently being evaluated in a multi-national study (the "Guard in Ischemia Against Necrosis [GUARDIAN]" study) involving 12,000 patients with acute coronary syndromes. Despite these exciting advances, however, much remains unknown regarding the molecular mechanisms that regulate cardiac sarcolemmal NHE activity, particularly in the setting of ischemia and reperfusion.

CARDIAC SARCOLEMMAL NHE ACTIVITY DURING ISCHEMIA AND REPERFUSION

The sarcolemmal NHE of cardiac myocytes comprises the ubiquitous NHE1 isoform of this multi-gene family[15]; the exchanger is quiescent at physiological values of intracellular pH but becomes activated in response to intracellular acidosis,[16,17] which is known to develop rapidly during myocardial ischemia.[18] Lazdunski and colleagues[16] were the first to propose a role for the sarcolemmal NHE in the pathogenesis of ischemia and reperfusion-induced injury in myocardium. According to the "Lazdunski Hypothesis," the sarcolemmal NHE is inactive during ischemia, despite the presence of intracellular acidosis, due to inhibition by extracellular acidosis. However, according to this hypothesis, the rapid normalization of extracellular pH upon reperfusion results in the generation of an outwardly directed H^+ gradient, leading to increased sarcolemmal NHE activity and thereby predisposing the myocardium to intracellular Na^+ and Ca^{2+} overload, with detrimental consequences. Consistent with this, several studies have shown that pharmacological inhibitors of NHE, given only at the time of reperfusion, can afford substantial cardioprotection.[19–22] Nevertheless, in other studies, such treatment protocols have been found to be ineffective[23–27] or to provide only partial protection,[28–32] relative to treatment before the onset of ischemia. In contrast, as reviewed previously,[10] there is widespread consensus among published studies regarding the significant cardioprotective benefit afforded by NHE inhibitors when given before the onset of ischemia (which in many cases[23–32] has been shown to be superior to that afforded by these drugs when given only during reperfusion), regardless of inter-study variations in models, severity of ischemia, and functional endpoints. On the basis of the available data, therefore, it is likely that the sarcolemmal NHE retains significant activity during ischemia as well as during subsequent reperfusion, and that exchanger activity during both periods is important in determining the ultimate extent of injury. Thus, the superior protection afforded by pre-ischemic treatment with NHE inhibitors in many studies probably arises from inhibition of exchanger activity during ischemia and early reperfusion. In this regard, the hypothesis that significant sarcolemmal NHE activity is retained during ischemia is supported by studies that have shown that intracellular Na^+ accumulation during ischemia is attenuated in the presence of NHE inhibitors.[33,34]

How does the sarcolemmal NHE retain its activity during ischemia in the face of the significant extracellular acidosis known to accompany intracellular acidosis? Firstly, it is important to note that, although the sarcolemmal NHE is undoubtedly inhibited by extracellular acidosis,[17,35] such inhibition is not absolute. Contrary to a common misconception, the primary regulator of NHE activity is not the trans-membrane H^+ gradient, but the intracellular pH, through the interaction of intracellular H^+ with the "H^+ sensor" site of the exchanger protein.[1] Indeed, work from the Vaughan-Jones laboratory has shown that the sarcolemmal NHE can remain active and extrude H^+ against an inwardly directed H^+ gradient, provided the intracellular pH is sufficiently low.[35] Secondly, certain processes associated with ischemia, such as the accumulation of lipid metabolites and the imposition of oxidant stress, may upregulate NHE activity, since exogenous lysophosphatidylcholine[36] and hydrogen peroxide[37,38] have both been shown recently to stimulate the sarcolemmal exchanger in cultured neonatal[37] and freshly isolated adult[36,38] rat ventricular myocytes. Finally, as discussed in some detail below, various endogenous, receptor-mediated pathways with relevance to ischemia have been shown to stimulate sarcolemmal NHE activity, apparently by increasing the sensitivity of the exchanger to intracellular H^+.

REGULATION OF CARDIAC SARCOLEMMAL NHE ACTIVITY BY ENDOGENOUS LIGANDS

Effects of Catecholamines

It is well established that myocardial ischemia results in the activation of the sympathetic nervous system, as well as the local release of norepinephrine within the ischemic zone.[39] Furthermore, there is evidence that α_1-adrenergic signaling is upregulated in ischemic myocardium.[40] In this regard, it is interesting to note that the majority of the published work on catecholamine-induced changes in sarcolemmal NHE activity has concentrated on the role of α_1-adrenoceptors (α_1-ARs). Thus, in isolated ventricular myocytes from the rat and guinea pig, it has been shown that α_1-AR agonists (such as phenylephrine[41,42] and 6-fluoronorepinephrine,[43] usually in the presence of a β_1-AR antagonist) increase sarcolemmal NHE activity, while α_1-AR antagonists (such as prazosin[42,43]) inhibit such NHE-stimulatory effects. Notably, α_1-adrenergic stimulation appears to retain its ability to increase sarcolemmal NHE activity in the presence of extracellular acidosis,[41] which has important implications for NHE regulation under ischemic conditions.

Due to the lack of selectivity of the pharmacological agents used in previous studies, it has not been possible until recently to draw any conclusions regarding the identity of the α_1-AR subtype(s) involved in α_1-adrenergic stimulation of sarcolemmal NHE activity. α_1-AR subtypes are classified by the International Union of Pharmacology[44] as α_{1A}-, α_{1B}-, and α_{1D}-ARs, which correspond respectively to the recombinant subtypes previously referred to as α_{1c}, α_{1b}, and α_{1d}-ARs (the last of which has also been referred to as the α_{1a}- or $\alpha_{1a/d}$-AR). Prompted by (1) the substantial evidence supporting a key role for sarcolemmal NHE activity in the pathogenesis of ischemia-reperfusion–induced injury (see above), (2) the reported ability of non-selective α_1-adrenergic agonists to increase sarcolemmal NHE activity[41–43] and (3) our earlier observation[45] that the pro-arrhythmic effect of the non-selective

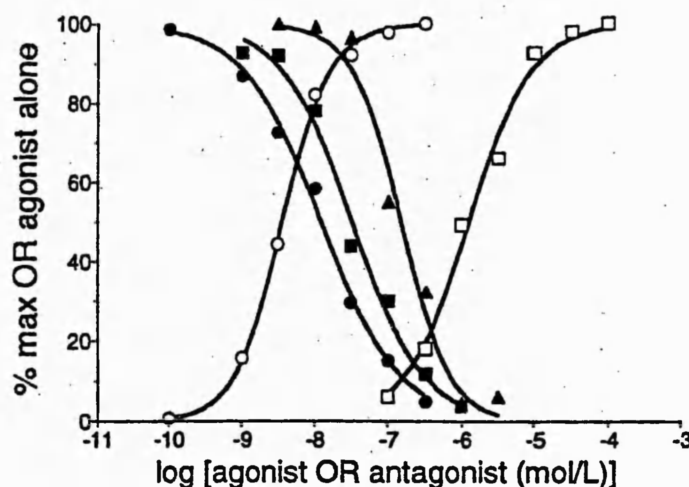

FIGURE 1. α_1-Adrenergic stimulation of sarcolemmal NHE activity in adult rat ventricular myocytes. Figure shows agonist dose-response curves for A616103 (○) and phenylephrine (□), as well as antagonist inhibition curves for prazosin (●), WB4101 (■), and 5-methylurapidil (▲) (all antagonists tested against 10 μmol/l phenylephrine). EC_{50} = 3.6 nmol/l for A61603 and 1.24 μmol/l for phenylephrine. IC_{50} = 12 nmol/l for prazosin, 32 nmol/l for WB4101, and 149 nmol/l for 5-methylurapidil. (Data from Yokoyama *et al.*[46])

α_1-adrenergic agonist phenylephrine could be reversed not only by NHE inhibition but also by an α_{1A}-AR–selective antagonist, we have recently attempted to delineate the roles of α_1-AR subtypes in regulating sarcolemmal NHE activity in adult rat ventricular myocytes.

FIGURE 1, adapted from our earlier study,[46] shows the stimulatory effects of phenylephrine and the α_{1A}-AR–selective agonist A61603 on sarcolemmal NHE activity and the inhibitory effects of the competitive antagonists prazosin, WB4101, and 5-methylurapidil on the response to 10 μM phenylephrine. Comparison of the relative potencies of these agents, as depicted in FIGURE 1, with their reported relative potencies and affinities at native[47] and recombinant[48] α_1-AR subtypes of rat origin provides evidence, for the first time, that α_1-adrenergic stimulation of sarcolemmal NHE activity is likely to be mediated selectively via the α_{1A}-AR subtype.[46] Since the α_{1A}-AR is the dominant α_1-AR subtype expressed in human myocardium[49] and myocardial hypertrophy appears to be accompanied by its transcriptional induction,[50] confirmation of α_{1A}-AR–mediated regulation of sarcolemmal NHE activity may not only provide a potential target for therapeutic intervention but may also reveal a mechanism for the increased susceptibility of hypertrophied myocardium to the consequences of ischemia and reperfusion (such as arrhythmias and contractile dysfunction).

In considering adrenergic regulation of the sarcolemmal NHE, it is important to note that β-adrenergic stimulation also affects exchanger activity. There is agreement among published studies that, in contrast to the effect of α_1-adrenergic stimulation, β_1-adrenergic stimulation inhibits sarcolemmal NHE activity in a variety of species,[42,51,52] probably through a cAMP-mediated pathway.[51–53] Therefore, the effects of the endogenous catecholamines norepinephrine and epinephrine on sar-

colemmal NHE activity may depend on the relative density or availability of α_1- versus β_1-ARs, which can be modulated by genetic factors (which may account for species-specific responses); accompanying disease (such as heart failure, which is associated with β_1-AR downregulation, or ischemia, which enhances α_1-AR signaling); or pharmacological therapy (in particular, the use of adrenergic antagonists).

Effects of Thrombin

The most common cause of acute myocardial ischemia in man is intracoronary thrombosis,[54] and thrombosis is associated with elevated levels of thrombin in the vicinity of the evolving thrombus.[55] Therefore, the biological actions of thrombin may be of pathophysiological significance during myocardial ischemia in the clinical setting. In this regard, it is now well-established that, in addition to its role in blood coagulation and thrombus formation, thrombin induces a variety of cellular responses through receptor-mediated pathways (for review see Coughlin[56]). Recently, we have published the first evidence that both thrombin and the synthetic thrombin receptor activating hexapeptide SFLLRN increase sarcolemmal NHE activity in adult rat ventricular myocytes.[57] The common ability of thrombin and SFLLRN to stimulate sarcolemmal NHE activity is consistent with this action being mediated by the thrombin receptor first cloned by Vu and colleagues[58] and is now known as protease-activated receptor 1 (PAR1), the mRNA for which we have shown to be expressed by adult rat ventricular myocytes.[57] However, recent findings suggest that other protease-activated receptors may also be involved. In this regard, Connolly and colleagues[59] have recently shown that in mice with targeted disruption of the PAR1 gene, responsiveness to thrombin is lost in some cell types (e.g., fibroblasts) but retained in others (e.g., platelets). This indicates the existence of additional thrombin receptor(s) and has led to the suggestion that different thrombin receptors may have tissue-specific roles.[59] Indeed, a second thrombin receptor (PAR3) has now been cloned and appears to be expressed in human myocardium.[60] Furthermore, there is preliminary evidence[61] for the expression of functional PAR2, a trypsin-activated receptor that is responsive also to the PAR1-activating peptide SFLLRN,[62] in neonatal rat ventricular myocytes. These developments necessitate verification of the hypothesis that cardiac sarcolemmal NHE is regulated by PAR1 and require determination of the roles, if any, of PAR2 and PAR3. Defining which of these receptors regulate sarcolemmal NHE activity clearly is critical to the potential therapeutic manipulation of this signaling pathway.

Effects of Endothelin

There is evidence that endothelin is released during myocardial ischemia and reperfusion[63] and that this release is accompanied by upregulation of endothelin receptors.[64] Therefore, the cellular actions of endothelin may also assume pathophysiological importance during myocardial ischemia and reperfusion. Within the context of the present article, it is notable that endothelin has been shown to stimulate sarcolemmal NHE activity, in isolated rat ventricular myocytes[65,66] as well as in canine cardiac Purkinje fibers.[52]

FUNCTIONAL IMPACT OF NHE-STIMULATORY LIGANDS IN ISCHEMIA AND REPERFUSION

What is the functional impact of receptor-mediated stimulation of sarcolemmal NHE activity in the setting of myocardial ischemia and reperfusion? Much of the evidence in this area has been obtained through the application of exogenous agonists in *in vitro* models of ischemia and reperfusion. Thus, work in Karmazyn's laboratory has shown that, in isolated rat hearts, both α_1-AR stimulation (in the presence of an adenosine antagonist)[67] and endothelin[68] exacerbate post-ischemic contractile dysfunction. Of particular relevance to the present article, the deleterious effects of both stimuli could be reversed by NHE inhibition,[67,68] thus implying a key role for the exchanger in the injurious mechanisms downstream of receptor activation. Consistent with this, Brunner and Opie[69] have shown recently that the deleterious effects of endothelin during ischemia and reperfusion could be attenuated not only by ET_A receptor antagonism but also by NHE inhibition. Furthermore, α_1-AR stimulation selectively within the ischemic zone has been shown by the authors' group[45] to exacerbate reperfusion-induced arrhythmias, with this proarrhythmic effect abolished by α_{1A}-AR antagonism or NHE inhibition, implicating the exchanger in the downstream mechanisms.

Within the context of ischemia and reperfusion, α_1-AR stimulation has also been implicated in the induction of ischemic preconditioning in rat myocardium.[70] Since ischemic preconditioning is associated with reduced intracellular acidosis during the prolonged ischemia,[71] it is possible that α_1-adrenergic stimulation of sarcolemmal NHE activity may contribute to this phenomenon. However, Gabel and colleagues[72] have shown recently that H^+ efflux during the prolonged ischemic period is not increased in preconditioned hearts. Furthermore, any stimulation of sarcolemmal NHE activity is unlikely to contribute to the cardioprotective mechanism(s) of ischemic preconditioning, since we have shown such protection to be retained (and indeed enhanced) in the presence of NHE inhibition.[73]

With regard to thrombin-induced effects during ischemia and reperfusion, Goldstein and colleagues[74] have shown that, in the canine heart *in vivo*, the incidence of malignant ventricular arrhythmias during acute ischemia is greater following thrombotic coronary occlusion than non-thrombotic balloon occlusion, implicating an arrhythmogenic role for factors (such as thrombin) associated with thrombus formation. Indeed, thrombin has now been shown to induce a potent arrhythmogenic effect during ischemia and reperfusion in isolated rat hearts,[75,76] in a manner that appears to be reversible by NHE inhibition.[76] A role for the sarcolemmal NHE in mediating the arrhythmogenic action of thrombin is supported by evidence that activation of the thrombin receptor exacerbates the intracellular accumulation of Na^+ during ischemia,[77] an observation that is consistent with stimulation of exchanger activity.

CONCLUSION

It is clear from the evidence discussed above that sarcolemmal NHE activity is modulated by a number of endogenous stimuli (e.g., catecholamines, thrombin, and endothelin), through receptor-mediated mechanisms, and that stimulation of the ex-

changer through these receptors may be of pathophysiological significance during ischemia and reperfusion. Undoubtedly, there are other, currently unidentified, receptors that also regulate sarcolemmal NHE activity in a positive or negative manner. The identification of such receptors and delineation of the intracellular signaling mechanisms that facilitate receptor-mediated regulation of sarcolemmal NHE activity may identify common pathways, which might represent fertile targets for the development of novel therapies for ischemic heart disease.

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Regulation of Cardiac Sarcolemmal Na^+/H^+ Exchanger Activity: Potential Pathophysiological Significance of Endogenous Mediators and Oxidant Stress

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Abstract. **The cardiac sarcolemmal Na^+/H^+ exchanger (NHE) extrudes one H^+ in exchange for one Na^+ entering the myocyte, utilizing for its driving force the inwardly directed Na^+ gradient maintained by the Na^+, K^+-ATPase. The exchanger is quiescent at physiological values of intracellular pH but becomes activated in response to intracellular acidosis. Recent evidence suggests that a variety of extracellular signals (e.g., adrenergic agonists, thrombin, endothelin, and oxidant stress) also modulate sarcolemmal NHE activity by altering its sensitivity to intracellular H^+. Because sarcolemmal NHE activity is believed to be an important determinant of the extent of myocardial injury during ischemia and reperfusion, regulation of exchanger activity by factors that are associated with ischemia is likely to be pathophysiological importance.**

Key Words: **NHE, catecholamines, oxidant stress, thrombin, endothelin, ischemia, reperfusion**

The plasma membrane Na^+/H^+ exchanger (NHE) is ubiquitous electroneutral exchanger that extrudes one H^+ in exchange for one Na^+, utilizing as its driving force the inwardly directed Na^+ gradient maintained by Na^+, K^+-ATPse [1,2]. The exchanger is thought to mediate a number of physiological functions in various cell types, including regulation of intracellular pH and cell volume (by virtue of its ability to transport H^+ and Na^+, respectively) and control of cell growth and proliferation (by mediating the actions of a number of mitogens and growth factors) [3]. Abnormalities in NHE activity have also been implicated in pathophysiological processes, such as renal acid-base disorders [3].

With respect to the cardiovascular system, increased NHE activity has been linked to hypertension [4], platelet activation [5], and the proliferative response of arterial smooth muscle cells to injury [6]. Additionally, recent evidence suggests that upregulation of NHE expression and/or activity may be associated with cardiac hypertrophy in both in vivo [7] and in vitro [8,9] models. However, as discussed in depth elsewhere in this issue, the strongest evidence for an important role for NHE in cardiac pathophysiology is that implicating the sarcolemmal exchanger in the unfavorable sequelae of ischemia and reperfusion, such as arrhythmias, contractile dysfunction, and infarction (for reviews, see Avkiran [10] and Fröhlich and Karmazyn [11]). Indeed, novel pharmacological inhibitors of the exchanger, such as HOE694 [12] and cariporide (HOE642) [13], have been developed for potential use in the therapy of ischemic heart disease [14]. Notably, the clinical efficacy of cariporide is currently being evaluated in a multinational study (the Guard in Ischemia Against Necrosis [GUARDIAN] study) involving 12,000 patients with acute coronary syndromes. Despite these exciting advances, much remains unknown regarding the molecular mechanisms that regulate cardiac sarcolemmal NHE activity, particularly in the setting of ischemia and reperfusion.

Cardiac Sarcolemmal NHE Activity During Ischemia and Reperfusion

The sarcolemmal NHE of cardiac myocytes comprises the ubiquitous NHE1 isoform of this multigene family [15]. The exchanger is quiescent at physiological values of intracellular pH but becomes activated in response to intracellular acidosis [16,17], which is known to develop rapidly during myocardial ischemia [18]. Lazdunski and colleagues [16] were the first to propose a role for sarcolemmal NHE in the pathogenesis of ischemia and reperfusion-induced injury in myocardium. According to the Lazdunski Hypothesis, sarcolemmal NHE is inactive during ischemia, despite the presence of intracellular acidosis, due to inhibition by *extracellular* acidosis. However, it states that rapid normalization of extracellular pH on reperfusion results in the generation of an outwardly directed H^+ gradient, leading to increased sarcolemmal NHE activity and thereby predisposing the myocardium to intracellular Na^+ and Ca^{2+} overload, with detrimental consequences. Consistent with this, several studies have shown that pharmacological inhibitors of NHE, given only at the time of reperfusion, can afford substantial cardioprotection [19–22]. Neverthe-

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less, in other studies such treatment protocols have been found to be ineffective [23–27] or to provide only partial protection [28–32] relative to treatment before the onset of ischemia.

In contrast, as reviewed previously [10], there is widespread consensus among published studies regarding the significant cardioprotective benefit afforded by NHE inhibitors when given before the onset of ischemia (which in many cases [23–32] has been shown to be superior to that afforded by these drugs when given only during reperfusion), regardless of interstudy variations in models, severity of ischemia, and functional endpoints. On the basis of the available data, therefore, it is likely that sarcolemmal NHE retains significant activity during ischemia as well as during subsequent reperfusion, and that exchanger activity during both periods is important in determining the ultimate extent of injury. Thus, the superior protection afforded by preischemic treatment with NHE inhibitors in many studies probably arises from inhibition of exchanger activity during ischemia *and* early reperfusion. In this regard, the hypothesis that significant sarcolemmal NHE activity is retained during ischemia is supported by studies that have shown intracellular Na^+ accumulation is attenuated in the presence of NHE inhibitors during both normothermic [33,34] and hypothermic [35] ischemia.

How does the sarcolemmal NHE retain its activity during ischemia in the face of the significant extracellular acidosis that is known to accompany intracellular acidosis? Firstly, it is important to note that although the sarcolemmal NHE is undoubtedly inhibited by extracellular acidosis [17,36], such inhibition is not absolute. Indeed, the primary regulator of NHE activity is *not* the transmembrane H^+ gradient, but intracellular pH, through the interaction of intracellular H^+ with the so-called H^+ sensor site of the exchanger protein [2]. Regarding cardiac NHE, work from the Vaughan-Jones laboratory has shown that sarcolemmal NHE can remain active and extrude H^+ against an inwardly directed H^+ gradient, provided the intracellular pH is sufficiently low [36]. Secondly, certain processes associated with ischemia, such as the accumulation of lipid metabolites, may upregulate NHE activity, because exogenous lysophosphatidylcholine has been shown recently [37] to stimulate the sarcolemmal exchanger in freshly isolated adult rat ventricular myocytes. Finally, as discussed in some detail later, various endogenous, receptor-mediated pathways with relevance to ischemia as well as oxidant stress have been shown to stimulate sarcolemmal NHE activity, apparently by increasing the sensitivity of the exchanger to intracellular H^+.

Regulation of Cardiac Sarcolemmal NHE Activity by Endogenous Factors

Effects of adrenergic stimulation

It is well established that myocardial ischemia results in activation of the sympathetic nervous system, as well as local release of norepinephrine within the ischemic zone [38]. Furthermore, there is evidence that α_1-adrenergic signaling is upregulated in ischemic myocardium [39]. In this regard, it is interesting to note that the majority of the published work on catecholamine-induced changes in sarcolemmal NHE activity has concentrated on the role of α_1-adrenoceptors (α_1-ARs). Thus, in isolated ventricular myocytes from the rat and guineapig, it has been shown that α_1-AR agonists (such as phenylephrine [40,41] and 6-fluoronorepinephrine [42], usually in the presence of a β_1-AR antagonist) increase sarcolemmal NHE activity, whereas α_1-AR antagonists (such as prazosin [41,42]) inhibit such NHE-stimulatory effects. Notably, α_1-adrenergic stimulation appears to retain its ability to increase sarcolemmal NHE activity in the presence of extracellular acidosis [40], which has important implications for NHE regulation under ischemic conditions.

Due to the lack of selectivity of the pharmacological agents used, it has not been possible until recently to draw any conclusions regarding the identity of the α_1-AR subtype(s) that are involved in α_1-adrenergic stimulation of sarcolemmal NHE activity. Prompted by (1) the substantial evidence supporting a key role for sarcolemmal NHE activity in the pathogenesis of ischemia and reperfusion-induced injury, (2) the reported ability of nonselective α_1-adrenergic agonists to increase sarcolemmal NHE activity [40–42], and (3) our earlier observation [43] that the proarrhythmic effect of the nonselective α_1-adrenergic agonist phenylephrine could be reversed not only by NHE inhibition but also by an α_{1A}-AR–selective antagonist, we have recently attempted to delineate the roles of α_1-AR subtypes in regulating sarcolemmal NHE activity in adult rat ventricular myocytes. This work has revealed that α_1-adrenergic stimulation of sarcolemmal NHE activity is likely to be mediated selectively via the α_{1A}-AR subtype [44]. Because the a1A-AR is the dominant α_1-AR subtype expressed in human myocardium [45] and myocardial hypertrophy appears to be accompanied by its transcriptional induction [46], confirmation of α_{1A}-AR–mediated regulation of sarcolemmal NHE activity may not only provide a potential target for therapeutic intervention but may also reveal a mechanism for the increased susceptibility of hypertrophied myocardium to the consequences of ischemia and reperfusion (such as arrhythmias and contractile dysfunction).

In considering adrenergic regulation of the sarcolemmal NHE, it is important to note that β-adrenergic stimulation also affects exchanger activity. There is agreement among published studies that, in contrast to the effect of α_1-adrenergic stimulation, β_1-adrenergic stimulation *inhibits* sarcolemmal NHE activity in a variety of species [41,47,48], probably through a cAMP-mediated pathway [47–49]. Therefore, the effects of the endogenous catecholamines norepinephrine and epinephrine on sarcolemmal NHE activity may depend on the relative density or availability of α_1- versus β_1-ARs, which can be modulated by genetic factors (which may

account for species-specific responses), accompanying disease (such as heart failure, which is associated with β_1-AR downregulation, ischemia, which enhances α_1-AR signaling), or pharmacological therapy (in particular, the use of adrenergic antagonists).

Effects of thrombin

The commonest cause of acute myocardial ischemia in humans is intracoronary thrombosis [50], and thrombosis is associated with elevated levels of thrombin in the vicinity of the evolving thrombus [51]. Therefore, the biological actions of thrombin may be of pathophysiological significance during myocardial ischemia in the clinical setting. In this regard, it is now well established that, in addition to its role in blood coagulation and thrombus formation, thrombin induces a variety of cellular responses through receptor-mediated pathways (for review, see Coughlin [52]).

Recently, we have published the first evidence that both thrombin and the synthetic thrombin receptor activating hexapeptide SFLLRN increase sarcolemmal NHE activity in adult rat ventricular myocytes [53]. The common ability of thrombin and SFLLRN to stimulate sarcolemmal NHE activity is consistent with this action being mediated by the thrombin receptor that was first cloned by Vu and colleagues [54] and is now known as *protease-activated receptor 1* (PAR1), the mRNA for which we have shown to be expressed by adult rat ventricular myocytes [53].

However, recent findings suggest that other protease-activated receptors may also be involved. In this regard, Connolly et al. [55] have recently shown that in mice with targeted disruption of the PAR1 gene, responsiveness to thrombin is lost in some cell types (e.g., fibroblasts) but retained in others (e.g., platelets). This indicates the existence of additional thrombin receptor(s) and has led to the suggestion that different thrombin receptors may have tissue-specific roles [55]. Indeed, a second thrombin receptor (PAR3) has been cloned and appears to be expressed in human myocardium [56]. Furthermore, there is preliminary evidence [57] for the expression of functional PAR2, a trypsin-activated receptor that is also responsive to the PAR1-activating peptide, SFLLRN [58], in neonatal rat ventricular myocytes. These developments necessitate verification of the hypothesis that cardiac sarcolemmal NHE is regulated by PAR1 and require determination of the roles, if any, of other protease-activated receptors. Defining the receptors that regulate sarcolemmal NHE activity clearly is critical to the potential therapeutic manipulation of this signaling pathway.

Effects of endothelin

There is evidence that endothelin is released during myocardial ischemia and reperfusion [59] and that this is accompanied by upregulation of endothelin receptors [60]. Therefore, the cellular actions of endothelin may also assume pathophysiological importance during myocardial ischemia and reperfusion. Within the context of the present article, it is notable that endothelin has been shown to stimulate sarcolemmal NHE activity in isolated rat ventricular myocytes [61,62] as well as in canine cardiac Purkinje fibers [48].

Effects of oxidant stress

For many years, increased oxidant stress has been implicated as a major contributing factor in the pathophysiology of ischemia and reperfusion-induced injury in the heart; however, despite intensive study in many laboratories, the cellular mechanisms responsible for this remain unclear. Recently, it was shown that contractile dysfunction during aerobic perfusion [63] or following ischemia and reperfusion [64] could be induced or exacerbated by the application of exogenous hydrogen peroxide (H_2O_2). These effects were reversed by the selective NHE inhibitor HOE 642 [63,64], thereby implicating NHE as the mediator. We have recently shown [65] that exogenous H_2O_2 can significantly increase sarcolemmal NHE activity, but only at acidic intracellular pH levels (6.7–6.9) that are readily attained during ischemia [66,67]. Based on the observation that the myocardium is subjected to increased oxidative stress during early reperfusion [68,69], it is tempting to suggest that subsequent contractile dysfunction may be partly mediated by oxidant stress–induced activation of sarcolemmal NHE.

The precise mechanism by which increased oxidant stress (exogenous H_2O_2) increases sarcolemmal NHE activity is not fully understood but has been proposed to involve a group of cytosolic protein kinases known as the extracellular regulated kinases (ERKs) [70]. The ERKs are members of the mitogen activated protein kinase (MAPK) superfamily of serine/threonine kinases, which also include other protein kinases, such as the p38 MAPK[71] and c-Jun N-terminus kinases (JNKs) [72]. p38 MAPK and JNKs are also activated by oxidant stress and various other cellular stresses, such as ischemia (p38 MAPK) and reperfusion (JNKs) [73]. In addition, there is recent evidence to suggest that in vascular smooth muscle cells, p38 MAPK may behave as a negative regulator of sarcolemmal NHE activity [74]. This suggests that, at least in some cell types, a complex inter relationship may exist between ERKs (activation of NHE) and p38 MAPK (inhibition of NHE), which may impact upon oxidant stress–induced regulation of exchanger activity. Obviously, further investigation and clarification of this concept is needed.

Functional Impact of NHE-Stimulatory Factors in Ischemia and Reperfusion

What is the functional impact of the NHE-regulatory factors discussed earlier in the setting of myocardial ischemia and reperfusion? Much of the evidence in this

area has been obtained through the application of the relevant exogenous stimuli in in vitro models of ischemia and reperfusion, and assessment of the resultant injury and dysfunction. As discussed later, the deleterious effects of these stimuli can be reversed by NHE inhibition, thus implying a key role for the exchanger in the downstream injurious mechanisms.

Endothelin

Khandoudi and coworkers [75] have demonstrated that the most potent of the endothelin isoforms, endothelin-1 (ET-1), is able to (1) depress contractile function, (2) increase resting tension, and (3) increase vascular resistance in the isolated rat heart. In addition, pretreatment with ET-1 prior to the onset of ischemia resulted in reduced recovery of contractile function during reperfusion. These effects of ET-1 were inhibited by administration of the NHE inhibitor methylisobutyl amiloride (MIA) prior to pretreatment with ET-1. Consistent with this, Brunner and Opie [76] have shown recently that the deleterious effects of endothelin during ischemia and reperfusion could be attenuated not only by ET_A receptor antagonism, but also by NHE inhibition. These observations strongly implicate NHE as a key mediator of the deleterious consequences induced by endothelin receptor stimulation.

Adrenergic stimulation

Phenylephrine-induced stimulation of α_1-ARs selectively within the ischemic zone has been shown by the authors' group [43] to exacerbate reperfusion-induced arrhythmias, with this proarrhythmic effect abolished by α_{1A}-AR antagonism or NHE inhibition, implicating the exchanger in the downstream mechanisms. Within the context of ischemia and reperfusion, α_1-AR stimulation has also been implicated in the induction of ischemic preconditioning in rat myocardium [77]. Because ischemic preconditioning is associated with reduced intracellular acidosis during the prolonged ischemia [78], it is possible that α_1-adrenergic stimulation of sarcolemmal NHE activity may contribute to this phenomenon. However, Gabel et al. [79] have shown recently that H^+ efflux during the prolonged ischemic period is not increased in preconditioned hearts. Furthermore, any stimulation of sarcolemmal NHE activity is unlikely to contribute to the cardioprotective mechanism(s) of ischemic preconditioning because we have shown such protection to be retained (and indeed enhanced) in the presence of NHE inhibition [80].

Thrombin

With regard to thrombin-induced effects during ischemia and reperfusion, Goldstein et al. [81] have shown that in the canine heart in vivo, the incidence of malignant ventricular arrhythmias during acute ischemia is greater following thrombotic coronary occlusion than nonthrombotic balloon occlusion, implicating an arrhythmogenic role for factors (such as thrombin) that are associated with thrombus formation. Indeed, thrombin has now been shown to induce a potent arrhythmogenic effect during ischemia and reperfusion in isolated rat hearts [82,83] in a manner that appears to be reversible by NHE inhibition [83]. A role for the sarcolemmal NHE in mediating the arrhythmogenic action of thrombin is supported by evidence that activation of the thrombin receptor exacerbates the intracellular accumulation of Na^+ during ischemia [84], an observation that is consistent with stimulation of exchanger activity.

Oxidant stress

Hoque and Karmazyn [63] have reported that the contractile dysfunction and impairment of myocyte metabolism induced by the perfusion of isolated rat hearts with H_2O_2 could be attenuated by selective NHE inhibitor HOE 642. In the setting of ischemia and reperfusion, recent evidence has suggested that the exacerbation of myocardial injury by H_2O_2 could be reversed by pretreatment of hearts with HOE 642 [64]. These findings suggest that an increase in sarcolemmal NHE activity may play a key role in the perturbations of cardiac function and metabolism induced by H_2O_2.

Concluding Comments

It is clear from the evidence discussed herein that sarcolemmal NHE activity is modulated by a number of endogenous stimuli (e.g., catecholamines, thrombin, endothelin, and oxidant stress), and that stimulation of the exchanger by these factors may be of pathophysiological significance during ischemia and reperfusion. Undoubtedly, there are other, currently unidentified factors that also regulate sarcolemmal NHE activity in a positive or negative manner. The identification of these factors and delineation of their intracellular signaling mechanisms may identify common pathways that might represent fertile targets for the development of novel therapies for ischemic heart disease.

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Cardiac Sarcolemmal Na^+/H^+ Exchanger Activity in Ischaemia: Potential Regulatory Factors

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The cardiac sarcolemmal Na^+/H^+ exchanger (NHE) extrudes one H^+ in exchange for one Na^+ entering the myocyte, utilizing for its driving force the inwardly directed Na^+ gradient that is maintained by the Na^+/K^+ ATPase. The exchanger is quiescent at physiological values of intracellular pH but becomes activated in response to intracellular acidosis. Recent evidence suggests that a variety of receptor-mediated stimuli (e.g. adrenergic agonists, thrombin and endothelin) and other factors (e.g. oxidant stress, lipid metabolites) that are associated with ischaemia also modulate sarcolemmal NHE activity, by altering its sensitivity to intracellular H^+. Since sarcolemmal NHE activity is believed to be an important determinant of the extent of myocardial injury during ischaemia and reperfusion, regulation of exchanger activity by endogenous factors that are associated with ischaemia is likely to be of pathophysiological importance.
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Key Words: Na^+/H^+ exchanger, ischaemia, acidosis, signal transduction, ion regulation.

Introduction

Na^+/H^+ exchangers (NHEs) are membrane proteins which transport H^+ in exchange for Na^+ in an electroneutral manner, utilizing for their driving force the transmembrane Na^+ gradient[1–3]. Six mammalian isoforms of NHE have so far been identified and these have been termed NHE-1 to NHE-6[4–7]. NHE-1 to NHE-4 are located in the plasma membrane, little is known about the localization and function of NHE-5, and NHE-6 appears to be the molecular homolog of the mitochondrial exchanger. NHE-1 and NHE-6 are ubiquitously distributed in tissue (the latter expressed abundantly in tissues with high mitochondrial content), whereas NHE-2 to NHE-5 show restricted expression[4–7]. Only NHE-1 and NHE-6 transcripts have so far been detected in the heart[4,7] and it is believed that the sarcolemmal NHE of cardiac myocytes is comprised predominantly, if not exclusively, of the NHE-1 isoform[8].

Recently, the sarcolemmal NHE has received attention as a potential mediator of various physiological and pathophysiological phenomena in the myocardium, such as increased myofilament sensitivity to Ca^{2+} in response to stimulation of a variety of G protein-coupled receptors[9–11] and the induction of hypertrophy by mechanical and neurohormonal stimuli[12–15]. However, perhaps the strongest evidence for an important role for the sarcolemmal NHE in cardiac pathophysiology is that implicating the exchanger in the unfavourable sequelae of ischaemia and reperfusion, such as arrhythmias, contractile dysfunction and infarction. Such evidence was obtained initially with non-specific NHE inhibitors (NHEIs) such as amiloride and its 5-amino-substituted derivatives (for reviews, see Avkiran[16] and Fröhlich and Karmazyn[17]) and has been substantiated by more recent studies with novel benzoylguanidine derivatives, such as HOE-694[18], HOE-642 (cariporide)[19], and EMD-85131[20]. These agents have proved to be of considerable investigative value since they are potent inhibitors of NHE[18–20], do not appear to inhibit other ion transportation[18,19] or pH regulatory[21] systems, and exhibit marked selectivity for the NHE-1 isoform of the exchanger[19,20,22]. Pre-clinical studies which have investigated the effects of these novel NHEIs on arrhythmias, contractile dysfunction and infarct size during myocardial ischaemia and reperfusion have been reviewed recently[23] and are discussed elsewhere in this issue.

Cardiac sarcolemmal NHE activity during ischaemia

The original hypothesis[24] that the sarcolemmal NHE may contribute to tissue injury and dysfunction during myocardial ischaemia and reperfusion, which is often referred to as the 'Lazdunski Hypothesis', proposed that sarcolemmal NHE activity was likely to be low during

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ischaemia. However, upon reperfusion, an increase in exchanger activity would arise from the rapid normalization of extracellular pH and the generation of an outwardly directed H^+ gradient, and could mediate reperfusion injury. As noted in a recent review[23], although this hypothesis is supported by studies which have shown a cardioprotective benefit with NHEIs when given shortly before or during reperfusion, it is not consistent with the majority of studies which have shown superior benefit when the NHEI is given prior to or early during ischaemia. The latter findings, alongside evidence that NHEIs attenuate intracellular Na^+ [25–27] and Ca^{2+} [26] accumulation during ischaemia in parallel with their cardioprotective effects, suggest that NHE activity is maintained during ischaemia and contributes substantially to the loss of ionic homeostasis and to the extent of injury.

How does the sarcolemmal NHE retain its activity during ischaemia in the face of the significant extracellular acidosis which is known to accompany intracellular acidosis? First, it is important to note that, although the sarcolemmal NHE is undoubtedly inhibited by extracellular acidosis[28,29], such inhibition is not absolute. Contrary to a common misconception, the primary regulator of NHE activity is not the transmembrane H^+ gradient, but the intracellular pH, through the interaction of intracellular H^+ with the so-called 'H^+ sensor' site of the exchanger protein[2]. Work from the Vaughan-Jones laboratory[29] has shown that the sarcolemmal NHE can remain active and extrude H^+ against an inwardly directed H^+ gradient, provided that the intracellular pH is sufficiently low. Second, certain processes associated with ischaemia, such as the release of neurohormonal mediators, the accumulation of lipid metabolites and the imposition of oxidant stress, may upregulate NHE activity by increasing the sensitivity of the exchanger to intracellular H^+ (this dual mechanism of regulation of sarcolemmal NHE activity is illustrated schematically in Fig. 1). This article will now outline the evidence supporting a potential role for such endogenous factors in maintaining sarcolemmal NHE activity during ischaemia.

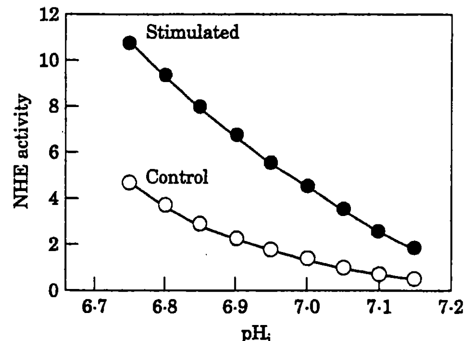

Figure 1 **Dual regulation of sarcolemmal Na^+/H^+ exchanger (NHE) activity in ventricular myocytes. NHE activity is regulated primarily by intracellular pH (pH_i) and increases as pH_i drops (open symbols). This relationship can be shifted in an alkaline direction by a variety of endogenous stimuli such as neurohormonal mediators, oxidant stress and lipid metabolites, such that the sarcolemmal NHE has greater activity at any given pH_i (solid symbols). See text for details.**

NHE regulation by neurohormonal mediators

Catecholamines

It is well established that myocardial ischaemia results in activation of the sympathetic nervous system as well as the local release of norepinephrine within the ischaemic zone[30]. Furthermore, there is evidence that α_1-adrenergic signalling is upregulated in ischaemic myocardium[31]. It is interesting to note that the majority of published work on catecholamine-induced changes in sarcolemmal NHE activity has concentrated on the role of α_1-adrenoceptors (α_1-ARs). Thus, in isolated ventricular myocytes from the rat and guinea-pig, it has been shown that α_1-AR agonists (such as phenylephrine[32,33] and 6-fluoro-norepinephrine[34], usually in the presence of a β_1-AR antagonist) increase sarcolemmal NHE activity, while α_1-AR antagonists (such as prazosin[33,34]) inhibit such NHE-stimulatory effects. In particular, α_1-adrenergic stimulation appears to retain its ability to increase sarcolemmal NHE activity in the presence of extracellular acidosis[32], which has important implications for NHE regulation under ischaemic conditions.

Owing to the lack of selectivity of the pharmacological agents used in previous studies, until recently it has not been possible to draw any conclusions about the identity of the α_1-AR subtype(s) that are involved in α_1-adrenergic stimulation of sarcolemmal NHE activity. Prompted by (1) substantial evidence supporting a key role for sarcolemmal NHE activity in the pathogenesis of ischaemia and reperfusion-induced injury[16], (2) the reported ability of non-selective α_1-adrenergic agonists to increase sarcolemmal NHE activity[32–34] and (3) the observation[35] that the proarrhythmic effect of the non-selective α_1-adrenergic agonist phenylephrine could be reversed not only by NHE inhibition but also by an α_{1A}-AR-selective antagonist, the author's laboratory has recently delineated the roles of α_1-AR subtypes in regulating sarcolemmal NHE activity in adult rat ventricular myocytes[36]. The relevant data provide evidence, for the first time, that α_1-adrenergic stimulation of sarcolemmal NHE activity is likely to be mediated selectively via the α_{1A}-AR subtype[36]. Since the α_{1A}-AR is the dominant α_1-AR subtype expressed in human myocardium[37] and myocardial hypertrophy appears to be accompanied by its transcriptional induction[38], confirmation of α_{1A}-AR-mediated regulation of sarcolemmal NHE activity may not only provide a potential target for therapeutic intervention but may also reveal a mechanism for the

increased susceptibility of hypertrophied myocardium to the consequences of ischaemia and reperfusion (such as arrhythmias and contractile dysfunction).

In considering adrenergic regulation of the sarcolemmal NHE, it is important to note that β-adrenergic stimulation also affects exchanger activity. Published studies agree that, in contrast to the effect of α_1-adrenergic stimulation, β_1-adrenergic stimulation inhibits sarcolemmal NHE activity in a variety of species[33,39,40], probably through a cAMP-mediated pathway[39–41]. Therefore, the effects of the endogenous catecholamines noradrenaline and adrenaline on sarcolemmal NHE activity may depend on the relative density or availability of α_1- compared with β_1-ARs. These can be modulated by genetic factors (which may account for species-specific responses), accompanying disease (such as heart failure, which is associated with β_1-AR downregulation, or ischaemia, which enhances α_1-AR signalling) or pharmacological therapy (in particular, the use of adrenergic antagonists). It is noteworthy that, at least in rat ventricular myocytes, the α_1-AR response appears to dominate, since noradrenaline produces a significant increase in sarcolemmal NHE activity (author's unpublished data).

Thrombin

The commonest cause of acute myocardial ischaemia in man is intracoronary thrombosis[42], and thrombosis is associated with elevated levels of thrombin in the vicinity of the evolving thrombus[43]. Therefore, the biological actions of thrombin may be of pathophysiological significance during myocardial ischaemia in the clinical setting. It is now well established that, in addition to its role in blood coagulation and thrombus formation, thrombin induces a variety of cellular responses through receptor-mediated pathways (for review, see Coughlin[44]). Recently, the author's laboratory has published the first evidence that both thrombin and the synthetic thrombin receptor activating hexapeptide SFLLRN increase sarcolemmal NHE activity in adult rat ventricular myocytes[45]. The common ability of thrombin and SFLLRN to stimulate sarcolemmal NHE activity is consistent with this action being mediated by the thrombin receptor that was first cloned by Vu *et al.*[46] and is now known as protease-activated receptor 1 (PAR1), the mRNA for which has been shown to be expressed in adult rat ventricular myocytes[45]. However, recent findings suggest that other protease-activated receptors may also be involved. Connolly *et al.*[47] have shown that in mice with targeted disruption of the PAR1 gene, responsiveness to thrombin is lost in some cell types (e.g. fibroblasts) but retained in others (e.g. platelets). This indicates the existence of additional thrombin receptor(s) and has led to the suggestion that different thrombin receptors may have tissue-specific roles[47]. Indeed, a second thrombin receptor (PAR3) has now been cloned and appears to be expressed in human myocardium[48]. Furthermore, there is preliminary evidence[49] for the expression of functional PAR2, a trypsin-activated receptor that is responsive also to the PAR1-activating peptide SFLLRN[50], in neonatal rat ventricular myocytes. These developments necessitate verification of the hypothesis that cardiac sarcolemmal NHE is regulated by PAR1 and require determination of the roles, if any, of PAR2 and PAR3. Defining which of these receptors regulate sarcolemmal NHE activity clearly is critical to the potential therapeutic manipulation of this signalling pathway.

Endothelin

There is evidence that endothelin is released during myocardial ischaemia and reperfusion[51] and that this is accompanied by an upregulation of endothelin receptors[52]. Therefore, the cellular actions of endothelin may also assume pathophysiological importance during myocardial ischaemia and reperfusion. Within the context of the present article, it is notable that endothelin has been shown to stimulate sarcolemmal NHE activity, in isolated rat ventricular myocytes[9,53] as well as in canine cardiac Purkinje fibres[40]. It is of interest that recent evidence suggests that responsiveness of the sarcolemmal NHE to stimulation by endothelin and angiotensin II (see below) may be altered in pressure overload-induced cardiac hypertrophy[53].

Angiotensin II

Several studies have shown that angiotensin-converting enzyme (ACE) inhibition can attenuate the detrimental consequences of myocardial ischaemia and reperfusion, evidently through reduced bradykinin breakdown[54–56]. Nevertheless, data indicating that inhibition of angiotensin II action via type 1 receptors, through either molecular biological[57,58] or pharmacological[57–61] means, can mimic at least some of the cardioprotective effects of ACE inhibition suggest that angiotensin II itself may be detrimental during ischaemia and reperfusion. This evidence, together with that suggesting a stimulation of sarcolemmal NHE activity in response angiotensin II[11,62,63], implicates the exchanger in the potential detrimental effects of this peptide hormone in the setting of myocardial ischaemia and reperfusion. Consistent with this, recent studies in isolated myocytes support a causal role for angiotensin II-mediated activation of the sarcolemmal NHE in cell injury during metabolic inhibition[64]. Nevertheless, it may be noted that angiotensin II does not appear to be such a potent stimulus for the sarcolemmal NHE when compared with the other mediators discussed above[11,53]. Furthermore, recent work in the author's laboratory suggests that subtypes 1 and 2 of the angiotensin II receptor (AT_1 and AT_2 receptors) may have opposing actions in the regulation of sarcolemmal NHE activity[65]. This dual regulation of the sarcolemmal NHE may have functional implications in disease conditions

that are associated with an altered AT_1:AT_2 receptor ratio, such as heart failure[66,67].

NHE regulation by other endogenous factors

Endogenous factors that have often been implicated in the pathogenesis of ischaemia and reperfusion-induced injury and dysfunction and particularly in the loss of ionic homeostasis, include the sarcolemmal accumulation of lipid metabolites[68] and the generation of oxidant stress[69]. In this context, it is interesting to note that exposure to exogenous lysophosphatidylcholine[70] and hydrogen peroxide[71,72] have both recently been shown recently to stimulate the sarcolemmal NHE in cultured neonatal[71] and freshly isolated adult[70,72] rat ventricular myocytes. Although the mechanism(s) underlying lysophosphatidylcholine-induced stimulation of sarcolemmal NHE activity remain(s) unknown, there is evidence to suggest that the hydrogen peroxide effect may occur via activation of specific intracellular signaling pathway(s), such as the extracellularly regulated kinase (ERK) cascade[71]. Thus, it appears that factors other than classical receptor-mediated stimuli may also regulate sarcolemmal NHE activity.

Impact of NHE-stimulatory factors in ischaemia and reperfusion

What is the potential impact, in the setting of myocardial ischaemia and reperfusion, of stimulation of sarcolemmal NHE activity by the factors discussed above? Much of the evidence in this area has been obtained through the application of exogenous stimuli in in vitro models of ischaemia and reperfusion. Thus, work in Karmazyn's laboratory has shown that both α_1-AR stimulation (in the presence of an adenosine antagonist)[73] and endothelin[74] exacerbate post-ischaemic contractile dysfunction in isolated rat hearts. It is particularly relevant to the present article that the deleterious effects of both stimuli could be reversed by NHE inhibition[73,74], thus implying a key role for the exchanger in the injurious mechanisms downstream of receptor activation. Consistent with this, Brunner and Opie[75] have recently shown that the deleterious effects of endothelin during ischaemia and reperfusion could be attenuated not only by ET_A receptor antagonism but also by NHE inhibition. Furthermore, α_1-AR stimulation selectively within the ischaemic zone has been shown by the author's group[35] to exacerbate reperfusion-induced arrhythmias; this proarrhythmic effect is nullified by α_{1A}-AR antagonism or NHE inhibition, implicating the exchanger in the downstream mechanisms.

Within the context of ischaemia and reperfusion, α_1-AR stimulation has also been implicated in the induction of ischaemic pre-conditioning in rat myocardium[76]. Since ischaemic pre-conditioning is associated with reduced intracellular acidosis during prolonged ischaemia[77], it is possible that α_1-adrenergic stimulation of sarcolemmal NHE activity may contribute to this phenomenon. However, Gabel *et al.*[78] have shown recently that the H^+ efflux during the prolonged ischaemic period is not increased in pre-conditioned hearts. Furthermore, any stimulation of sarcolemmal NHE activity is unlikely to contribute to the cardioprotective mechanism(s) of ischaemic pre-conditioning, since such protection is retained (and indeed enhanced) in the presence of NHE inhibition[79].

In studying thrombin-induced effects during ischaemia and reperfusion, Goldstein *et al.*[80] have shown that the incidence of malignant ventricular arrhythmias in the canine heart in vivo during acute ischaemia is greater following thrombotic coronary occlusion than non-thrombotic balloon occlusion. This implicates an arrhythmogenic role for factors (such as thrombin) that are associated with thrombus formation. Thrombin has now been shown to induce a potent arrhythmogenic effect during ischaemia and reperfusion in isolated rat hearts[81,82] in a manner that appears to be reversible by NHE inhibition[82]. A role for the sarcolemmal NHE in mediating the arrhythmogenic action of thrombin is supported by evidence that activation of the thrombin receptor exacerbates the intracellular accumulation of Na^+ during ischaemia[83], an observation consistent with stimulation of exchanger activity.

The role of increased sarcolemmal NHE activity in the potential detrimental effects of angiotensin II during myocardial ischaemia and reperfusion has not been characterized. However, the recent observation that AT_1 and AT_2 receptors may mediate opposing actions in the regulation of sarcolemmal NHE activity[65] raises the possibility that selective antagonists of the AT_1 receptor may be of benefit during ischaemia/reperfusion. They can inhibit NHE stimulation both by blocking AT_1-mediated excitatory actions and by enhancing AT_2-mediated inhibitory actions.

Finally, it is noteworthy that the cardiodepressant effects of low-dose lysophosphatidylcholine[70] and low-dose hydrogen peroxide in combination with myocardial ischaemia and reperfusion[84] could both be attenuated by pharmacological NHE inhibition. This suggests that the detrimental effects of these factors on myocardial integrity and function either may be mediated through increased sarcolemmal NHE activity or that they may require an active exchanger.

Conclusion

It is apparent from the evidence discussed above that sarcolemmal NHE activity is modulated by a variety of endogenous factors (e.g. catecholamines, thrombin, endothelin, angiotensin II, oxidant stress, lysophosphatidylcholine) and that stimulation of the exchanger by these factors may be of pathophysiological significance during ischaemia and reperfusion. Undoubtedly,

there are other, currently unidentified, factors that also regulate sarcolemmal NHE activity in a positive or negative manner. The identification of such factors, characterization of their potential interplay, and delineation of the signalling mechanisms that underlie their effects on sarcolemmal NHE activity may identify common pathways. The latter might, in turn, represent fertile new targets for therapeutic manipulation of the exchanger, particularly in ischaemic heart disease.

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Sarcolemmal Na^+/H^+ Exchanger Activity and Expression in Human Ventricular Myocardium

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OBJECTIVES To determine sarcolemmal Na^+/H^+ exchanger (NHE) activity and expression in human ventricular myocardium.

BACKGROUND Although the sarcolemmal NHE has been implicated in various physiological and pathophysiological phenomena in animal studies, its activity and expression in human myocardium have not been studied.

METHODS Ventricular myocardium was obtained from unused donor hearts with acute myocardial dysfunction (n = 5) and recipient hearts with chronic end stage heart failure (n = 11) through a transplantation program. Intracellular pH (pH_i) was monitored in enzymatically isolated single ventricular myocytes by microepifluorescence. As the index of sarcolemmal NHE activity, the rate of H^+ efflux at a pH_i of 6.90 ($J_{H6.9}$) was determined after the induction of intracellular acidosis in bicarbonate-free medium. Na^+/H^+ exchanger isoform 1 (NHE1) expression in ventricular myocardium was determined by immunoblot analysis.

RESULTS Human ventricular myocytes exhibited readily detectable sarcolemmal NHE activity after the induction of intracellular acidosis, and this activity was suppressed by the NHE1-selective inhibitor HOE-642 (cariporide) at 1 μmol/L. Sarcolemmal NHE activity of myocytes was significantly greater in recipient hearts ($J_{H6.9}$ = 1.95 ± 0.18 mmol/L/min) than it was in unused donor hearts ($J_{H6.9}$ = 1.06 ± 0.15 mmol/L/min). In contrast, NHE1 protein was expressed in similar abundance in ventricular myocardium from both recipient and unused donor hearts.

CONCLUSIONS Sarcolemmal NHE activity of human ventricular myocytes arises from the NHE1 isoform and is inhibited by HOE-642. Sarcolemmal NHE activity is significantly greater in recipient hearts with chronic end-stage heart failure than it is in unused donor hearts, and this difference is likely to arise from altered posttranslational regulation. (J Am Coll Cardiol 2000; 36:534–40) © 2000 by the American College of Cardiology

The sarcolemmal Na^+/H^+ exchanger (NHE) of cardiac myocytes is believed to be the ubiquitous NHE isoform 1 (NHE1) of the multigene NHE family (1) and contributes significantly to the integrated control of intracellular pH (pH_i) in this cell type (2). Na^+/H^+ exchanger isoform 1 activity is regulated primarily by pH_i through the interaction of H^+ with a "H^+-sensor" site on the exchanger's membrane domain in a manner that results in exchanger activation in response to intracellular acidosis (3,4). Consistent with this, the cardiac sarcolemmal NHE is relatively quiescent at physiological pH_i, but its activity increases progressively as pH_i declines (2). Sarcolemmal NHE activity is also modulated by a variety of neurohormonal stimuli such as $alpha_1$-adrenergic agonists (5,6), endothelin (7), thrombin (8) and angiotensin II (9) through receptor-mediated mechanisms. These agents appear to increase sarcolemmal NHE activity by increasing the pH_i-sensitivity of the exchanger, which is the mechanism known to underlie growth factor-induced stimulation of NHE1 (3,4).

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Recently, the sarcolemmal NHE has received attention as a potential mediator of various physiological and pathophysiological phenomena in myocardium such as inotropic responses to a variety of agonists (7,10–13) and muscle stretch (14) and the induction of hypertrophy by mechanical (15) and neurohormonal (16) stimuli. In addition, work with the novel NHE1-selective inhibitors in our laboratory (17–20) and by others (for recent reviews, see Avkiran [21] and Karmazyn et al. [22]) has provided support for the hypothesis that sarcolemmal NHE activity is an important determinant of the severity of arrhythmias, contractile dysfunction and tissue necrosis during myocardial ischemia and reperfusion. Recent evidence suggests that NHE activity may also be involved in the induction of myocyte apoptosis during ischemia and reperfusion (23,24) and metabolic inhibition and recovery (25). These experimental findings have instigated trials with NHE inhibitors in clinical settings of myocardial ischemia and reperfusion, such as the recent GUARDIAN (Guard during Ischemia Against Necrosis) trial in patients with acute coronary syndromes (26).

Although significant advances have been made, as described above, in understanding of the regulation and roles of the sarcolemmal NHE, these have been achieved exclusively through the use of myocardial tissue and cells from a variety of animal species. As a consequence, the applicability to man of many of the findings is unconfirmed, and little is

Abbreviations and Acronyms

$beta_i$	= intrinsic buffering power
dpH_i/dt	= rate of recovery of pH_i
J_H	= rate of H^+ efflux
$J_{H6.9}$	= rate of H^+ efflux at pH_i 6.90
NCE	= Na^+/Ca^{2+} exchanger
NHE	= Na^+/H^+ exchanger
NHE1	= Na^+/H^+ exchanger isoform 1
pH_i	= intracellular pH

known regarding sarcolemmal NHE activity and expression in human myocardium. We have used a microepifluorescence technique to determine, for the first time, sarcolemmal NHE activity in ventricular myocytes isolated from explanted human hearts. In addition, we determined NHE expression in ventricular myocardium of those hearts by immunoblot analysis.

METHODS

Human ventricular myocytes. Ventricular myocardium was obtained from explanted human hearts comprising 11 recipient hearts with chronic end-stage heart failure (eight with ischemic heart disease and three with dilated cardiomyopathy) and five donor hearts that were not used for transplantation due to a low ejection fraction, as described recently (27). Table 1 shows the characteristics of the individuals from whom ventricular tissue was obtained over a period of 15 months and the number and ventricular origin of the cells from each preparation that were used in the microepifluorescence studies. The mean age of patients from whom recipient hearts were obtained was 49.3 ± 3.2 years (n = 11), which was significantly greater ($p < 0.05$, unpaired *t* test) than that of the individuals from whom the unused donor hearts originated (33.8 ± 6.2 years, n = 5).

Table 1. Origin of Human Ventricular Myocytes Used in the Study

Heart No.	Status	Age (yr)/ Gender	Cells Studied
1	Recipient (IHD)	55/M	4 (RV)
2	Unused donor	22/F	12 (RV)
3	Unused donor	19/F	16 (RV)
4	Recipient (DCM)	20/M	10 (RV)
5	Recipient (DCM)	50/M	7 (LV)
6	Recipient (IHD)	48/M	11 (LV)
7	Unused donor	42/M	7 (LV)
8	Recipient (IHD)	49/M	4 (RV)
9	Recipient (IHD)	47/M	4 (RV)
10	Recipient (IHD)	55/M	5 (RV)
11	Unused donor	34/F	4 (LV)
12	Unused donor	52/F	4 (LV)
13	Recipient (IHD)	59/M	6 (RV)
14	Recipient (IHD)	56/F	4 (LV)
15	Recipient (IHD)	48/M	10 (RV)
16	Recipient (DCM)	55/M	4 (LV)

DCM = dilated cardiomyopathy; IHD = ischemic heart disease; LV = left ventricular; RV = right ventricular.

Myocytes were isolated by enzymatic digestion of left or right ventricular myocardium, as described in detail previously (28), and only rod shaped cells were used in the microepifluorescence studies. Of the 112 myocytes listed in Table 1, 87 (54 from recipient hearts and 33 from unused donor hearts) were used for determination of sarcolemmal NHE activity. The remainder were used for in situ calibration of the pH-sensitive fluorescent dye carboxy-seminaphthorhodafluor-1 (C-SNARF-1), estimation of intrinsic buffering power ($beta_i$) and determination of the NHE-inhibitory efficacy of HOE-642 (cariporide), a potent NHE1-selective inhibitor (29), which we have shown to inhibit sarcolemmal NHE activity in rat ventricular myocytes (19) and which was tested in the GUARDIAN trial (26).

Measurement of pH_i and $beta_i$. The microepifluorescence-based approach that was used to monitor pH_i in single cells has been described in detail previously (8) and used in our earlier studies with rat ventricular myocytes (5,6,8,9,19,30). In brief, cells loaded with C-SNARF-1 were placed on a glass coverslip in a 100 μL chamber and fluorescence recordings made using a dual-emission photometer system (D104C; Photon Technology International Inc.) during continuous superfusion (3.5 mL/min) with bicarbonate-free Tyrode's solution (34°C). Calibration was with nigericin-containing solutions, and $beta_i$ was estimated during stepwise removal of extracellular NH_4Cl, as described (8). The calibration curve was obtained by nonlinear least squares fit of normalized emission ratios; this gave best-fit values for pK and *a* of 7.08 and −1.46, respectively, which are similar to the values previously obtained in rat ventricular myocytes (8).

Determination of sarcolemmal NHE activity. The rate of acid efflux (J_H) was used as the index of sarcolemmal NHE activity, as in our previous work (5,6,8,9,19,30). After 5 to 10 min of superfusion with Tyrode's solution, myocytes were subjected to intracellular acidosis (in order to activate the sarcolemmal NHE) by transient (5 min) exposure to 30 mmol/L NH_4Cl and its subsequent washout (14 min). Since pH_i was lowered to ≤6.90 upon NH_4Cl washout in all cells that were subjected to this protocol, J_H was estimated at a pH_i of 6.90 and termed $J_{H6.9}$.

In experiments in which the NHE inhibitor HOE-642 was used, cells were subjected to two consecutive acid pulses (as described above) separated by 10 min. During the second acid pulse, HOE-642 (1 μmol/L) was included in the superfusate during exposure to NH_4Cl and the first 7 min of NH_4Cl washout; HOE-642 was subsequently removed from the Tyrode's solution to assess the reversibility of drug action. With the same protocol, myocyte contraction was monitored using a video edge-detection system, as described before (31).

Determination of NHE and Na^+/Ca^{2+} exchanger expression. Myocardial expression of NHE1 was determined at protein level by immunoblot analysis. In order to avoid potential problems with differential recoveries of mem-

branes from unused donor and recipient heart samples, immunoblot analysis was conducted using unfractionated tissue homogenates as described recently (32). Na^{+}/Ca^{2+} exchange (NCE) expression was also determined as a positive control for the presence of sarcolemmal protein in the samples. Ventricular tissue samples (approximately 0.2 g) obtained from regions without overt signs of fibrosis or damage were rapidly thawed, weighed and homogenized for 3 to 4 min in lysis buffer (sorbitol [5%], histidine [pH 7.4; 25 mmol/L], Na_2EDTA [50 mmol/L], KCl [50 mmol/L], leupeptin [1 μg/μL], PMSF [0.5 mmol/L] and benzamidine [1 mmol/L]). For NHE analysis, 0.5% SDS and 0.1% beta-mercaptoethanol were added to 25 μL of sample containing 100 μg of protein. After boiling for 5 min, 55 μL of lysis buffer and 5 μL of polyoxyethylene-8-lauryl ether (Sigma, Poole, United Kingdom) were added to the sample. After incubation at 37°C for 15 h, 50 μL of 3× SDS-sample buffer was added and the sample boiled for 10 min. For NCE analysis, SDS-sample buffer (×1) was added directly to an aliquot of tissue homogenate to obtain a final protein concentration of 2 μg/μL and the sample boiled for 10 min. After centrifugation, all samples (100 μg protein) were subjected to electrophoresis using a 7.5% SDS-polyacrylamide gel, and the separated proteins were transferred to polyvinylidene difluoride membranes. Immunoblot analysis was performed using mouse monoclonal antibody for NHE1 (1:500 dilution; #MAB3140, Chemicon International Inc., Harrow, United Kingdom) or NCE (1:500 dilution; #C2C12, Cambridge BioScience, United Kingdom) in conjunction with antimouse secondary antibody and enhanced chemiluminescence (Amersham Pharmacia Biotech, Little Chalfont, United Kingdom).

Statistical analysis. For each heart, $J_{H6.9}$ was determined in up to 11 cells of either left or right ventricular origin (Table 1), and an average value was obtained. Data for unused donor and recipient groups are expressed as mean ± SEM, with the n values representing the number of hearts in each group. The unpaired *t* test was used to compare $J_{H6.9}$ in recipient versus unused donor hearts, and $p < 0.05$ was considered significant.

RESULTS

The rate of recovery of pH_i (dpH_i/dt) after an intracellular acid load is determined not only by the J_H but also by the $beta_i$ at the relevant pH_i (33). Therefore, to obtain accurate estimates of J_H (as the index of sarcolemmal NHE activity), it was necessary to determine $beta_i$ in both populations of myocytes. Our data revealed no difference in $beta_i$ between myocytes from recipient and unused donor hearts (Fig. 1). Linear least squares regression analysis of all data points gave the equation $beta_i = -33.7 \cdot pH_i + 260.1$, which is very similar to the equation that describes the relationship between pH_i and $beta_i$ in rat ventricular myocytes (8). Basal pH_i values were not significantly different between myocytes from recipient (7.31 ± 0.02) and unused donor (7.29 ± 0.03) hearts, and both groups of cells acidified to a similar extent upon washout of NH_4Cl, with minimal pH_i values of 6.70 ± 0.03 and 6.71 ± 0.03, respectively. Myocytes from recipient hearts exhibited faster recovery from acidosis, as illustrated by the representative recordings shown in Figure 2A. Quantitative analysis of such data revealed that $J_{H6.9}$ was significantly greater in myocytes from recipient hearts than it was in cells from unused donor hearts (Fig. 2B).

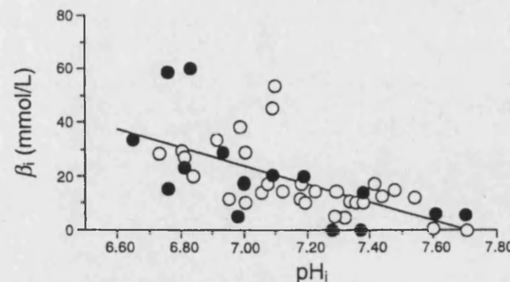

Figure 1. The relationship between pH_i and β_i in human ventricular myocytes obtained from unused donor hearts **(open symbols)** and recipient hearts with end-stage heart failure **(solid symbols)**. Linear least squares regression analysis of all points gave the equation $\beta_i = -33.7 \cdot pH_i + 260.1$. β_i = intrinsic buffering power; pH_i = intracellular pH.

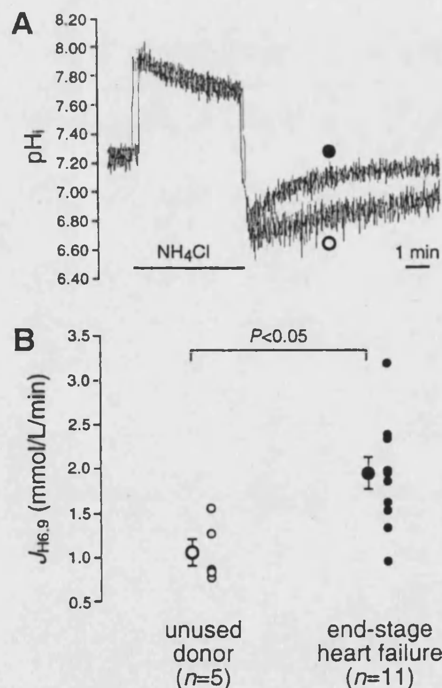

Figure 2. **(A)** Representative single-cell pH_i recordings during acid pulses and **(B)** individual and mean $J_{H6.9}$ values in ventricular myocytes obtained from unused donor hearts **(open symbols)** and recipient hearts with end-stage heart failure **(solid symbols)**. In **(B)**, n indicates the number of hearts in each group. pH_i = intracellular pH; $J_{H6.9}$ = rate of H^{+} efflux at pH_i 6.90.

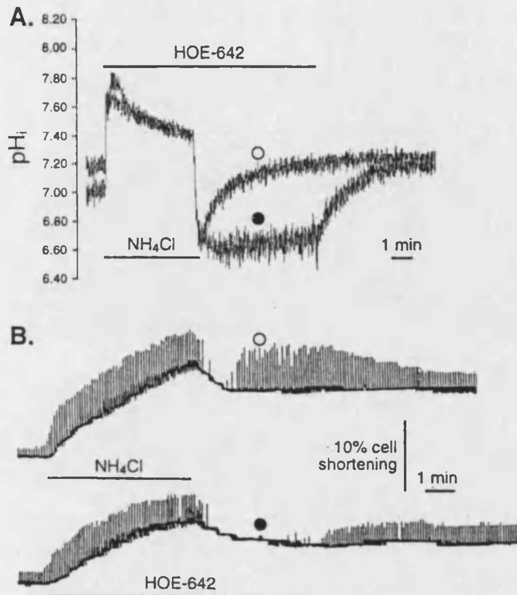

Figure 3. Representative recordings of **(A)** pH_i and **(B)** cell contraction in human ventricular myocytes from recipient hearts with end-stage heart failure during two consecutive acid pulses. The first acid pulses **(open symbols)** were under control conditions whereas during the second acid pulses **(solid symbols)** HOE-642 (1 μmol/L) was present during exposure to NH_4Cl and the first 7 min of NH_4Cl washout, as indicated by the **horizontal bars**. The baseline changes in **(B)** reflect changes in resting cell length. pH_i = intracellular pH.

To confirm that, under the conditions used, recovery of pH_i from acidosis in human ventricular myocytes occurred predominantly by H^+ efflux through the sarcolemmal NHE, we exposed myocytes to intracellular acidosis in the presence of HOE-642. As illustrated by the representative recordings shown in Figure 3A, although there was rapid recovery from acidosis under control conditions, such recovery was markedly suppressed in the presence of HOE-642. The effect of HOE-642 was rapidly reversible, such that when the inhibitor was removed from the superfusion solution, pH_i recovered from acidosis at a rate comparable with that seen under control conditions (Fig. 3A). Figure 3B illustrates that the changes in pH_i were paralleled by changes in the amplitude of cell contraction. Thus, intracellular alkalosis during NH_4Cl exposure was associated with an increase in contraction amplitude, while intracellular acidosis after NH_4Cl washout was accompanied by a reduction in contraction amplitude. Furthermore, HOE-642 depressed the recovery of cell contraction after NH_4Cl washout, in parallel with its inhibitory effect on pH_i recovery from acidosis.

Immunoblot analysis of a random selection of ventricular myocardium from unused donor and recipient hearts revealed that the 110 kDa NHE1 protein was expressed in similar abundance in all samples (Fig. 4A). Na/Ca^{2+} exchanger expression was also readily detected in all samples as

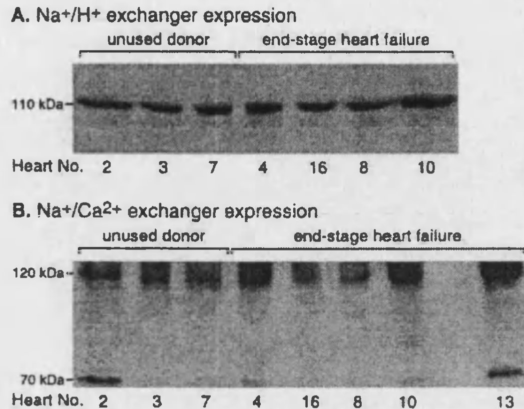

Figure 4. Autoradiograms illustrating protein expression of **(A)** the Na^+/H^+ exchanger (NHE1 isoform) and **(B)** the Na^+/Ca^{2+} exchanger in ventricular samples from unused donor hearts and recipient hearts with end-stage heart failure. Heart numbers relate to Table 1; in **(B)** the lane between heart numbers 10 and 13 contained size markers.

two proteins of 120 and 70 kDa; these have been shown previously to represent the intact NCE and a proteolytic fragment, respectively, in human myocardium (32). In contrast to NHE1, however, in three of the five recipient hearts that were studied (heart numbers, 4, 10 and 13 in Table 1), NCE protein was present in markedly greater abundance (Fig. 4B). This difference in NCE abundance, which is consistent with earlier reports (27,34), did not arise from differential protein loading since Coomassie blue staining (not shown) revealed comparable loading of samples.

DISCUSSION

Sarcolemmal NHE activity in human ventricular myocytes. This study is the first to measure sarcolemmal NHE activity in ventricular myocytes from human hearts. Our data show that, after the induction of intracellular acidosis, sarcolemmal NHE activity is readily detectable in human myocytes, as has been shown to be the case in ventricular myocytes from other species (2,8,11). Interestingly, sarcolemmal NHE activity in ventricular myocytes from unused donor hearts appeared to be lower than that in ventricular myocytes from normal rat hearts, measured using the same equipment and methodology. Thus, mean $J_{H6.9}$ was 1.06 ± 0.15 mmol/L/min in five unused donor hearts (33 cells) in this study, but 2.76 ± 0.26 mmol/L/min in 37 rat cells randomly selected from those studied during an overlapping period (5). This suggests the existence of species-specific differences in the expression or regulation of the sarcolemmal NHE.

Our finding that pH_i recovery from acidosis in human ventricular myocytes was inhibited by 1 μmol/L HOE-642 confirms that such recovery arose from H^+ efflux through the sarcolemmal NHE. Furthermore, this finding indicates that the sarcolemmal NHE of human ventricular myocytes

is indeed NHE1 since at 1 μmol/L HOE-642 is a selective inhibitor of this isoform (29). This is consistent with work by Fliegel and colleagues (35) who cloned NHE1 from a human cardiac cDNA expression library. We also found that changes in pH_i, induced by NH_4Cl pulses in the absence or presence of HOE-642, produced parallel changes in the amplitude of myocyte shortening. This is in keeping with the established importance of pH_i in regulating myocardial contractility (36) and consistent with the recent observations of Ito et al. (37) on the effects of NH_4Cl pulses on cell shortening in rat ventricular myocytes.

Unused donor versus recipient myocardium. An interesting and potentially important finding of this study is the significantly greater sarcolemmal NHE activity of myocytes in recipient hearts with chronic end-stage heart failure relative to unused donor hearts with acute myocardial dysfunction. That the abundance of NHE1 protein was similar in ventricular tissue from unused donor hearts and recipient hearts with end-stage heart failure suggests that posttranslational mechanisms are likely to be responsible for this difference in sarcolemmal NHE activity. Although these mechanisms cannot be identified on the basis of the present findings, it is interesting to note recent evidence that the activities of protein kinase C (38) and Ca^{2+}/calmodulin dependent kinase (39) are increased in human myocardium with end-stage heart failure since both kinases have been proposed as stimulatory regulators of sarcolemmal NHE activity in rat ventricular myocytes (6–9,40).

In addition to a potential role for the kinase-mediated signaling pathways outlined above, it may be argued that an altered intracellular Na^+ concentration, arising from an increased NCE activity (see below), could also contribute to the greater sarcolemmal NHE activity in recipient hearts with end-stage heart failure. This is unlikely, however, since recent work in sheep Purkinje fibers (41) has shown that variation in the intracellular Na^+ concentration is not a physiologically important regulator of NHE activity in the heart.

Ventricular myocytes from human myocardium with end-stage heart failure exhibit varying degrees of hypertrophy (42). Therefore, the possibility that the observed difference in $J_{H6.9}$ between recipient and unused donor hearts may simply reflect a difference in the myocyte membrane surface area to volume ratio needs to be considered. In this context, a recent paper (43) has reported the first direct measurements of membrane surface area (measured by cell capacitance) and cell volume (measured by confocal microscopy) in control versus hypertrophied ventricular myocytes. The findings of that study (43), which used rat ventricular myocytes, have revealed that the membrane surface area to volume ratio remains constant over a threefold increase in cell volume, with no significant difference in this ratio between control and hypertrophied cells. Therefore, the difference in $J_{H6.9}$ observed in this study between unused donor versus recipient hearts is unlikely to be an artefact that arises from myocyte hypertrophy in the latter; instead, it is likely to reflect a true difference in sarcolemmal NHE activity.

It is notable that, although sarcolemmal NHE activity of myocytes was significantly greater in recipient hearts with end-stage heart failure under conditions of intracellular acidosis, basal pH_i was not altered. This may indicate that, at physiological values of pH_i (>7.10), the sarcolemmal NHE of myocytes was quiescent in both recipient and unused donor hearts, as is the case in ventricular myocytes from a variety of animal species (2,8,11). Maximum NHE activity could not be determined in this study because it was not possible to lower pH_i below approximately 6.70 without compromising myocyte viability.

Potential clinical relevance of findings. The NHE phenotype of healthy human myocardium is unknown and may differ from that of the unused donor hearts used in this study. However, if it is assumed that our novel data reflect increased sarcolemmal NHE activity in end-stage heart failure, then this change could have important (patho)physiological consequences. In particular, the greater sarcolemmal NHE activity of failing myocardium may increase its susceptibility to injury and dysfunction during ischemia and reperfusion, in view of the proposed role of the exchanger in this setting (see introduction). Indeed, experimental studies have suggested that failing myocardium is more susceptible to contractile dysfunction (44) and ventricular fibrillation (45) during ischemia and reperfusion. In this context, it is important to note that: (1) the mechanisms that underlie the detrimental effects of increased NHE activity during myocardial ischemia and reperfusion are thought to involve Ca^{2+} influx through NCE, operating in reverse mode (46), and (2) expression of NCE protein is increased in failing human myocardium (as shown in previous studies [27,34] and confirmed here), and this is accompanied by greater NCE activity (47). In the light of our findings, the question of whether increased NHE activity contributes to the development of heart failure also needs to be addressed, particularly in view of the in vitro data that pharmacological NHE inhibition attenuates the development of hypertrophy in response to mechanical and neurohormonal stimuli in neonatal (15) and adult (16) rat ventricular myocytes.

Our data may also have wider clinical relevance because they represent the first direct evidence that human ventricular myocytes express a functional NHE1 protein whose activity is inhibited by HOE-642 in a readily reversible manner. This NHE1-selective inhibitor was used in the recent GUARDIAN trial, whose primary objective was to determine whether NHE inhibition decreases the combined incidence of mortality and myocardial infarction (both Q-wave and non-Q-wave) in patients with acute coronary syndromes (26). The preliminary results of this trial, as presented at the 48th Scientific Sessions of the American College of Cardiology (48), have shown no significant reduction in the composite incidence of death and myocardial infarction in response to drug treatment in the overall study population. Nevertheless, with the highest dose of

HOE-642 (120 mg intravenously three times a day), there were significant reductions in the composite incidence of death and myocardial infarction in patients undergoing surgical revascularization and in the incidence of Q-wave myocardial infarction in the other patient populations (48). Although many factors may have contributed to these findings, including the presence or absence of timely reperfusion (without which NHE inhibition would not be expected to provide significant benefit [21]), it would be important to determine whether an NHE-inhibitory concentration (≥1 μmol/L) of HOE-642 was maintained in the circulation during the period of risk in the various study groups.

Concluding comments. Our present findings have shown that ventricular myocytes from explanted human hearts exhibit sarcolemmal NHE activity, which arises from the NHE1 isoform and is inhibited by HOE-642 in a reversible manner. Such activity is significantly greater in recipient hearts with chronic end-stage heart failure than it is in unused donor hearts with acute myocardial dysfunction. This difference in sarcolemmal NHE activity occurs in the absence of a difference in NHE1 protein expression in recipient versus donor myocardium, which suggests the involvement of posttranslational regulatory mechanisms. Identification of the relevant molecular mechanisms and determination of the functional significance of the observed difference in sarcolemmal NHE activity require further investigation.

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REVIEW ARTICLE

Na^+/H^+ Exchange Inhibitors for Cardioprotective Therapy: Progress, Problems and Prospects

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Extensive pre-clinical work indicates that inhibition of the sarcolemmal Na^+/H^+ exchanger (NHE) affords significant protection to myocardium subjected to ischemia and reperfusion, predominantly through reduced intracellular accumulation of Na^+ and consequently Ca^{2+}. In contrast, recent clinical studies with the NHE inhibitors cariporide and eniporide in patients with evolving myocardial infarction (MI) and those at risk of MI have provided mixed and somewhat contradictory data. The experimental evidence suggests that the key mechanism through which NHE inhibitors afford protection consists in slowing the progression of myocardial injury during ischemia and thereby enhancing myocardial salvage by reperfusion. It follows from this that, to obtain maximum cardioprotective benefit, 1) the NHE inhibitor must be present in jeopardized myocardium, at a concentration sufficient to inhibit NHE activity, before (or as soon as possible after) the onset of ischemia, and 2) ischemia must be terminated by timely reperfusion. Thus, in the GUARDIAN trial, the cardioprotective efficacy of cariporide was limited to the subset of high-risk patients who underwent coronary artery bypass graft surgery, in whom both prerequisites could be readily fulfilled. In contrast, no cardioprotective benefit was observed in the ESCAMI trial, in which eniporide was administered late as an adjunct to reperfusion therapy in patients with evolving MI. Ongoing clinical studies will determine whether NHE inhibition will find therapeutic application in the setting of cardiac surgery, while pre-clinical investigations continue to test the potential of NHE inhibitors in the treatment of other cardiovascular diseases such as heart failure. (J Am Coll Cardiol 2002;39:747–53) © 2002 by the American College of Cardiology Foundation

Since the latter part of the 20th century, investigation of the mechanisms that contribute to myocardial injury and dysfunction during ischemia and reperfusion has been a major occupation of cardiovascular investigators. This effort has been based on the expectation that such an understanding may lead to the development of novel therapeutic approaches that can be used, in conjunction with reperfusion, to enhance the salvage and functional recovery of ischemic myocardium. Interest has variably focused on the roles and manipulation of metabolic changes, inflammatory responses, oxidative stress and ionic perturbations (1). Most recently, the powerful adaptive response of the myocardium that is triggered by ischemic or pharmacologic preconditioning has received widespread attention (2). Despite much investigative effort, however, no single therapy has been adopted clinically for protection of the ischemic/reperfused myocardium, a goal that is commonly referred to as "cardioprotection". The therapeutic arsenal that is currently available to physicians in combating ischemic heart disease targets the coronary vasculature and circulatory elements, and includes drugs that inhibit the development of atherosclerosis and stabilize existing lesions, drugs that suppress intracoronary thrombosis, and drugs and interventions that re-institute coronary perfusion. However, this arsenal is deficient in direct cardioprotective agents that specifically target the myocardial cells. A safe and effective new therapy that slows the progression of myocardial ischemic injury and/or attenuates the detrimental consequences of reperfusion ("reperfusion injury") would therefore be expected to synergize with existing therapies and provide considerable benefit.

A NOVEL PHARMACOLOGIC APPROACH TO CARDIOPROTECTION

Among the wide range of putative cardioprotective drugs that have been tested in experimental studies of myocardial ischemia and reperfusion, those that specifically target and inhibit the Na^+/H^+ exchanger (NHE) isoform 1, a ubiquitously expressed protein that is the molecular homolog of the cardiac sarcolemmal NHE, have shown particular promise. This growing family of NHE inhibitors, which include cariporide (3), eniporide (4) and zoniporide (5), have been found to afford substantial protection in animal models of myocardial ischemia and reperfusion, with an unusual degree of conformity between different investiga-

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Abbreviations and Acronyms

CABG	= coronary artery bypass graft
CK	= creatine kinase
CK-MB	= creatine kinase-MB isoform
ECG	= electrocardiogram
ESCAMI	= *E*valuation of the *S*afety and *C*ardioprotective Effects of Eniporide in *A*cute *M*yocardial *I*nfarction
EXPEDITION	= Na^+/H^+ *E*xchange Inhibition to *P*revent Coronary *E*vents in Acute Cardiac Con*ditions*
GUARDIAN	= *Guard* During *I*schemia *A*gainst *N*ecrosis
MI	= myocardial infarction
NCX	= Na^+/Ca^{2+} exchanger
NHE	= Na^+/H^+ exchanger
PCI	= percutaneous coronary intervention

tors, species and models (see recent reviews by Avkiran [6] and Karmazyn et al. [7]). Indeed, some laboratory findings suggest that NHE inhibition may provide cardioprotective benefit that is equivalent, or perhaps even superior, to that afforded by ischemic preconditioning (8–10).

The potential mechanisms that are likely to underlie the protection afforded by NHE inhibitors during myocardial ischemia and reperfusion have been reviewed recently (11). As illustrated in Figure 1 , the available experimental evidence suggests that such protection is likely to arise primarily from the attenuation of intracellular Na^+ accumulation during ischemia, which in turn would attenuate intracellular Ca^{2+} accumulation (through reduced Ca^{2+} efflux and/or increased Ca^{2+} influx via the sarcolemmal Na^+/Ca^{2+} exchanger) during both ischemia and subsequent reperfusion (11). In some settings additional mechanisms, such as the attenuation of neutrophil accumulation (12,13) and coronary endothelial dysfunction (14) within the jeopardized tissue, may also contribute to the protection afforded by NHE inhibitors, through the inhibition of NHE activity in non-myocardial cells (11).

The apparent therapeutic potential of NHE inhibitors, coupled with the perceived need for cardioprotective therapy in the management of ischemic heart disease, has led to the initiation of clinical studies with these agents within a remarkably short time. This is best illustrated by the observation that the first experimental data with cariporide were not published in peer-reviewed literature until 1995 (3), but by 1997 the drug was already being administered to patients with ischemic heart disease in the combined phase II/phase III GUARDIAN (Guard During Ischemia Against Necrosis) trial (15). The recent publication of the results of the GUARDIAN trial (16) and other clinical studies with NHE inhibitors (17,18) provides an opportunity to evaluate these results in relation to the pre-clinical knowledge base, to outline the likely prerequisites for effective utilization of NHE inhibitors in combating myocardial ischemia and to consider the prospective applications of these drugs.

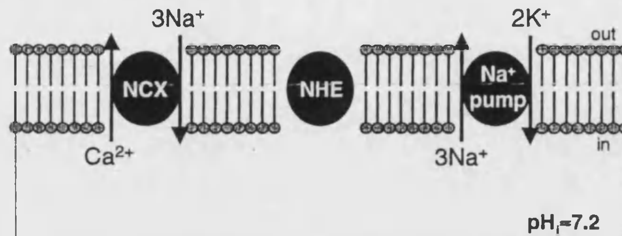
CLINICAL STUDIES WITH NHE INHIBITORS: RESULTS AND REFLECTIONS

A recent issue of the *Journal of the American College of Cardiology* includes a report of the main findings of a clinical study with eniporide (18), which follows the earlier publication of the results of two other clinical studies with cariporide (16,17). Two of these studies (17,18) involved patients with acute myocardial infarction (MI) in whom the NHE inhibitor was used as an adjunct to reperfusion therapy (by primary coronary angioplasty or thrombolysis), and the other study (16) tested the cardioprotective efficacy of NHE inhibition in a large number of patients at risk of MI during spontaneous or iatrogenic ischemia.

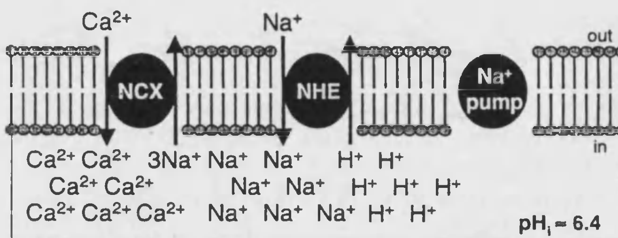
NHE inhibition in patients with MI. Rupprecht et al. (17) determined the effects of cariporide in patients with anterior MI who were expected to receive reperfusion therapy by primary coronary angioplasty within 6 h of the onset of symptoms. One hundred patients were randomized to receive placebo or 40 mg cariporide as an intravenous bolus 10 min before reperfusion, and placebo or drug administration was completed within approximately 4 h after the onset of symptoms. Cardiac enzymes (creatine kinase [CK], creatine kinase-MB isoform [CK-MB] and lactate dehydrogenase) were determined in blood samples obtained before and 4, 12, 24, 36 and 72 h after reperfusion in 85 patients (placebo n = 43, cariporide n = 42). Left ventricular function was determined by contrast ventriculography before treatment and at three-week follow-up in 46 patients (placebo n = 21, cariporide n = 25). The main findings of the study were that, after reperfusion: 1) ejection fraction remained unchanged in the placebo group but increased in the cariporide group, such that the change from baseline to follow-up was greater in the latter group ($p = 0.045$); 2) regional left ventricular wall-motion abnormalities tended to resolve in both groups, but the change from baseline to follow-up was greater in the cariporide group ($p = 0.045$); and 3) the cumulative release of CK-MB (the area under the curve) was reduced in the cariporide group relative to the placebo group ($p = 0.047$). The authors concluded that these data were consistent with the notion that reperfusion injury contributed to MI in humans and should be a target for interventions such as NHE inhibition (17). They further suggested that large-scale clinical trials were warranted to establish this therapeutic principle (17).

The results of such a trial, in the form of the ESCAMI (Evaluation of the Safety and Cardioprotective Effects of Eniporide in Acute Myocardial Infarction) trial, have been reported recently (18). This study employed a two-stage design and recruited patients with anterior or inferior MI who were expected to receive reperfusion therapy, by primary coronary angioplasty or thrombolysis (at the physician's discretion), within 6 h of the onset of symptoms.

A. non-ischemic



B. ischemic



C. ischemic + NHE inhibitor

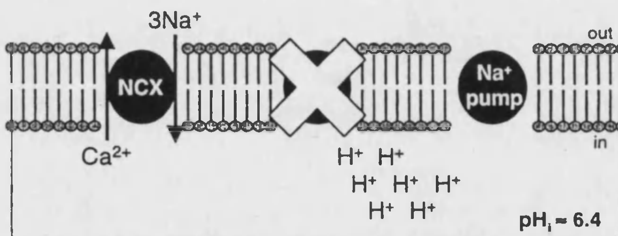


Figure 1. Potential mechanism through which Na^+/H^+ exchanger (NHE) inhibition preserves intracellular ion homeostasis and thereby myocardial integrity and function after ischemia and reperfusion. **(A)** Under basal conditions, NHE is relatively quiescent, the Na^+/K^+ ATPase (Na^+ pump) utilizes ATP to extrude Na^+, and the bidirectional Na^+/Ca^{2+} exchanger (NCX) works predominantly in forward (Ca^{2+} efflux) mode. **(B)** During ischemia, NHE becomes activated in response to intracellular acidosis and possibly by other NHE-stimulatory factors (26). The resulting influx of Na^+, occurring in the presence of ischemia-induced attenuation of Na^+ pump activity, causes the intracellular accumulation of Na^+. Such a rise in the intracellular Na^+ concentration during ischemia alters the reversal potential of the NCX in a manner that inhibits its operation in forward mode but favors its operation in reverse (Ca^{2+} influx) mode, thus producing intracellular Ca^{2+} accumulation (Ca^{2+} overload) during both ischemia and subsequent reperfusion. **(C)** NHE inhibitors are likely to afford a cardioprotective effect during ischemia and reperfusion by inhibiting this sequence at an early stage, through the limitation of Na^+ influx during ischemia. Note that the illustration has been simplified for clarity, and that mechanisms other than NHE activity are also likely to contribute to the intracellular accumulation of Na^+ and consequently Ca^{2+} during ischemia and reperfusion.

Blood samples were collected before and 4, 8, 12, 16, 24, 36, 48, 60 and 72 h after the start of reperfusion therapy for assessment of α-hydroxybutyrate dehydrogenase, CK and CK-MB content. Ejection fraction or regional wall motion were not assessed. In stage 1, patients (n = 430) were randomized to receive placebo (n = 88) or 50 mg (n = 86), 100 mg (n = 91), 150 mg (n = 74) or 200 mg (n = 91) eniporide, as a 10-min intravenous infusion that had to be completed at least 10 min before the start of coronary angioplasty or within 15 min after the start of thrombolysis. Data from this stage indicated reductions in cumulative enzyme release of approximately 15% with 100 mg eniporide and 30% with 150 mg eniporide, although no effect was seen with the 200 mg dose. On the basis of these findings, the study was extended into stage 2, during which additional patients (n = 959) were randomized to receive placebo (n = 322) or 100 mg (n = 321) or 150 mg (n = 316) eniporide in an identical manner to stage 1. However, the results of stage 2 of the trial, when considered alone or in combination with those of stage 1, revealed no effect of eniporide on cumulative enzyme release. Furthermore, this lack of effect of active treatment persisted across various predefined subgroups (e.g., reperfusion by thrombolysis [n = 590] vs. coronary angioplasty [n = 363]; anterior [n = 389] vs. inferior [n = 513] infarction; early [≤4 h from the onset of symptoms, n = 696] vs. late [>4 h from the onset of symptoms, n = 229]

reperfusion). Thus, the overall results of this study oppose the hypothesis that NHE inhibition, used as an adjunct to reperfusion, reduces MI by attenuating reperfusion injury.

Clearly, the outcome of the ESCAMI trial with eniporide (18) contradicts the findings of the earlier study with cariporide (17) with respect to the effects of NHE inhibition, as an adjunct to reperfusion therapy, on cardiac enzyme release. This difference in outcome is unlikely to have a pharmacologic basis, because eniporide is a more potent NHE inhibitor than cariporide (19) and the doses of eniporide used in the ESCAMI trial (18) were up to five times greater than the dose of cariporide used by Rupprecht et al. (17). It might be argued that NHE inhibition during reperfusion reduces myocardial enzyme release only in a highly selected group of patients who have large anterior infarcts and receive reperfusion by primary coronary angioplasty (17). However, this is also unlikely because, in the ESCAMI study, cumulative enzyme release did not differ between placebo and eniporide treatment even in the subgroup of patients who had anterior infarcts and received reperfusion by primary coronary angioplasty (18). The most likely explanation for the apparent discrepancy appears to be a chance finding in the study by Rupprecht et al. (17), arising as a consequence of the small sample size. Indeed, such a finding is also likely to have been responsible for the ultimately misleading results of stage 1 of the ESCAMI trial (18).

Is the lack of efficacy of NHE inhibition as an adjunct to reperfusion in patients with acute MI surprising, given what is known about the nature of reperfusion injury and the data from extensive pre-clinical work with NHE inhibitors? First, the existence of "lethal" reperfusion injury (defined as myocardial cell death arising from reperfusion rather than from the preceding period of ischemia) and its clinical relevance in the setting of acute MI are open to debate (20,21). Second, as reviewed in depth previously (6), the majority of available pre-clinical data show that NHE inhibitors limit infarct size dramatically when given before or soon after the onset of ischemia, but not when administered shortly before or at the time of reperfusion, suggesting that NHE activity does not contribute significantly to any lethal reperfusion injury. Indeed, even in the early experiments in pigs that demonstrated a limitation of infarct size with NHE inhibition from shortly before reperfusion, significantly greater benefit was observed when the NHE inhibitor was administered before the onset of ischemia (22). A recent study by Klein et al. (23), in pigs that were instrumented to receive residual flow (through an extracorporeal circuit) during a 60-min period of regional ischemia, has sought to determine definitively the key period during which NHE activity contributes to MI. As illustrated in Figure 2 , this study showed that infarct size measured after 24 h of reperfusion was significantly reduced by the intracoronary infusion of cariporide during the first 30 min of ischemia or throughout the entire 60 min of ischemia plus the first 10 min of reperfusion, but not by such infusion during the last 15 min of ischemia plus the first 10 min of reperfusion (23). In the light of such data and with the benefit of hindsight, a strong case may be made that the negative overall outcome of the ESCAMI trial was in fact predictable and that the unexpected findings were the positive indications from the earlier, smaller clinical studies. Indeed, when considered together, the clinical and pre-clinical data reinforce the concept that NHE activity during early ischemia (rather than during reperfusion) is the principal determinant of the extent of myocardial injury, such that early (ideally pre-ischemic) treatment is a prerequisite

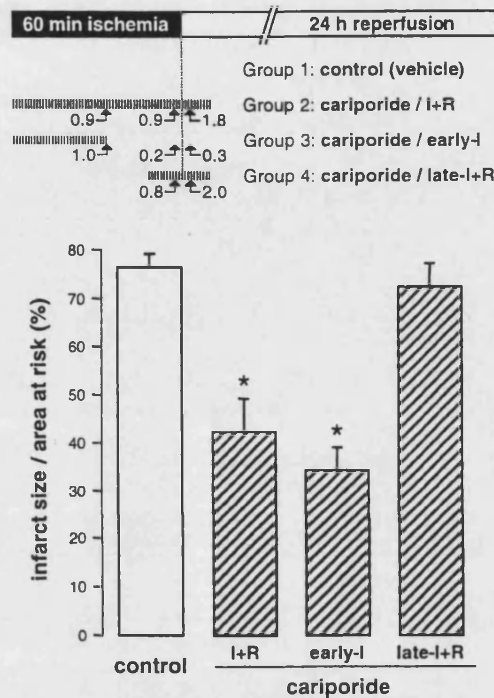


Figure 2. Effects on infarct size of intracoronary infusion of the Na⁺/H⁺ exchanger (NHE) inhibitor cariporide during various periods, in pig hearts subjected to 60 min of regional low-flow ischemia and 24 h of reperfusion. Infarct size was measured at the end of 24 h of reperfusion by both histochemical and histologic methods. The top panel illustrates the experimental protocol, with the vertically hatched bars indicating the periods of cariporide infusion and the arrows showing the coronary sinus cariporide concentrations (in μmol/l) after 30 min of ischemia, immediately before reperfusion and immediately after reperfusion, in the various study groups. Note that a minimum concentration of approximately 1 μmol/l cariporide is required for effective inhibition of sarcolemmal NHE activity in cardiac ventricular myocytes (24). As shown, infarct size was significantly limited by the intracoronary infusion of cariporide during the first 30 min of ischemia or throughout the entire 60 min of ischemia plus the first 10 min of reperfusion. In contrast, infusion of cariporide during the last 15 min of ischemia plus the first 10 min of reperfusion provided no benefit, even though the coronary sinus cariporide concentrations at the end of ischemia and the beginning of reperfusion were sufficient to inhibit NHE activity. Thus, NHE activity during early ischemia, rather than that during late ischemia and early reperfusion, appears to be the principal determinant of the extent of myocardial infarction. I = ischemia; R = reperfusion, * = $p < 0.05$ versus control. The figure is based on data from Klein et al. (23).

to obtain maximum cardioprotective benefit with NHE inhibitors (6,11).

NHE inhibition in patients at risk of MI. The GUARDIAN trial (16) recruited three cohorts of patients who were defined as sharing a common risk of MI during hospitalization: these were patients with unstable angina or non-Q-wave MI (n = 5,233), patients undergoing high-risk percutaneous coronary intervention (PCI) (n = 3,439), and patients undergoing high-risk coronary artery bypass graft (CABG) surgery (n = 2,918). The study was designed with the combined objectives of selecting an effective dose (the usual objective of a phase II trial) and determining clinical efficacy (the usual objective of a phase III trial). As such, patients were randomized to receive placebo (n = 2,910) or 20 mg (n = 2,909), 80 mg (n = 2,888) or 120 mg (n = 2,883) cariporide every 8 h as an intravenous infusion for two to seven days after randomization. The results indicated no significant effect of active treatment on the primary end point, which was all-cause mortality or MI (diagnosed by electrocardiogram and CK-MB criteria) between randomization and 36-day follow-up. Nevertheless, subgroup analysis revealed that in patients undergoing high-risk CABG surgery, there was a significant reduction in the combined incidence of death and MI with the 120 mg dose of cariporide (from 16.2% in the placebo group to 12.1%, $p = 0.03$), and this benefit was maintained after six months (16). Intriguingly, the 120 mg dose of cariporide also tended to reduce the incidence of Q-wave MI in the other two entry diagnostic groups (16).

A laudable feature of the design of the GUARDIAN trial is the attempt to treat patients with the NHE inhibitor before the onset of an episode of myocardial ischemia that might culminate in infarction, reflecting the knowledge gained from pre-clinical work. However, the pre-clinical work also indicates that the key mechanism through which NHE inhibitors afford protection is by delaying the progression of myocardial injury during ischemia and thereby enhancing myocardial salvage by reperfusion (6). It follows from this that a further prerequisite to obtain maximum cardioprotective benefit with NHE inhibitors is timely reperfusion, in whose absence severely ischemic myocardium will eventually succumb to infarction regardless of treatment (6). It is probably not a coincidence therefore that the prerequisites of early treatment and timely reperfusion are both fulfilled in the setting of CABG surgery, where significant cardioprotective benefit was afforded by NHE inhibition in the GUARDIAN trial (16). As discussed by Théroux et al. (16), in the other entry diagnostic groups included in the GUARDIAN trial, evolving new infarcts would be reperfused only if ST-segment elevation developed (in patients admitted with unstable angina or non-Q-wave MI) or abrupt vessel closure ensued (in patients undergoing high-risk PCI). It is reasonable to speculate that the tendency in these cohorts toward a reduced incidence of Q-wave MI in response to high-dose cariporide treatment (16) may be a reflection of infarct size limitation by NHE inhibition in patients who exhibited such events and therefore received reperfusion therapy.

In addition to the presence or absence of timely reperfusion, a further issue to consider in interpreting the data from the GUARDIAN trial is the lack of a discernible dose-response relationship, even among patients who underwent CABG surgery (16). This raises the possibility that the optimal dose of cariporide may be higher than the maximum dose used in the GUARDIAN trial, barring unacceptable adverse effects. Indeed, pharmacokinetic modeling based on information from the GUARDIAN trial has indicated that, for therapeutic efficacy, a threshold plasma drug concentration of 550 ng/ml (approximately 1.4 μmol/l) needs to be exceeded during the period of risk (16). The model has also predicted that this would have occurred in only 67% of patients who received the highest (120 mg) dose of cariporide before CABG surgery (16). Of relevance to this issue, our recent studies in rat ventricular myocytes indicate that, in the presence of a physiologic Na^+ concentration, ≥ 1 μmol/l cariporide is required for effective inhibition of sarcolemmal NHE activity (24). Thus, future clinical studies will need to consider using higher doses or modified drug administration protocols to ensure that the myocardial drug concentration during the period of risk is sufficient for effective inhibition of sarcolemmal NHE activity.

NHE INHIBITORS FOR CARDIOPROTECTION IN ACUTE ISCHEMIA: WHAT NEXT?

The positive findings of the GUARDIAN trial in the setting of CABG surgery were revealed by retrospective analysis of subgroup data (16). Clearly, therefore, there is an important need for further trial(s) that are designed prospectively to determine whether NHE inhibition affords cardioprotective benefit in patients undergoing high-risk CABG surgery. In an effort to fulfill this need, the objective of the recently initiated EXPEDITION (Na^+/H^+ Exchange Inhibition to Prevent Coronary Events in Acute Cardiac Conditions) trial is to test the hypothesis that NHE inhibition results in a reduction in myocardial injury in such patients, using a modified dosing regimen of cariporide. If the hypothesis is proven by the eventual findings of the EXPEDITION trial, then it will be necessary to consider whether there are other clinical settings in which NHE inhibitors may be given to patients before the onset of myocardial ischemia, in order to preserve myocardial viability during such ischemia and improve salvage by subsequent reperfusion. An immediate application may be in non-CABG cardiac surgery where the heart is also subjected to global ischemia and reperfusion. Additionally, consideration needs to be given to the possibility of using NHE inhibitors for perioperative cardioprotection in patients who are at high risk of cardiac events, such as MI, during non-cardiac surgery.

In the non-surgical sphere, it may be opportune to

determine whether long-term NHE inhibition affords functional and/or symptomatic benefit in patients with stable angina through direct myocardial protection. Recent data in conscious pigs subjected to repetitive cycles of sublethal regional ischemia (2 min) and reperfusion (8 min) have shown that the ensuing regional contractile dysfunction is attenuated by NHE inhibition (25). In addition to a potential benefit during the transient episodes of ischemia, NHE inhibition in patients with angina would be expected to provide further benefit if spontaneous coronary occlusion ensues, by delaying the progression of ischemic injury and thereby enhancing myocardial salvage by subsequent reperfusion, as discussed earlier.

NHE INHIBITORS FOR CARDIOPROTECTION BEYOND ACUTE ISCHEMIA?

Emerging experimental evidence suggests that sarcolemmal NHE activity may play an important pathophysiologic role beyond its contribution to the development of myocardial injury during acute ischemia and reperfusion. In this context, it is notable that although sarcolemmal NHE activity is regulated primarily by intracellular pH, it is also subject to regulation by a variety of neurohormonal mediators (26). Intriguingly, several myocardial G protein-coupled receptors whose activation by their cognate ligands has been associated with the induction of a hypertrophic phenotype in vitro, such as α_1-adrenergic (27), endothelin (28), angiotensin II (29) and thrombin (30) receptors, have also been shown to mediate increased sarcolemmal NHE activity (31–35). Furthermore, the induction of myocyte hypertrophy in vitro, in response to neurohormonal (36) or mechanical (37) stimuli, has been shown to be attenuated by NHE inhibition. Importantly, recent in vivo evidence in the rat indicates that NHE inhibition with cariporide attenuates myocardial hypertrophy and the development of heart failure after infarction, independently of infarct size limitation or afterload reduction (38,39). Of potential clinical relevance, our recent work has shown that ventricular myocytes from explanted human hearts with end-stage heart failure exhibit significantly greater sarcolemmal NHE activity than their counterparts from unused donor hearts, probably through altered posttranslational regulation of the exchanger (40). Thus, the available evidence points toward a causal or permissive role for sarcolemmal NHE activity in the development of cardiac hypertrophy and its progression to heart failure, suggesting that NHE inhibitors might find a novel therapeutic application in this setting.

Before the therapeutic potential of NHE inhibitors in the management of heart failure is tested in clinical trials, further experimental investigation may need to be performed. At present, the distal mechanism(s) through which NHE regulates myocardial growth and remodeling are unclear, and there is some controversy regarding the relative contributions of Na^+ influx versus H^+ efflux as mediators of such regulation. In addition, there is a paucity of information on the efficacy of NHE inhibition relative to, or in combination with, established therapies such as angiotensin-converting enzyme inhibition and beta-adrenergic receptor blockade in animal models of heart failure. In this context, a potential advantage of NHE inhibition over other therapies may be the direct attenuation of myocyte hypertrophy and remodeling in the absence of significant hemodynamic effects (39).

CONCLUSIONS

The mixed results of recent clinical studies with NHE inhibitors contrast with the highly encouraging data from pre-clinical investigations and serve to highlight the enormous challenge of translating therapeutic potential into therapeutic reality. Nevertheless, a valid case may be made that the design of the clinical studies that have been completed to date did not reflect optimally the current knowledge from extensive pre-clinical work, particularly on the mechanism of action of NHE inhibitors. Indeed, with perfect hindsight, it is relatively straightforward to provide a scientific rationale for the disappointing outcomes of these clinical studies.

On the positive side, the clinical studies have shown that NHE inhibitors are well tolerated by patients (at least over a short period of treatment) and have provided a strong indication that, in the appropriate setting, they can indeed protect the human myocardium from injury during ischemia and reperfusion. Furthermore, animal experiments continue to support the cardioprotective potential of these agents in acute myocardial ischemia and have indicated that NHE inhibition may provide benefit beyond the preservation of acutely ischemic and reperfused myocardium. The very considerable challenge remains the translation of this potential into reality. This goal may be achieved only through pre-clinical work that improves our understanding of the pathophysiologic roles of the NHE system and the consequences of its inhibition, and complementary clinical trials that reflect such understanding.

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