Chapter 13
Early Hypertrophic Signals After Myocardial Stretch. Role of Reactive Oxygen Species and the Sodium/Hydrogen Exchanger

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Abstract In this chapter the enhanced activity of the cardiac Na^+/H^+ exchanger (NHE-1) after myocardial stretch is considered a key step of the intracellular signaling pathway leading to the slow force response to stretch as well as an early signal for the development of cardiac hypertrophy. We propose that the chain of events triggered by stretch begins with the release of small amounts of angiotensin II which in turn induce the release/formation of endothelin. The actions of these hormones trigger the production of mitochondrial reactive oxygen species that enhances NHE-1 activity, causing an increment in the intracellular Na^+ concentration which promotes the increase in intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) through the Na^+/Ca^{2+} exchanger. This $[Ca^{2+}]_i$ increase would trigger cardiac hypertrophy by activation of widely recognized Ca^{2+}-dependent intracellular signaling pathways.

Keywords Myocardium · Stretch · Sodium/hydrogen exchanger · Reactive oxygen species · Hypertrophy

13.1 Introduction

Adding electrons to oxygen produces sequentially: (1) superoxide anion (O_2^-), (2) hydrogen peroxide (H_2O_2), (3) hydroxil radical (OH^-) and finally water (H_2O) (Boveris, 1998). While H_2O_2 is not a free radical, this very reactive and membrane permeant molecule is included among the reactive oxygen species (ROS), together with the oxygen radicals O_2^- and OH^-. Mitochondria are the main source of ROS production, although NADPH oxidase and Xanthine Oxidase may also contribute to ROS formation (Giordano, 2005). The enzyme responsible for NO production

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(Nitric Oxide Synthase, NOS) can also generate O_2^- under certain oxidative stress conditions (Takimoto et al., 2005).

During many years ROS were considered deleterious agents, but in the last years evidences of their effects as second messengers have emerged (D'Autreaux and Toledano, 2007). Furthermore, the concept that free radicals in the heart could be "friend or foe" depending on the magnitude, duration or timing of the redox signal has been recently suggested (Downey and Cohen, 2008). The cardiac Na^+/H^+ exchanger (NHE-1) is a target for ROS through the activation of kinases (Sabri et al., 1998; Snabaitis et al., 2002). ROS, kinases activation, and NHE-1 hyperactivity are three early hypertrtophic signals after myocardial stretch and/or stimulation by growth factors. Interestingly, inhibition of ROS, NHE-1 or growth factors results in regression of cardiac hypertrophy. The discussion of how these three factors are linked among them and how they are linked to other well known hypertrophic signals constitutes the aim of this chapter.

13.2 NHE-1 and Myocardial Stretch

In 1998 Bluhm et al. published the results obtained with an elegant theoretical ionic model of a ventricular myocyte used to analyze the changes in sarcolemmal ion fluxes following step changes in cardiac muscle length. They suggested that a sudden increase in muscle length might induce changes in sarcolemmal Na^+ influx leading to an increase in $[Na^+]_i$ and a concomitant increase in systolic Ca^{2+} entry through the Na^+/Ca^{2+} exchanger (NCX). However, the mechanism by which the increase in $[Na^+]_i$ takes place was not proposed. Since the NHE-1 is an important Na^+ entry pathway in cardiomyocytes, the possible role played by the exchanger will be analyzed in detail.

The finding of a stretch-induced myocardial alkalization in cat papillary muscles bathed with a bicarbonate-free medium was the first piece of evidence provided by our laboratory referent to NHE-1 activation by myocardial stretch and the main role played by this exchanger in the early signals leading to hypertrophy (Cingolani et al., 1998). The absence of bicarbonate in the medium allowed us to analyze the role of NHE-1 without the influence of bicarbonate-dependent intracellular pH (pH_i)-regulatory mechanisms. The stretch-induced myocardial alkalization was suppressed by either angiotensin II (Ang II) type 1 (AT_1) or endothelin (ET) type A (ET_A) receptors blockade, suggesting the involvement of these receptors in the stretch-induced activation of NHE-1 (Cingolani et al., 1998). In accordance with this, Sadoshima and co-authors (1993) have initially reported the release of Ang II after stretching cultured neonatal cardiomyocytes. They showed that the addition of the surrounding medium from stretched to non-stretched cardiomyocytes promoted hypertrophy, and that Ang II was the autocrine mediator of this effect. These authors also suggested that Ang II is stored in secretory vesicles in myocytes and released within 1 min by mechanical stretch (Sadoshima et al., 1993). Contemporarily, Ito et al. (1993) found in the same type of preparation that Ang II promotes the release/formation of ET-1, demonstrating that ET-1 is an autocrine factor in the

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mechanism of Ang II-induced cardiac hypertrophy. In addition, Yamazaki et al. (1996) found that, together with an increase in NHE-1 activity, stretch induced a rise in the concentration of ET-1 constitutively secreted from cardiomyocytes to the culture medium. The same authors showed that NHE-1 inhibition partially attenuated the stretch-induced mitogen-activated protein kinase (MAPK) activation. Our main contribution was to demonstrate the existence of a stretch-triggered autocrine/paracrine release of Ang II/ET leading to NHE-1 activation in an adult cardiac multicellular preparation (Cingolani et al., 1998; Alvarez et al., 1999; Perez et al., 2001). This finding allowed us to propose the hypothetical scheme depicted in Fig. 13.1. The proposed chain of events begins with the release of preformed Ang II and ends with an increase in the Ca^{2+} transient through reverse mode of NCX (NCX_{rev}) activation and/or forward mode of NCX ($NCX_{forward}$) inhibition secondary to the NHE-1 activation-mediated rise in $[Na^+]_i$. If we analyze the potential effects of NHE-1 activation on myocardial contractility, we should consider two different mechanisms: Na^+-triggered increase in the Ca^{2+} transient through NCX, and an increase in pH_i that would increase the contractile force by increasing myofilament Ca^{2+} responsiveness. Considering the latter possibility, it is important to emphasize that little or no change in pH_i is detected when the stimulating effect of stretch, exogenous Ang II or ET-1 on NHE-1 is studied in the presence of bicarbonate buffers (Cingolani et al., 1998; Alvarez et al., 1999; Perez et al., 2001, 2003; Aiello et al., 2005; Luers et al., 2005) The explanation for the lack of change in pH_i can be found in the fact that growth factors like Ang II and ET-1 simultaneously activate at least two opposing pH_i-regulatory mechanisms: the alkalinizing

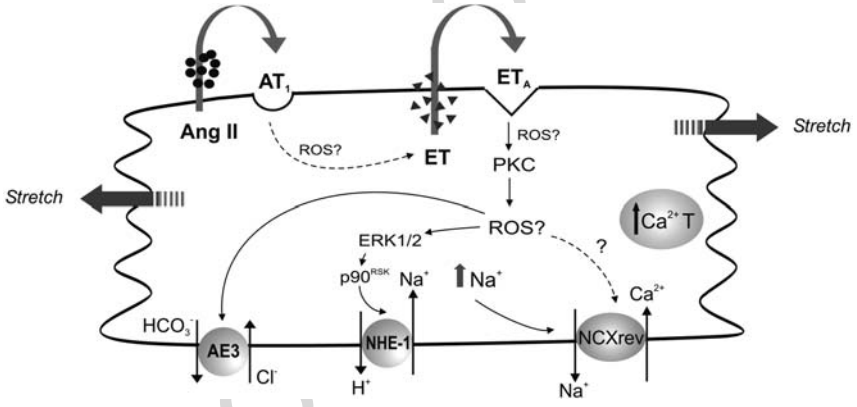

Fig. 13.1 A representation of the proposed autocrine/paracrine cascade of events following myocardial stretch. Endogenous Ang II is released from the myocytes activating AT_1 receptors in an autocrine fashion. Stimulation of AT_1 induces the release/formation of ET, which simultaneously activates NHE-1 and Cl^-–HCO_3^- exchanger through ET_A receptors. The activation of Cl^-– HCO_3^- exchanger prevents the expected intracellular alkalization due to NHE-1 activation but does not prevent the rise in $[Na^+]_i$. The increase in $[Na^+]_i$ drives the NCX in its reverse mode and this, together with a probable direct action on the exchanger, leads to the increase in Ca^{2+} transient ($Ca^{2+}T$)

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NHE-1 and the acidifying Na^+–independent Cl^-–HCO_3^- anion exchanger (Ganz et al., 1988; Thomas, 1989; Camilion de Hurtado et al., 1998; de Hurtado et al., 2000; Alvarez et al., 2001; Cingolani et al., 2003a; Perez et al., 2003). The scheme in Fig. 13.1 illustrates the fact that Ang II – through release/formation of ET-1, simultaneously stimulates NHE-1 and Cl^-–HCO_3^- exchanger, thus minimizing the changes in pH_i but without affecting the increase in $[Na^+]_i$ that follows NHE-1 activation. Therefore, NHE-1 activation can be detected as a pH_i increase only if bicarbonate is absent in the medium. We emphasize this point because the absence of changes in pH_i after growth factor stimulation in bicarbonate media is not widely recognized, though it was reported by Ganz et al. in 1988 in mesangial cells and a call for attention was published by Thomas (1989) in a letter to Nature one year later. More recently, Schafer et al. (2002) demonstrated that the hypertrophic response of cardiomyocytes to α- and β-adrenergic stimulation requires NHE-1 activation but not cellular alkalization. In summary, although there is enough evidence to suggest a direct correlation between activation of cellular acid extrusion mechanisms and proliferation, there is also enough evidence to state that proliferation can occur without changes in pH_i, and that changes in pH_i do not necessarily induce proliferation (Schafer et al., 2002; Ganz et al., 1988, 1990; Shrode et al., 1997). There is no agreement in the literature about the role played by the NHE-1 in growth and viability. While some authors report that NHE-1-deficient transgenic mice can grow at normal rate (Grinstein et al., 1989), others have shown that these animals exhibit growth retardation and are subject to slow-wave epilepsy (54–56).

The effects of myocardial stretch, exogenous Ang II and ET-1 on pH_i and $[Na^+]_i$ in cat papillary muscles are illustrated in Fig. 13.2. In these experiments, low doses of exogenous Ang II or ET-1 that probably reproduced those released after stretch

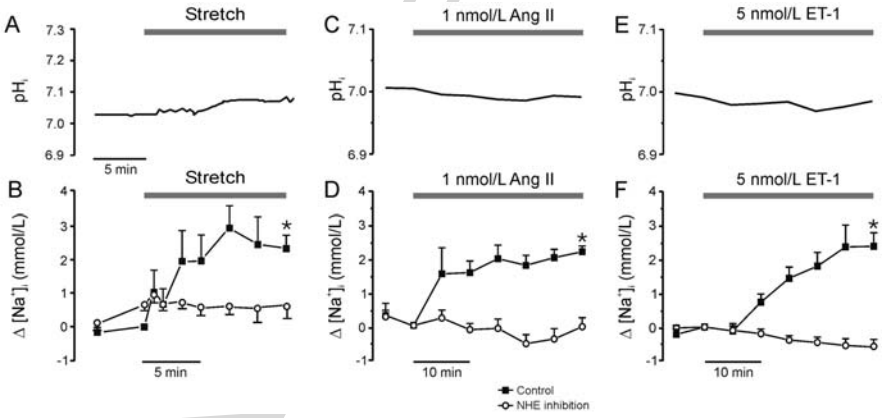

Fig. 13.2 Representative experiments showing that in the presence of bicarbonate, NHE-1 activation by stretch (**Panel A**), exogenous Ang II (**Panel C**) or ET-1 (**Panel E**) does not change pH_i. The same interventions promoted an increase in $[Na^+]_i$ that was prevented by NHE-1 blockade (pooled results of **Panels B, D** and **F**). * Indicates $P<0.05$ vs. NHE inhibition. Modified from Perez et al. (2003) with permission

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did not affect pH_i but significantly increased $[Na^+]_i$. This rise in $[Na^+]_i$ was suppressed by NHE-1 inhibition. The ET receptor blockade exerted the same inhibitory effect after myocardial stretch and after the addition of exogenous Ang II or ET-1 (Perez et al., 2001, 2003). The role played by the Cl^-–HCO_3^- exchanger in preventing intracellular alkalization after myocardial stretch is better visualized by repeating the intervention in a bicarbonate medium before and after inhibition of the anion-exchanger with specific antibodies (see Fig. 13.3) (Cingolani et al., 2003a).

Under these conditions, an increase in pH_i takes place only after Cl^-–HCO_3^- exchanger inhibition. It is not clear whether changes in pH_i after the addition of growth factors or stretch stimulation localized to certain subcellular spaces within the myocyte may occur in the presence of bicarbonate-dependent mechanisms. The fact that an increase in pH_i stimulates protein synthesis (Fuller et al., 1990) does not necessarily mean that intracellular alkalization occurs after myocardial stretch, Ang II or ET-1 stimulation (Ganz et al., 1988; Schafer et al., 2002; Cingolani et al., 2005). We would like to emphasize that our proposal is valid for the concentration used by us. Higher concentrations of Ang II and/or ET-1 can trigger mechanisms other than those described herein.

It is known that the increase in $[Na^+]_i$ can induce an increase in $[Ca^{2+}]_i$ through the NCX as a result of a decrease in Ca^{2+} efflux (decreased forward mode) and/or an increase in Ca^{2+} entry (increased reverse mode). As mentioned before, the increase in $[Na^+]_i$ induced by stretch or by exogenous low doses of Ang II or ET-1 was prevented by blocking NHE-1 (Fig. 13.2) (Alvarez et al., 1999; Perez et al., 2001, 2003; Aiello et al., 2005). The increase in myocardial $[Na^+]_i$ detected in our experiments was ~3–6 mmol/L. In line with this, increases of similar magnitude were detected by Baartscheer et al. (2005) in the myocardium of rabbit failing hearts with enhanced activity of NHE-1 and by Luers et al. (2005) after stretching rabbit

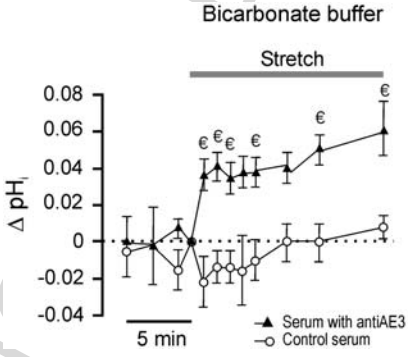

Fig. 13.3 When Cl^-– HCO_3^- exchanger activity is inhibited by a specific antibody against it, the slow increase in force after stretch is even greater than when the anion exchanger is operative, due to a rise in pH_i despite the presence of extracellular bicarbonate. Under this condition, the increase in myofilament responsiveness increases developed force in addition to the effect of the augmented Ca^{2+} transient. € P <0.05 vs. Control serum. Modified from Cingolani et al. (2003a) with permission

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myocardium. This increase in $[Na^+]_i$ shifts the reversal potential of NCX to a more negative voltage, thus allowing the NCX to operate in reverse mode for a longer period of time during the action potential and promoting Ca^{2+} influx to the cell which should be reflected by changes in contractility. As reported by Bers et al. (2003), cardiomyocytes have a limited capacity to buffer increases in $[Na^+]_i$ and the NCX is more sensitive than the Na^+/K^+ ATPase pump to a change in $[Na^+]_i$ of this magnitude.

Calculation of the estimated reversal potential of NCX in cat papillary muscles gives a value of –34 mV which is of the same order of magnitude as those estimated by other authors (Kusuoka et al., 1993; Bers, 2001), if we assume 10 mmol/L $[Na^+]_i$, 140 mmol/L extracellular Na^+, 1.5 mmol/L extracellular Ca^{2+} and a 150 nmol/L diastolic $[Ca^{2+}]_i$. The quick rise in sub-membrane $[Ca^{2+}]_i$ due to the Ca^{2+} transient that shifts the NCX reversal potential to even more positive voltages (Bers and Despa, 2006) would lead to a minimal contribution of the NCX_{rev} to basal contractility under normal conditions (Perez et al., 2001, 2003; Aiello et al., 2005). This in accordance to what we have shown that NCX_{rev} inhibition with 5 μmol/L KB-R7943 did not affect basal contractility or the increase in contractility of ∼20% promoted by rising extracellular Ca^{2+} from 1.35 to 1.9 mmol/L (Fig. 13.4) in cat papillary muscle. However, these results are in contrast to those obtained by Kurogouchi et al. (2000) in the dog myocardium that showed that KB-R7943 promoted a pronounced negative inotropic effect, discrepancy that might depend on the model and/or species used in each study. However, in isolated cat ventricular myocytes a decrease in basal inotropism of approximately 20% was detected after 1 μmol/L KB-R7943 (Cingolani et al., 2006). Therefore, this compound seems to exert greater negative inotropic effect in isolated myocytes.

The approximately 3–6 mmol/L increase in $[Na^+]_i$ induced by stretch (34), exogenous Ang II (Perez et al., 2003) or ET-1 (Aiello et al., 2005) in our experimental conditions certainly changes the scenario by shifting the reversal potential of NCX from –34 to –55 mV, allowing operation of the NCX reverse mode during a longer fraction of the action potential plateau. In line with the above-mentioned effect of stretch, Ang II and ET on $[Na^+]_i$, we detected a negative shift of the NCX reversal potential of –5 and –15 mV after treating isolated patch-clamped cat myocytes with 1 and 10 nmol/L ET-1, respectively (Aiello et al., 2005). Considering these experimental results, estimation of the ET-1-induced increase in $[Na^+]_i$ gives values of approximately 1.6 and 5.0 mmol/L for 1 and 10 nmol/L ET-1, respectively. These values are of the same order of magnitude as those measured in the bulk of the cytosol by epifluorescence in papillary muscles after addition of 5 nmol/L ET-1 (Perez et al., 2003). However, it is important to note that the increase in $[Na^+]_i$ in the isolated myocytes might reflect changes of this ion in a space in which intracellular dialysis with the solution of the patch pipette cannot maintain $[Na^+]$ at a constant level. The increase in $[Na^+]_i$ would tend to increase Ca^{2+} influx through reverse mode NCX during systole and to reduce Ca^{2+} extrusion via forward mode NCX during diastole that should necessarily end with an increase in the force of contraction as reported by us (Alvarez et al., 1999; Perez et al., 2001, 2003; Aiello et al., 2005).

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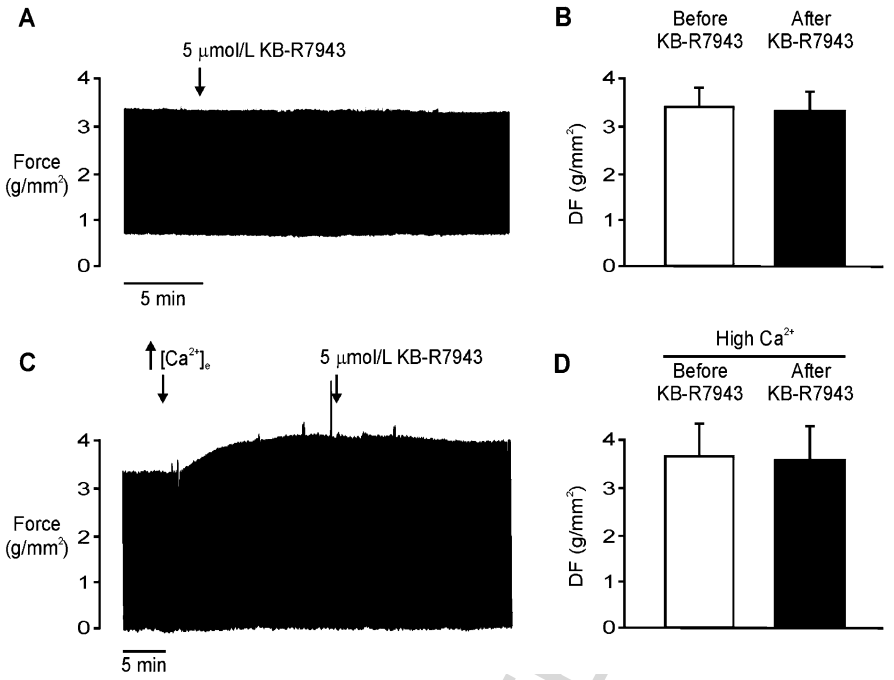

Fig. 13.4 Original force records showing the lack of effect of 5 μmol/L KB-R7943 (NCXrev blocker) on basal contractility (A, extracellular Ca^{2+}=1.35 mmol/L) and on the increase in contractility of ∼20% promoted by increasing extracellular Ca^{2+} from 1.35 mmol/L to 1.9 mmol/L (C). Overall results of developed force (DF, in g/mm^2) for each type of experiments (B, n=6 and D, n=4). These results also strongly suggest that KB-R7943 at this concentration does not exert non-specific actions which may affect contractility. Reproduced from Perez et al. (Cingolani et al., 2003a) with permission

We have reported an increase in the Ca^{2+} transient amplitude of about 12% during the slow force response without changes in diastolic Ca^{2+} (Alvarez et al., 1999; Perez et al., 2001), result that coincides with that reported by Kentish and Wrzosek (1998). The reported lack of participation of the sarcoplasmic reticulum in this mechanism (Bluhm and Lew, 1995; Hongo et al., 1995; Kentish and Wrzosek, 1998) further supports the notion that the NCX_{rev} is one possible mechanism involved in the increase in Ca^{2+} transient.

The question that now arises is if this increase in $[Ca^{2+}]_i$ secondary to the increase in $[Na^+]_i$ is the only mechanism responsible for the positive inotropic effect when Ang II or ET are involved in the mechanism. Figure 13.5 shows that the developed force increases linearly with the increase in $[Na^+]_i$ caused by Na^+/K^+-ATPase inhibition, and that this increase is blunted by KB-R7943 (Fig. 13.5, inset). However, when $[Na^+]_i$ increases because of ET-1-induced activation of NHE-1 (Aiello et al., 2005), the increase in developed force lies above the linear relationship (Fig. 13.5). In addition, if ET-1 is applied when the rise in $[Na^+]_i$ caused by Na^+/K^+-ATPase inhibition reached a steady state in the presence

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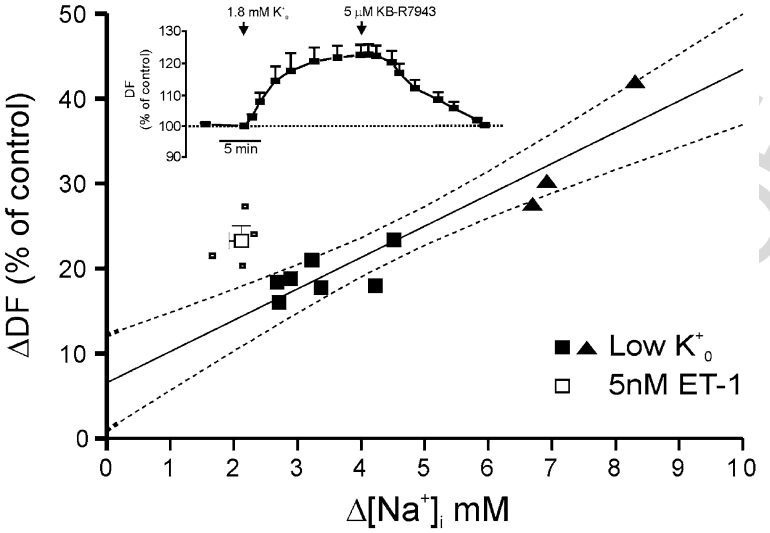

Fig. 13.5 The increase in $[Na^+]_i$ induced by partial inhibition of Na^+/K^+ ATPase by lowering extracellular K^+ (*squares*: 1.8 mmol/L; triangles: 0.9 mmol/L) increased developed force (DF) as a function of $[Na^+]_i$. This effect may be assigned to activation of NCXrev, because it was reverted by KB-R7943 (5 μmol/L; *inset*). However, when $[Na^+]_i$ levels were augmented by ET-1-induced NHE activation, the results lied above the relationship, suggesting that factors additional to the rise in $[Na^+]_i$ have taken place. Modified from Aiello et al. (2005) with permission

of NHE-1 inhibition, the peptide still produces a positive inotropic effect that is completely reversed by either inhibition of NCX_{rev} or protein kinase C (PKC) (Aiello et al., 2005). Patch-clamp experiments in isolated myocytes showed that ET-1 increases the NCX current and negatively shifts the NCX reversal potential (Aiello et al., 2005). Taken together, these data suggest that ET-1 is driving the reverse mode of the NCX by an NHE-1-mediated increase in $[Na^+]_i$ and by a direct stimulatory effect on the NCX, possibly by a PKC-dependent phosphorylation mechanism (Aiello et al., 2005). It is important to mention that PKC is a well known target of intracellular ROS (Juhaszova et al., 2004; Costa and Garlid, 2008). Thus, increased production of ROS by Ang II and/or ET-1 could stimulate PKC and might lead to the activation of both transporters, the NHE-1 and/or the NCX (Fig. 13.6).

Interestingly, experiments performed by Eigel et al. (2004) in guinea pig ventricular myocytes demonstrated that ROS activate NCX directly (Fig. 13.6). On the other hand, it was reported that Ang II or myocardial stretch, via AT_1 receptors stimulation, induces a ROS-mediated reduction of the transient outward potassium current (I_{to}) by a signaling pathway involving NADPH oxidase activation (Zhou et al., 2006). Moreover, Lu et al. (2008), recently reported that I_{to}, the slow delayed outward K^+ current (I_{Kslow}) and the steady-state K^+ current (I_{ss}) are phosphorylated and inhibited by p90RSK after ROS activation of this enzyme. Thus, decreased potassium currents would lead to a prolongation of action potential duration, which may eventually increase Ca^{2+} influx through NCX_{rev} (Fig. 13.6).

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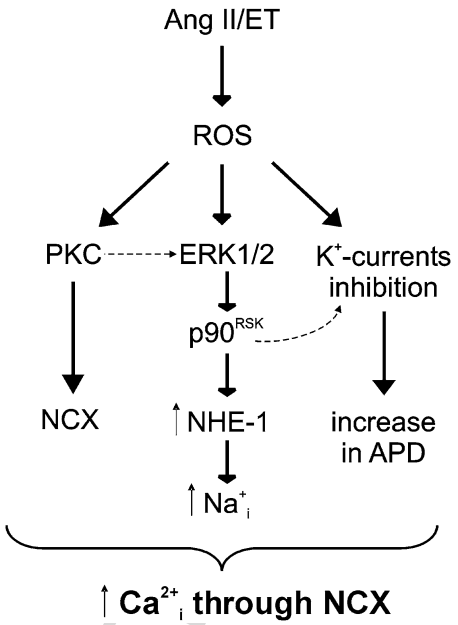

Fig. 13.6 Potential intracellular pathways mediated by ROS after stimulation of the autocrine Ang II/ET crosstalk. ROS generated by this autocrine mechanism might trigger the three different ROS-dependent pathways depicted in the Figure: **(a)** $[Na^+]_i$-independent and PKC-dependent pathway by direct stimulation of NCX; **(b)** $[Na^+]_i$-dependent pathway, consistent with a negative shift of the NCX reversal potential after a rise in $[Na^+]_i$ due to NHE-1 activation and **(c)** prolongation of the action potential duration due to inhibition of K^+ currents

In summary, it may be suggested that the reverse mode of cardiac NCX is modulated by myocardial stretch or, equivalently, by the Ang II/ET network, through the three different ROS-dependent pathways depicted in Fig. 13.6: (a) an $[Na^+]_i$-dependent pathway, consistent with a negative shift of the NCX reversal potential after a rise in $[Na^+]_i$ due to NHE-1 activation; (b) an $[Na^+]_i$–independent and protein kinase C-dependent pathway by direct stimulation of NCX; and (c) a prolongation of the action potential duration. All these intracellular pathways appear to be contributing in concert to the increase in Ca^{2+} after stretch.

The fact that Ang II triggers the beginning of the cascade of events leading to the slow force response has not been confirmed in all their steps. Activation of the NHE-1 after stretch has been confirmed in different species by several authors (Yamazaki et al., 1998; Alvarez et al., 1999; Calaghan and White, 2004; von Lewinski et al., 2004; Luers et al., 2005). However, the pathway leading to its activation is controversial. The release of Ang II and activation of the AT_1 receptors by stretch proposed by us in rat and cat myocardium (Cingolani et al., 1998; Alvarez et al., 1999; Perez et al., 2001), though reported in isolated rat myocytes (Sadoshima et al., 1993; Leri et al., 1998), was not confirmed by other investigators in ferret multicellular preparations (Calaghan and White, 2001). The role played by ET has been reported by Calaghan and White in ferret (Calaghan and White, 2001) and by us in rat (Alvarez et al., 1999) and cat myocardium (Cingolani et al., 1998; Perez et al., 2001), but it was not found in rabbit (Luers et al., 2005) or failing human myocardium (von Lewinski et al., 2004).

Whether the discrepancies are a matter of species differences is not apparent to us yet, but in any case, they leave open the possibility that under different

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experimental conditions some other mechanisms may be triggered by stretch. In this regard, another report by Calaghan and White (2004) shows activation of stretch-activated channels in addition to NHE-1 activation after myocardial stretch in rat ventricular myocytes and papillary muscles; Isenberg et al. (2005) proposed that myocardial stretch increases $[Na^+]_i$ and $[Ca^{2+}]_i$ in cell organelles partly by their influx through the stretch-activated channels, but they were unable to prevent the increase in $[Na^+]_i$ by cariporide. Interestingly, Hongo et al. (1996) demonstrated that the slow force response can be also detected in isolated cardiomyocytes, but they did not detect an increase in $[Na^+]_i$ during its development. In the same work, the authors also reported that L-type Ca^{2+} current is not involved in the slow force response. Vila Petroff et al. (2001) presented evidence that stretch activates the PI-3-kinase pathway to phosphorylate the endothelial isoform of nitric oxide synthase. Then nitric oxide stimulates Ca^{2+} release from the sarcoplasmic reticulum and promotes the slow force response. Unfortunately, the results of Vila-Petroff et al. (2001) could not be reproduced by other authors either in papillary muscle or isolated myocytes (Calaghan and White, 2004). This was certainly expected since the mechanism proposed by these authors requires a functional sarcoplasmic reticulum and the possible role of the sarcoplasmic reticulum in the slow force response has been clearly ruled out by several authors including Bluhm and Lew (1995), Hongo et al. (1995) and Kentish and Wrzosek (1998).

Another important aspect to consider in order to clarify the failure of detecting if ET is participating in the slow force response to stretch is to analyse the pharmacological intervention used to prove it. In this regard, Endoh et al. have clearly shown that high doses of the non-specific ET receptor antagonist TAK044 were necessary to prevent the inotropic effect of ET in the myocardium (Endoh et al., 1998). In our hands, either TAK044 or the selective ET_A receptor antagonist BQ123 (Fig. 13.7) blunted the slow force response (Alvarez et al., 1999; Perez et al., 2001). However, if based on the works of Calaghan and White (2001) and our own results (Cingolani et al., 1998; Alvarez et al., 1999; Perez et al., 2001) the role of ET after stretch is accepted in addition to the well known fact that Ang II induces release/formation of ET as shown in different studies by us (de Hurtado et al., 2000; Aiello et al., 2002; Perez et al., 2003; Cingolani et al., 2006) and others (Dohi et al., 1992; Imai et al., 1992; Chua et al., 1993; Ito et al., 1993; Fujisaki et al., 1995; Barton et al., 1997; Rajagopalan et al., 1997; Serneri et al., 1999; Muller et al., 2000; Ficai et al., 2001; Ortiz et al., 2001; Muller et al., 2002; Seccia et al., 2003), the rationale to accept our proposed chain of events seems to be plausible.

Regarding the identification of the ET isoform (s) that could be participating in the response to stretch, experiments in cat papillary muscles from our own laboratory showed an increase in ET-3 mRNA after stretch (Ennis et al., 2005). However, we should bear in mind that Tamamori et al. (1996) reported that, in cultured neonatal cardiomyocytes, ET-3 triggers the synthesis and release of ET-1, which in turn mediates a hypertrophic response. Therefore, though speculative, we should consider the possibility that the stretch of multicellular preparations triggers ET-3 release that might be responsible for the inotropic response and for the sequential release/formation of ET-1, which would induce cell growth. Moreover, while stretch could sequentially induce the release of ET-3 and ET-1, it is possible that exogenous

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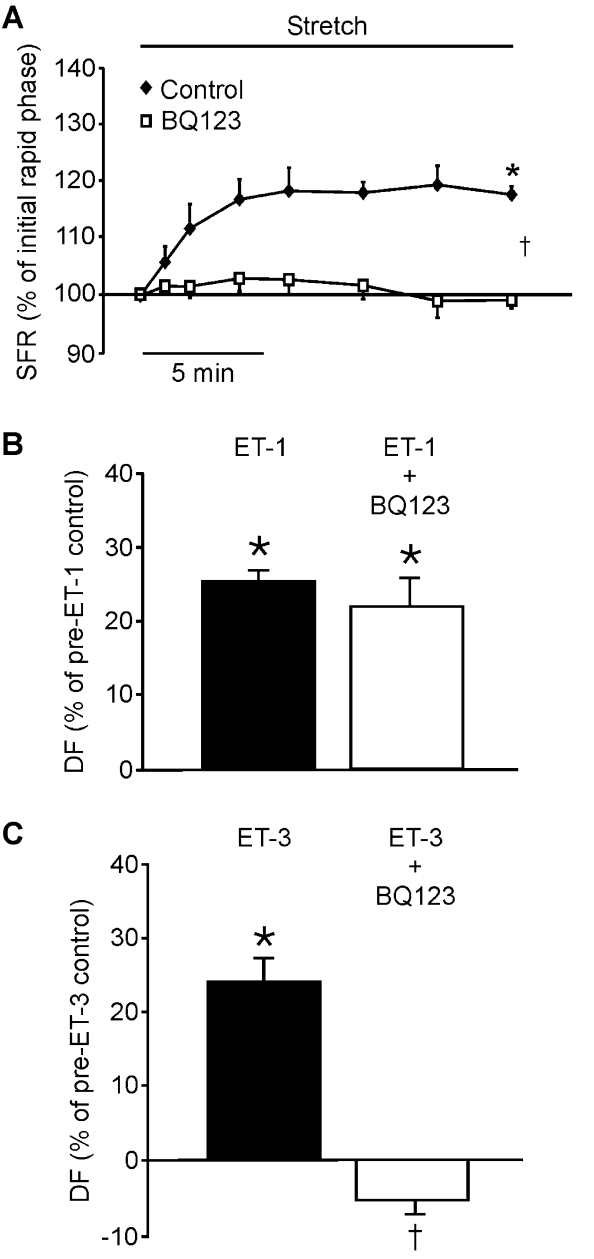

Fig. 13.7 **Panel A**: The stretch of rat papillary muscles promotes a slow force response (SFR) which stabilized after 10–15 min in a value ~20% greater initial phase. The ET_A blocker BQ123 canceled the SFR (the SFR was expressed as percent of the initial rapid phase). * Indicates $P<0.05$ vs. initial rapid phase, † indicates $P<0.05$ between curves. **Panel B** shows the lack of effect of BQ123 on the positive inotropic effect of 5 nmol/L ET-1. Comparative averaged results of developed force (DF) (expressed as percent of the pre-ET-1 value) after 30 min of incubation under both experimental conditions are shown. * Indicates $P<0.05$ vs. pre-ET-1 value. **Panel C**: Addition of 5 nmol/L ET-3 to a papillary muscle increased DF in a similar magnitude to the same dose of ET-1. This time, however, BQ123 canceled the increase in DF, suggesting that this may be the isoform involved in the SFR. DF was expressed as percent of the pre-ET-3 value after 30 min of incubation with the peptide. * Indicates $P<0.05$ vs. pre-ET-3 value, † indicates $P<0.05$ vs ET-3 alone. Modified from Ros et al. (2005) with permission

Ang II induces the release of ET-1 that in turn mediates, in this case, the increase in contractility. Supporting these speculations, we demonstrated, working with cat papillary muscles, that the same concentration of the ET_A blocker BQ123 (300 nmol/L) was able to cancel the slow force response to stretch and the inotropic effect induced by ET-3, but not that induced by ET-1 (Fig. 13.7) (Ros et al., 2005). However, we

need to mention that we have recently demonstrated that the positive inotropic effect and the increase in ROS production induced by ET-1 in isolated cat ventricular myocytes were effectively blocked by 300 nmol/L BQ123 (De Giusti et al., 2008).

We can state that myocardial stretch-induced NHE-1 activation and the role of the NCX in increasing Ca^{2+} transient are confirmed facts. Considering the results of other investigators and our own (Cingolani et al., 1998; Alvarez et al., 1999; Perez et al., 2001; Calaghan and White, 2004; Cingolani et al., 2005; Luers et al., 2005) together with those from the experiments in isolated neonatal cardiomyocytes (Yamazaki et al., 1998), we can conclude that NHE-1 activation induced by myocardial stretch constitutes a relevant intracellular signal leading to myocardial hypertrophy. A recent publication support the idea that activation of NHE-1 is sufficient to generate Ca^{2+} signals that induce cardiac hypertrophy and failure (Nakamura et al., 2008). This signaling pathway can be also evoked by equipotent doses of exogenous Ang II or ET-1 (Perez et al., 2003). Since it has been demonstrated that Ang II induces the release of ET-1 (see below), at least in some species, which in turn induces ROS formation and NHE-1 activation, the physiological chain of events depicted in Fig. 13.8 seems plausible.

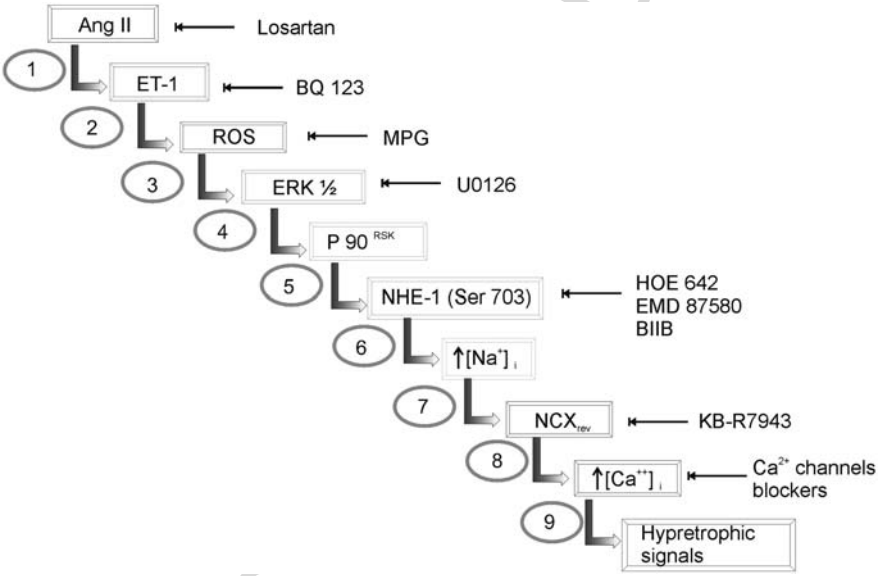

Fig. 13.8 Intracellular mechanisms triggered by 1 nmol/L Ang II. The figure schematizes the sequential steps that take place after activation of AT_1 receptors by Ang II, effect that can be blocked by the AT_1 blocker Losartan. Step 1: release of endogenous ET-1. Step 2: Increased ROS production after ET_A receptors activation, effect that can be blocked by the ET_A antagonist BQ123 and the ROS scavenger MPG. Step 3: Activation of the MAP kinase ERK 1/2 by ROS, effect that can be blocked by the MEK inhibitor U0126. Step 4: Phosphorylation and activation of P90RSK. Step 5: Phosphorylation and activation of the NHE-1, which can be blocked by the NHE-1 inhibitors HOE 642 (cariporide), EMD 87580 and BIIB. Step 6: Increase in the intracellular concentration of Na^+. Step 7: Activation of the reverse mode of the NCX, effect that can be inhibited by the blocker of the NCX_{rev}, KB-R7943. Step 8: Increase in the Ca^{2+} transient. Step 9: This increase in intracellular Ca^{2+} might lead to cardiac hypertrophy

13.3 Evidences for the ANG II-Induced Release of ET-1 Autocrine Mechanism

Many cardiovascular effects initially thought to be mediated by Ang II were in fact reported to be due to the paracrine/autocrine action of endogenous ET-1 released by the octapeptide (Ito et al., 1993; Rajagopalan et al., 1997; Liang and Gardner, 1998; Ortiz et al., 2001). The effects of stretch, which were mediated by the action of endogenous ET released by Ang II described above, were reflected by results obtained in cat papillary muscles. Since this is a multicellular preparation, it was not possible to elucidate if the action of ET-1 was paracrine or autocrine. However, working with isolated cat ventricular myocytes, we also reported that the increase in I_{NCX} induced by Ang II was blocked by ET receptors blockers, suggesting an autocrine interaction between these two hormones (Aiello et al., 2002). Accordingly, more recently we also showed that Ang II induced a concentration-dependent increase in sarcomere shortening of cat myocytes, which was downward shifted after ET receptors blockade (Fig. 13.9). This shift decreased the maximal effect of Ang II by approximately 30% and cancelled the effect of 1 nmol/L Ang II (Fig. 13.9). Therefore, these findings demonstrate that the increase in contractility induced by 1 nmol/L Ang II is entirely due to an autocrine pathway involving an ET isoform.

Further evidence that Ang II induces the release/production of ET from the myocyte was obtained in RT-PCR experiments performed in isolated cat myocytes

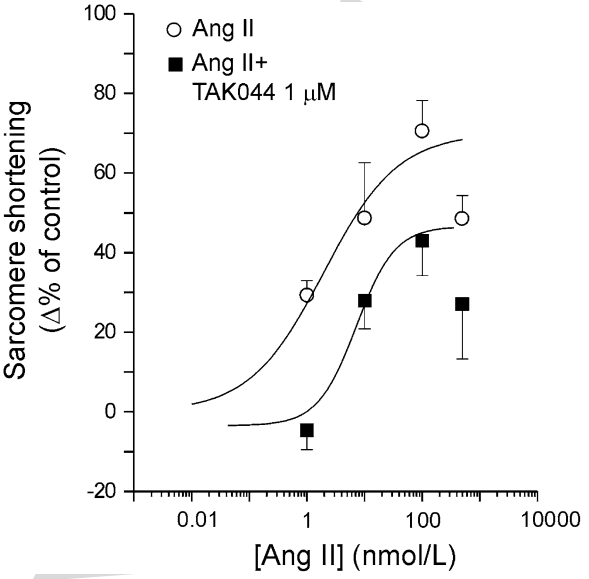

Fig. 13.9 Dose-response curve for different concentrations of Ang II, from 1 to 500 nmol/L, in the absence or presence of TAK044 (1 μmol/L). The maximal positive inotropic effect was obtained with 100 nmol/L Ang II. TAK044 shifted the dose-response curve to the right, and completely blocked the inotropic effect of 1 nmol/L Ang II indicating that this effect was entirely due to the action of the endogenous ET released/produced by Ang II. However, the data suggest that concentrations higher than 1 nmol/L are activating other mechanisms than the autocrine signal triggered by Ang II. Modified from Cingolani et al. (2006) with permission

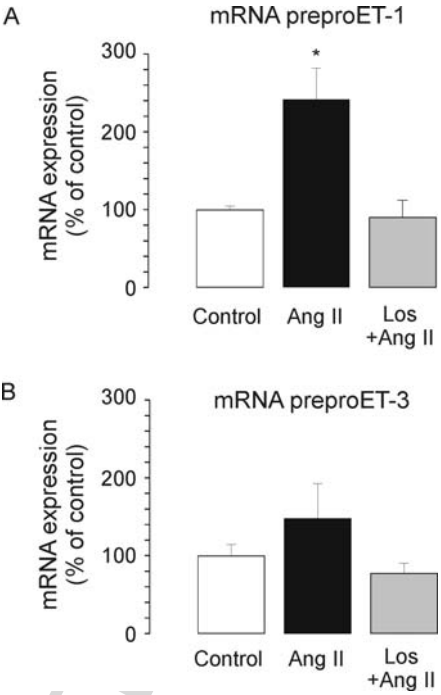

Fig. 13.10 Real-time RT-PCR. **Panel A** shows a significant increase in the expression of mRNA of preproET-1 induced by 1 nmol/L Ang II. This increase was prevented by losartan. The mRNA levels for preproET-3 (**Panel B**) does not change with 1 nmol/L Ang II. * Indicates $p<0.05$ vs. control. Modified from Cingolani et al. (2006) with permission

exposed to 1 nmol/L Ang II. Following 15 minutes of exposure to Ang II, isolated cat ventricular myocytes showed a significant increase in the expression of preproET-1 mRNA but not in that of preproET-3 mRNA (Fig. 13.10) (Cingolani et al., 2006). Although these RT-PCR experiments do not elucidate the mechanism by which Ang II induces the release/production of ET-1, they suggest that Ang II increases the "de novo" production of ET-1 in the isolated myocytes. It seems unlikely that the increase in preproET-1 mRNA levels in 15 min upon Ang II exposure could explain the acute positive inotropic effect induced by this peptide during that time frame, since production of mature ET-1 to be released by the myocyte would require a longer time period. However, it appears valid to assume, if the translation efficiency is not altered, that the Ang II-induced increase in ET-1 mRNA during this time reflects an increase in ET-1 synthesis secondary to the decrease in its intracellular pools due to its release, a mechanism probably leading to restore these intracellular pools.

13.4 The Slow Force Response as the Mechanical Counterpart of the Autocrine Mechanism Triggered by Stretch: the Anrep's Phenomenon

It is well known that two consecutive phases characterize the increase in force after myocardial stretch: A rapid and immediate one and the slow force response. The initial rapid change in force is induced by an increase in myofilament Ca^{2+}

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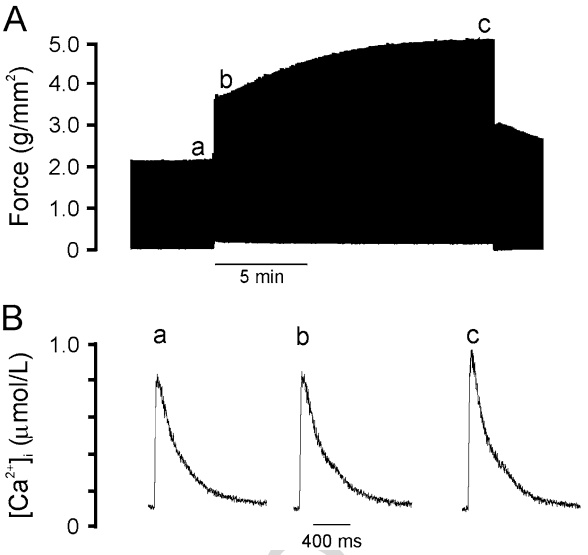

Fig. 13.11 After stretching a papillary muscle from 92 to 98% of Lmax, a sudden increase in force immediately occurs (a to b, **Panel A**), due to an increase in myofilament Ca^{2+} responsiveness. After that, a progressive increase in force develops during the next 10–15 min, the slow force response (SFR) (b to c), that is due to an increase in the Ca^{2+} transient (**Panel B**). Modified from Cingolani et al. (2001) with permission

responsiveness without changes in the Ca^{2+} transient whose underlying mechanisms are beyond the scope of this review (Fig. 13.11). The slow force response, in turn, is due to a progressive increase in the Ca^{2+} transient without changes in myofilament Ca^{2+} responsiveness during this phase (Fig. 13.11) (Allen and Kurihara, 1982; Kentish and Wrzosek, 1998; Alvarez et al., 1999). The increase in the Ca^{2+} level appears to result from the autocrine/paracrine mechanism described in the previous section. While the initial change in force after stretch seems to express the Frank-Starling mechanism, the slow force response may conceivably be the expression of Anrep's phenomenon.

In 1912, Von Anrep observed that when aortic pressure was elevated, ventricular volume initially increased and then declined to the starting volume. It appeared to him that an influence operating soon after myocardial dilatation caused an increase in myocardial contractility. His interpretation was that perhaps, the decrease in the flow to the adrenal glands induced the release of catecholamines and the consequent positive inotropic effect. In 1959, experiments by Rosenblueth et al. (1959) indicated that an increase in coronary perfusion pressure was not necessarily concomitant with the return of the heart to its initial volume. In 1960, Sarnoff et al. coined the term "pressure-induced homeometric autoregulation" to define the decrease in left ventricular end diastolic volume that follows an increase in diastolic volume due to a sudden increase in afterload. On the other hand, since the experiments of Sarnoff et al. (1960) were performed in isolated hearts, the study served to rule out the possibility of a role played by catecholamines in the described phenomenon. Interestingly, Sarnoff defined as "homeometric autoregulation" a phenomenon occurring in an organ which was not attributable to an influence by nerves or chemicals in its vicinity, paving the way for the idea of an autocrine/paracrine mechanism after cardiac stretching (Sarnoff et al., 1960). The existence of a real change in contractility during the homeometric autoregulation was challenged by the possibility of changes

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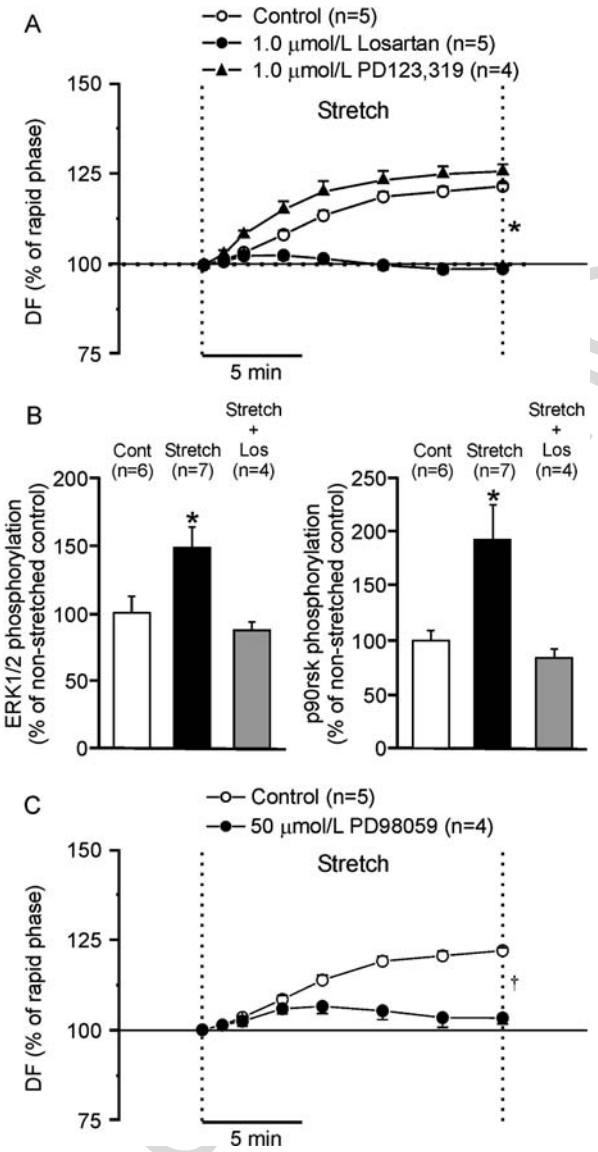

Fig. 13.12 Suppression of the slow force response (expressed as percent of initial rapid phase) after AT_1 but not AT_2 receptors blockade (Losartan and PD123, 319 respectively) (**Panel A**). Myocardial stretch significantly increased ERK1/2 and p90RSK phosphorylation, effect cancelled by losartan (Los) (**Panel B**). Inhibition of MEK (a kinase upstream ERK1/2 and downstream RAS) by PD98059 cancelled slow force response (expressed as percent of the initial rapid phase) (**Panel C**). *Indicates $P < 0.05$ vs. non-stretched control (cont); † indicates $P < 0.05$ control vs. PD98059. DF = developed force. Modified from Caldiz et al. (2007) with permission

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in coronary blood flow distribution (Monroe et al., 1972). However, in 1973 Parmley and Chuck reproduced for the first time the contractile effect of stretch in isolated strips of ventricular myocardium. They showed that when the length of the muscle was increased, there were corresponding rapid and slow increases in the developed force. Since the slow force response to the change in length was still present in isolated muscles from animals treated with reserpine, those authors also ruled out the possibility of catecholamines released by nerve endings as having a role in the mechanism.

We and other authors have provided evidence that activation of NHE-1 after stretch play a key role in the development of the slow force response (Alvarez et al., 1999; Perez et al., 2001; Calaghan and White, 2004; von Lewinski et al., 2004; Luers et al., 2005), however, there is no agreement in the role played by Ang II and ET in NHE-1 activation (Sadoshima et al., 1993; Leri et al., 1998; Alvarez et al., 1999; Calaghan and White, 2001; Perez et al., 2001). Ang II is an octapeptide acting through its own G coupled receptors AT_1 and AT_2. $G_\alpha q$-$\beta\gamma$ activated by either Ang II or ET-1 targets the NHE through extracellular signal-regulated protein kinases 1/2 (ERK1/2)-p90 ribosomal S6 kinase (p90RSK). We showed that the slow force response was abolished by AT_1 receptors blockade (Alvarez et al., 1999; Perez et al., 2001) (Caldiz et al., 2007) but not by AT_2 receptors blockade (Caldiz et al., 2007) as shown in Fig. 13.12A. These results support the notion that Ang II is released after stretch and triggers the intracellular signaling pathways leading to slow force response. We should keep in mind that the release of Ang II from the cell after stretch and its link with ET-1 has been previously demonstrated (Ito et al., 1993; Sadoshima et al., 1993). Furthermore, a significant increase in ERK1/2 and p90RSK kinase phosphorylation can be detected after 15 minutes of stretch, effects that are both cancelled by AT_1 receptors blockade with losartan as shown in Fig. 13.12B (Caldiz et al., 2007). Finally, inhibition of MEK (a kinase that is upstream of ERK1/2 and downstream of RAS kinases) by PD98059 abolished the slow force response to stretch (Fig. 13.12C).

13.5 Role of ROS After Stretch, ANG II and ET-1

Ang II and ET-1 are well known activators of the NADPH oxidase (Lavigne et al., 2001; Giordano, 2005; Kimura et al., 2005b) and through this action it has been reported the phenomenon called “ROS-induced ROS-release”, by which a small amount of ROS triggers a greater ROS production from the mitochondria (Fig. 13.13) (Zorov et al., 2000; Brandes, 2005; Kimura et al., 2005a). The possibility that this mechanism participates in the chain of events following stretch was examined. Figure 13.14A shows that stretch -in addition to its mechanical effect- induces an increase in intracellular ROS formation of approximately 30% above baseline levels. Furthermore, scavenging of ROS by N-(2-mercaptopropionyl)-glycine (MPG) or EUK8 inhibited both stretch-induced increase in ROS (Fig. 13.14A) and the slow force response (Fig. 13.14B). We

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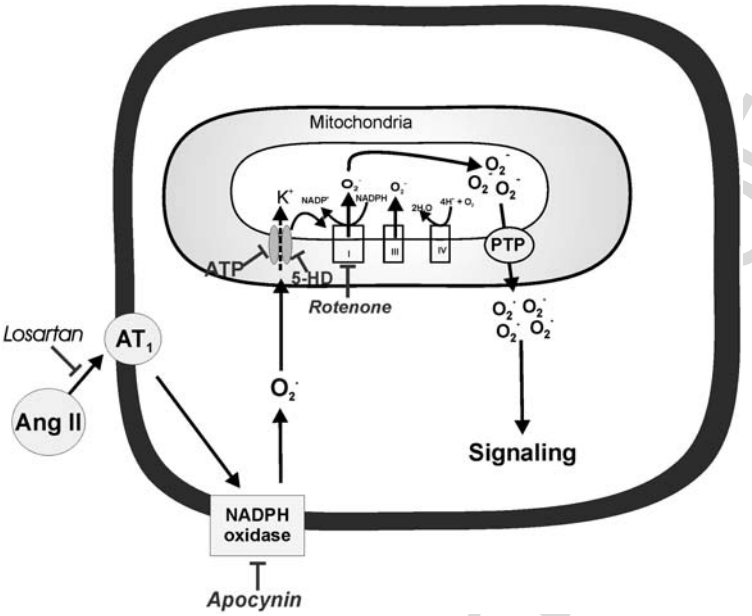

Fig. 13.13 The proposed "ROS-induced ROS-release mechanism". Stimulation of cardiac myocytes with Ang II leads via the action of AT_1 receptor to the assembly and activation of NADPH oxidase. The subsequently generated O_2^- stimulate mK_{ATP} channels, which augments the production of more O_2^- by the electron transport chain and allows the mitochondrial permeability transition pore (MPT) to open, facilitating the efflux of large amounts of O_2^- into the cytoplasm. O_2^- (or H_2O_2) can then act as signaling molecules in the cytosol (i.e. activating MAP kinases)

also found that the scavenging of ROS inhibited the increase in $[Na^+]_i$ that occurs in response to the stretch (Fig. 13.14C). We may hypothesize that activation of NAPDH oxidase after stretch would produce a small amount of O_2^-, which may open the ATP-sensitive mitochondrial potassium (mK_{ATP}) channels and produce a larger amount of O_2^- responsible for generating the slow force response. Therefore, if these assumptions were correct, the slow force response should be abolished by either NADPH oxidase inactivation or blockade of mK_{ATP} channels. As shown in Fig. 13.15A, slow force response was abolished after inhibition of NADPH oxidase inhibition (apocynin or diphenyleneiodonium chloride, DPI) or after blockade of mKATP channels (5-hydroxydecanoate, 5HD, or glibenclamide). The NHE-1-induced increase in $[Na^+]_i$ underlying the slow force response was also abolished by these interventions (Fig. 13.15B).

Ang II induced the production of O_2^- in a concentration-dependent manner in cat cardiac slices (Fig. 13.16A). Interestingly, the Ang II-induced concentration-dependent increase in O_2^- was very similar to the above shown (Fig. 13.9) concentration-dependent inotropic response curve (Fig. 13.16A), suggesting a potential correlation between Ang II-induced ROS production and positive

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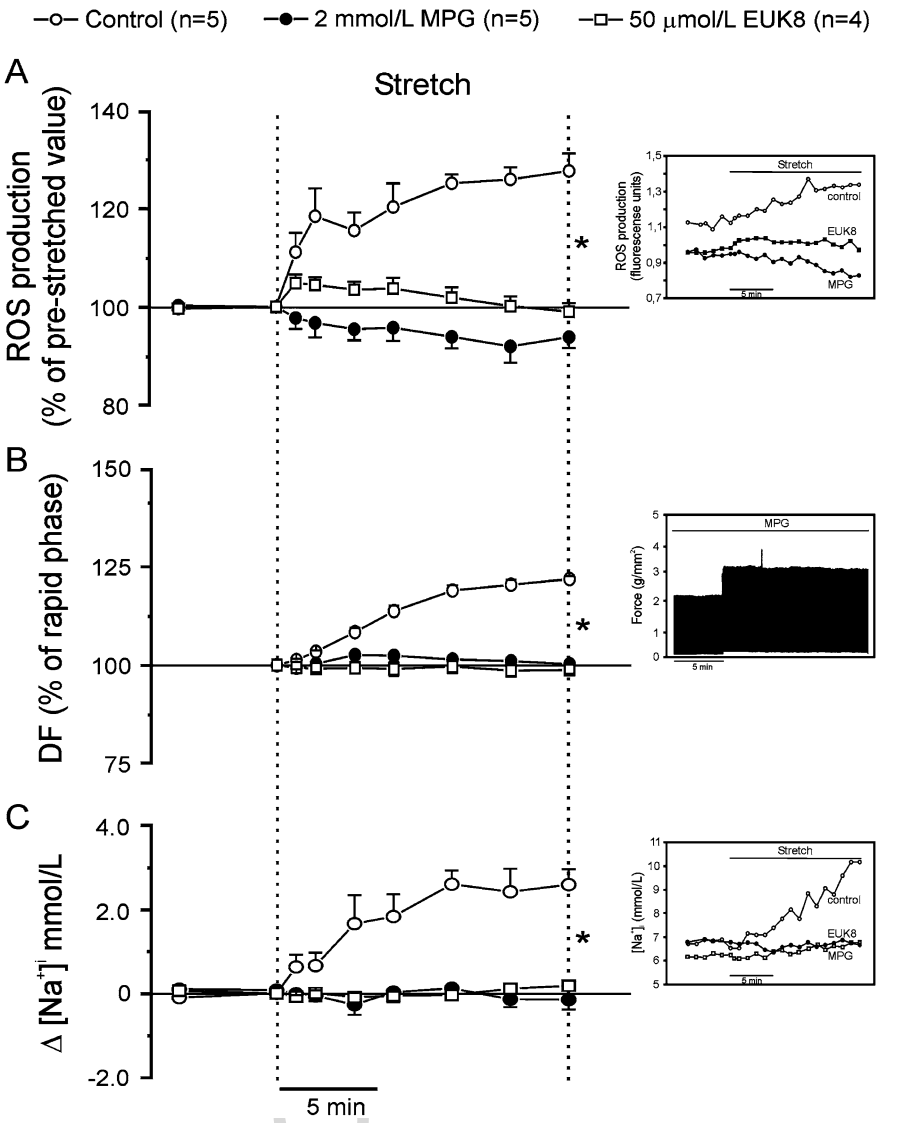

Fig. 13.14 Myocardial stretch induced an intracellular ROS increase of ~30% above the baseline levels that was cancelled by the ROS scavengers MPG and EUK8 (**Panel A**). MPG and EUK8 also cancelled the slow force response (expressed as percent of initial rapid phase) (**Panel B**). Furthermore, ROS scavenging also blunted stretch-induced increase in $(Na^+)_i$ (**Panel C**). *Insets* show original raw data. *Indicates $P < 0.05$ control vs. MPG and EUK8. DF = developed force. Modified from Caldiz et al. (2007) with permission

inotropy. The O_2^- production augmented by 1 nmol/L Ang II was abolished by AT_1 receptors blockade (losartan), ROS scavenging (MPG), NADPH oxidase inhibition (apocynin) and mK_{ATP} channels blockade (5HD or glibenclamide) as

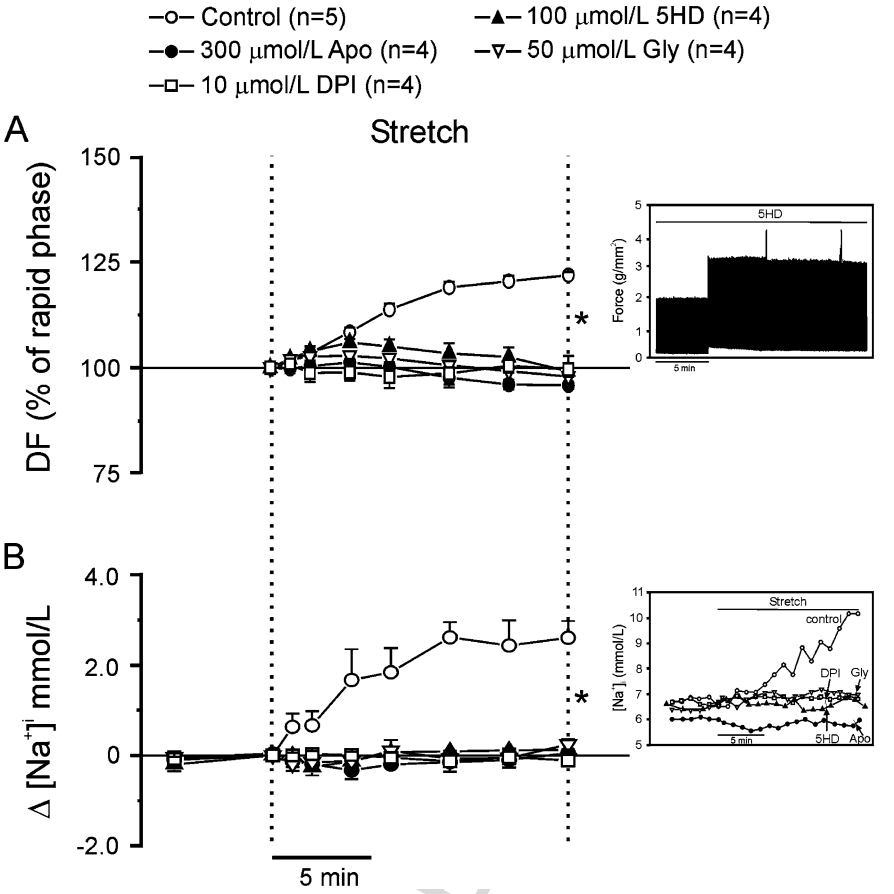

Fig. 13.15 NADPH oxidase inhibition by apocynin (Apo) or diphenyleneiodonium chloride (DPI) as well as mK_{ATP} channels blockade with 5-hydroxydecanoate (5HD) or glybenclamide (Gly) abolished slow force response (expressed as percent of initial rapid phase) (**Panel A**). All these interventions also cancelled NHE-1-mediated increase in $[Na^+]_i$ that accompanied the slow force response (**Panel B**). Insets show original raw data. * Indicates $P < 0.05$ control vs. all other groups. DF = developed force. Modified from Caldiz et al. (2007) with permission

shown in Fig. 13.16B. This Ang II-induced O_2^- production was also blunted by the non selective ET receptors blocker TAK044 and by the selective ET_A receptors antagonist BQ123 (unpublished observations), indicating that this effect is, in fact, mediated by endogenous ET released by Ang II. Consistently, MPG, apocynin, glybenclamide and 5HD also blocked the production of O_2^- induced by exogenous ET-1 in isolated cat ventricular myocytes (De Giusti et al., 2008) (Fig. 13.17). In line with these experiments, the ET-1-induced positive inotropic effect in cat ventricular myocytes was inhibited by these blockers (De Giusti et al., 2008) (Fig. 13.18), indicating that the "ROS-induced ROS-release" mechanism triggered

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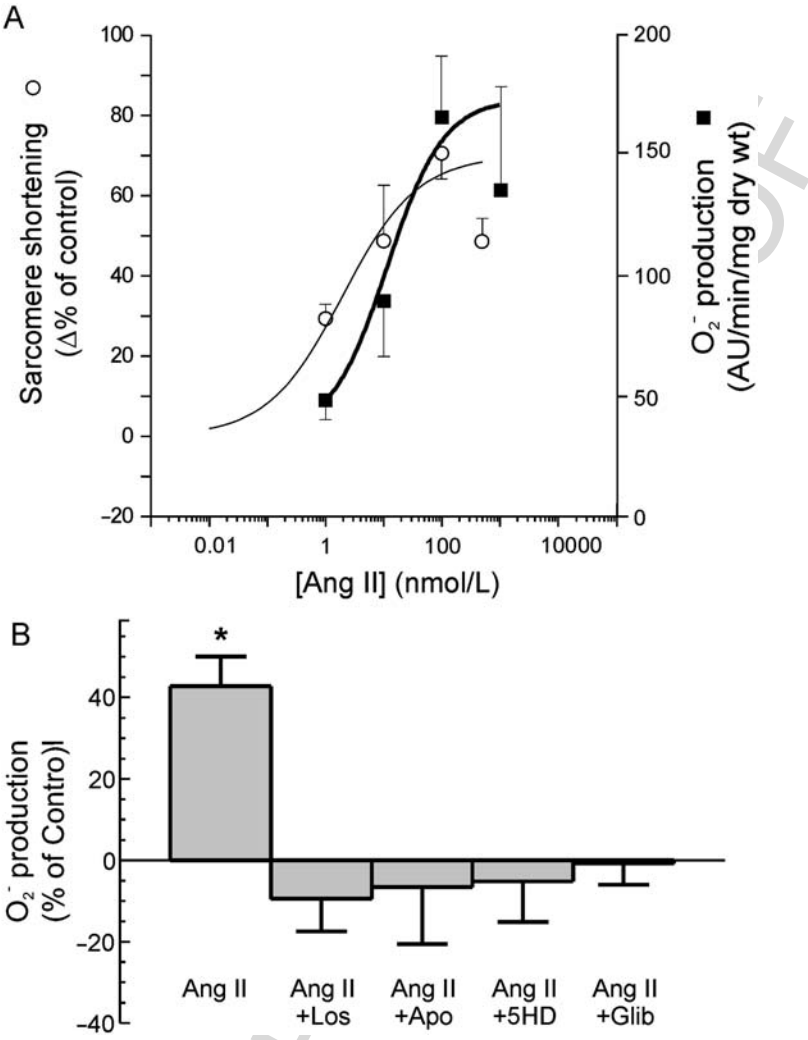

Fig. 13.16 **Panel A**: Ang II dose-response curves for the inotropic response and the production of O_2^-. The effect of different concentrations of Ang II on O_2^- production was assessed in cardiac tissue slices. Values of O_2^- production are expressed as the difference from control. The Ang II-induced concentration-dependent increase in O_2^- was very similar to the concentration-dependent inotropic response curve, suggesting a potential correlation between Ang II-induced ROS production and positive inotropy. **Panel B**: Superoxide production induced by 1 nmol/L Ang II (n = 34) in the absence and presence of 1 μmol/L losartan (Los, n = 8); 2 mmol/L MPG (n = 3); 300 μmol/L apocynin (Apo, n = 7); 100 μmol/L 5-hydroxydecanoate (5HD, n = 10) and 50 μmol/L glibenclamide (Gly, n = 6), expressed as percent of control values without additions and after 15 min of incubation. * Indicates $P < 0.05$ vs. control. Modified from Caldiz et al. (2007) and Garciarena et al. (2008) with permission

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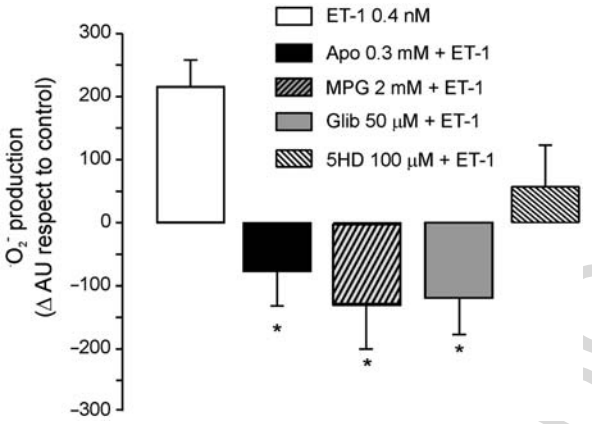

Fig. 13.17 Effect of MPG, the NADPH oxidase blocker, apocynin, and the mK_{ATP} blockers, glibenclamide and 5HD, on the ET-1-induced O_2^- production. Average increase in O_2^- after 15 min of 0.4 nmol/L ET-1 ($n = 12$). This increase was prevented by apocynin (Apo, 0.3 mmol/L; $n = 13$), MPG (2 mmol/L; $n = 12$) and glibenclamide (Glib, 50 μmol/L; $n = 14$) and attenuated by 5HD (100 μmol/L; $n = 15$) indicating that ET-1 is inducing the formation of O_2^- by activation of the NADPH oxidase, which in turn release O_2^- from the mitochondria after opening mK_{ATP} channels (ROS-induced-ROS-release). The results were expressed as the values in AU min $^{-1}$ 105 cells $^{-1}$ obtained in the presence of drugs minus control. *Indicates $p < 0.05$ vs. ET-1. Modified from De Giusti et al. (2008) with permission

by ET-1 participates in the inotropic response, being the release of mitochondrial ROS a step in the signaling cascade involved in this pathway.

The ET-1-induced positive inotropic effect observed in cat ventricular myocytes was also cancelled by the PKC inhibitor, chelerythrine, indicating that this kinase is involved in the intracellular pathway of this effect (De Giusti et al., 2008) (Fig. 13.19). However, the exact site of action of this enzyme in the chain of effects is unknown. One of these possible sites could be the activation of NADPH oxidase, since PKC activation is a critical step in the phosphorylation of the NADPH oxidase subunit p47phox and the subsequent assembly of this enzyme (Seshiah et al., 2002). However, the participation of PKC downstream NADPH oxidase activation can also be responsible for the effects of ET-1 on contractility. In relation to this matter, it is important to mention that the stimulation of the NHE-1 after PKC activation by ROS has been previously reported (Snabaitis et al., 2002). In addition, PKC can act upstream or downstream mK_{ATP} channels since PKC stimulation of mK_{ATP} channels (Sato et al., 1998) and PKC activation by mitochondrial ROS produced after mK_{ATP} channels opening (Juhaszova et al., 2004) have been reported. Moreover, a feed-forward mechanism in which mitochondrial swelling leads to activation of PKC, which stimulates mK_{ATP} channels and further increases mitochondrial swelling, has been also proposed (Juhaszova et al., 2004). Finally, the possibility that different PKC isoforms are acting upstream and downstream the production of ROS and/or the activation of mK_{ATP} channels might also be considered.

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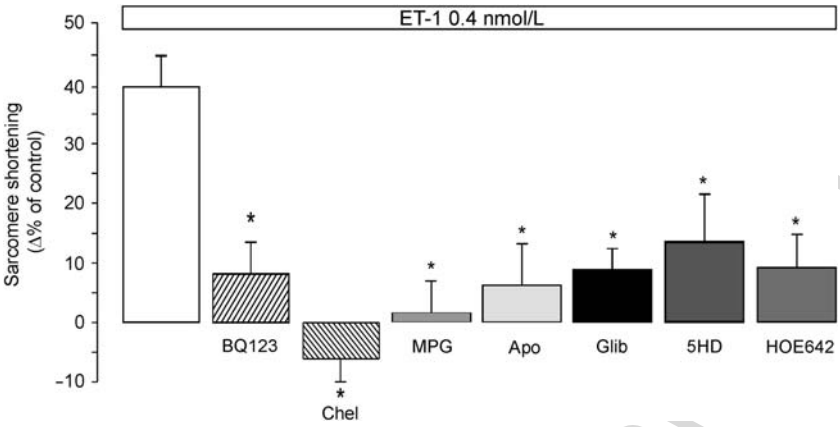

Fig. 13.18 The ET-1-induced positive inotropic effect is blunted by ET_A receptors, PKC and NADPH oxidase blockade, ROS scavenging, mK_{ATP} blockers and NHE inhibition. The average changes in SL shortening, expressed as delta percent of the control, with 0.4 nmol/L ET-1 ($n = 10$), and with the same concentration of ET-1 but in the presence of 0.3 μmol/L BQ123 ($n = 9$), 2 μmol/L chelerythrine (Chel, $n = 6$), 2 mmol/L MPG ($n = 8$), 0.3 mmol/L apocynin (Apo, $n = 13$), glibenclamide (Glib, 50 μmol/L, $n = 6$), 5HD (500 μmol/L, $n = 9$) and 5 μmol/L HOE642 (cariporide, $n = 7$) are shown. The positive inotropic effect induced by ET-1 was inhibited by BQ123, indicating that this effect is due to stimulation of the ET_A receptor. Prevention of the ET-1-induced increase in contractility with Chel suggests the participation of PKC in the intracellular pathway. Since MPG, Apo, Glib and 5HD also abolished this positive inotropic effect, the results suggest the participation of ET-1-induced ROS production by NADPH oxidase and the participation of mitochondrial ROS in this effect. Furthermore, the positive inotropic effect induced by ET-1 was inhibited by HOE642, indicating that this effect is mediated by NHE stimulation. *Indicates $p < 0.05$ vs. ET-1. Modified from De Giusti et al. (2008) with permission

The ET-1-induced positive inotropic effect was inhibited by NHE blockade with HOE642 (Fig. 13.18). Additionally, ET-1 was able to increase the proton flux (J_H) carried by the NHE during the recovery of intracellular acidosis induced by ammonium pulses and this effect was inhibited by scavenging ROS with MPG (De Giusti et al., 2008). These data are in line with previous results that have shown activation of the NHE after exogenous addition of H_2O_2 and stimulation of the MAPK ERK 1/2 pathway (Snabaitis et al., 2002) (Sabri et al., 1998). Consistently, ERK 1/2 phosphorylation induced by 1 nmol/L Ang II was cancelled by MPG, apocynin, glibenclamide, 5HD and the inhibitor of the complex I of the electron transport chain, rotenone (Fig. 13.19) (Garciarena et al., 2008), indicating that mitochondrial ROS released after NADPH oxidase activation are responsible for this effect. Interestingly, ERK 1/2 phosphorylation was also inhibited by cariporide (HOE642) (Fig. 13.19), suggesting that this compound is acting at a mitochondrial site, as also suggested by other authors (Juhaszova et al., 2004; Toda et al., 2007). In addition, it has been also demonstrated in cardiac slices that the Ang II-induced mitochondrial O_2^- formation was cancelled by cariporide and two other NHE-1 blockers, BIIB723 and EMD87580 (Fig. 13.20A) (Garciarena et al., 2008). Parallel in vitro experiments determined that these inhibitors were unable to decrease O_2^- formation

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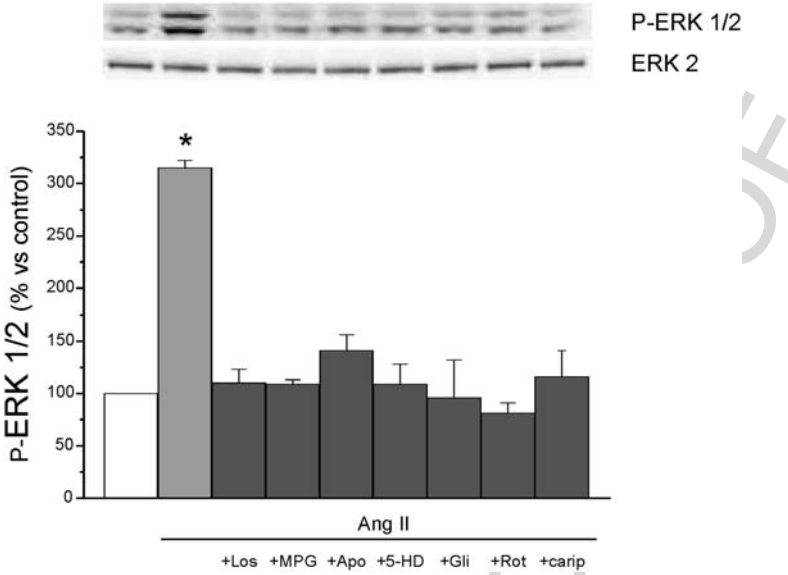

Fig. 13.19 Ang II-induced phosphorylation of ERK 1/2. Ang II (1 nmol/L) induced an increase in ERK1/2 phosphorylation in isolated cat ventricular myocytes that was prevented by losartan (Los, 1 μmol/L), MPG (2 mmol/L), apocynin (Apo, 300 μmol/L), 5-HD (100 μmol/L), glibenclamide (Gli, 50 μmol/L), rotenone (Rot, 10 μmol/L), and cariporide (carip, 10 μmol/L) (n = 4). No changes in total ERK1/2 was observed. $^{*}p < 0.05$ vs. all other groups, ANOVA. Modified from Garciarena et al. (2008) with permission

induced by PMS and NADH in a range that includes the values of chemiluminescence obtained with 1–100 nmol/L Ang II (Fig. 13.21A) (Garciarena et al., 2008), indicating that they were not acting as ROS scavengers. Moreover, the production of mitochondrial O_2^- induced by the mK_{ATP} opener diazoxide was also inhibited by cariporide (Fig. 13.20B). Thus, it seems likely that cariporide is targeting the mitochondria and blunting ROS formation which, in addition to the direct blocking effect

Fig. 13.20 **Panel A**: The stimulatory effect of 30 min-incubation with Ang II on O_2^- production by cardiac tissue slices was prevented by three different NHE-1 inhibitors; cariporide (carip, 10 μmol/L; $n = 12$), BIIB723 (BIIB, 1 μmol/L; $n = 3$) and EMD87580 (EMD, 5 μmol/L; $n = 4$). Values are the difference from the control after 15 min in the presence of lucigenin. **Panel B**: The increase in the chemiluminescence signal observed with 100 μmol/L Diaz ($n = 17$) was of a similar magnitude to that induced by 1 nmol/L Ang II and it was prevented by 5-HD (100 μmol/L; $n = 5$), carip (10 μmol/L; $n = 5$) and cyclosporine A (CsA) 2 μmol/L ($n = 5$). $^{*}p < 0.05$ vs. all other groups, ANOVA. **Panel C**: MPTP formation inhibition suppressed the stimulatory action of Ang II on mitochondrial ROS production. CsA (0.5, 1 and 2 μmol/L) prevented the effect of Ang II ($n = 4$). 2 μmol/L CsA did not affect control chemiluminiscence signal. Values are the difference from the control after 15 min in the presence of lucigenin expressed as the mean ± SE. None of the inhibitors used had an effect on the control chemiluminescence signal. $^{*}p < 0.05$ vs. all other groups, ANOVA. Modified from Garciarena et al. (2008) with permission

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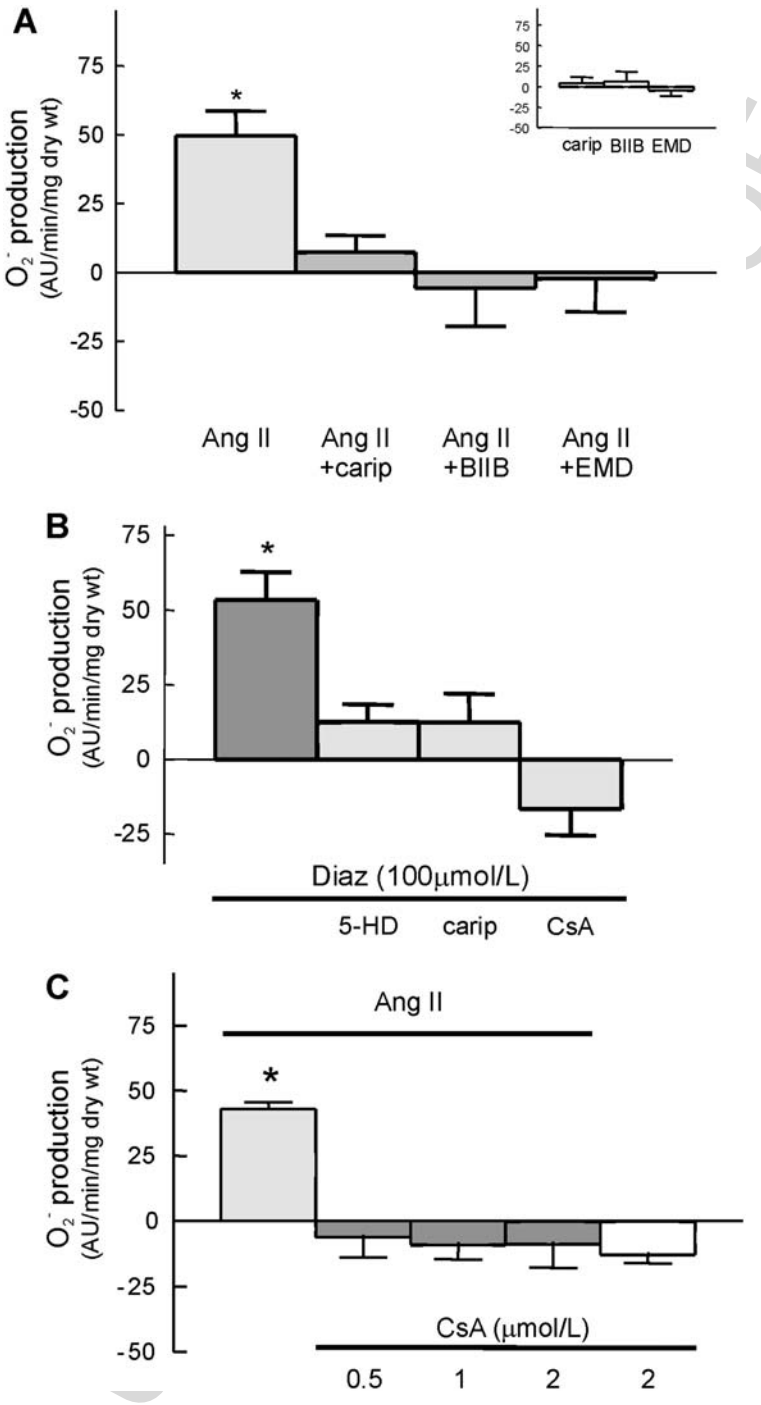

Fig. 13.20 (continued)

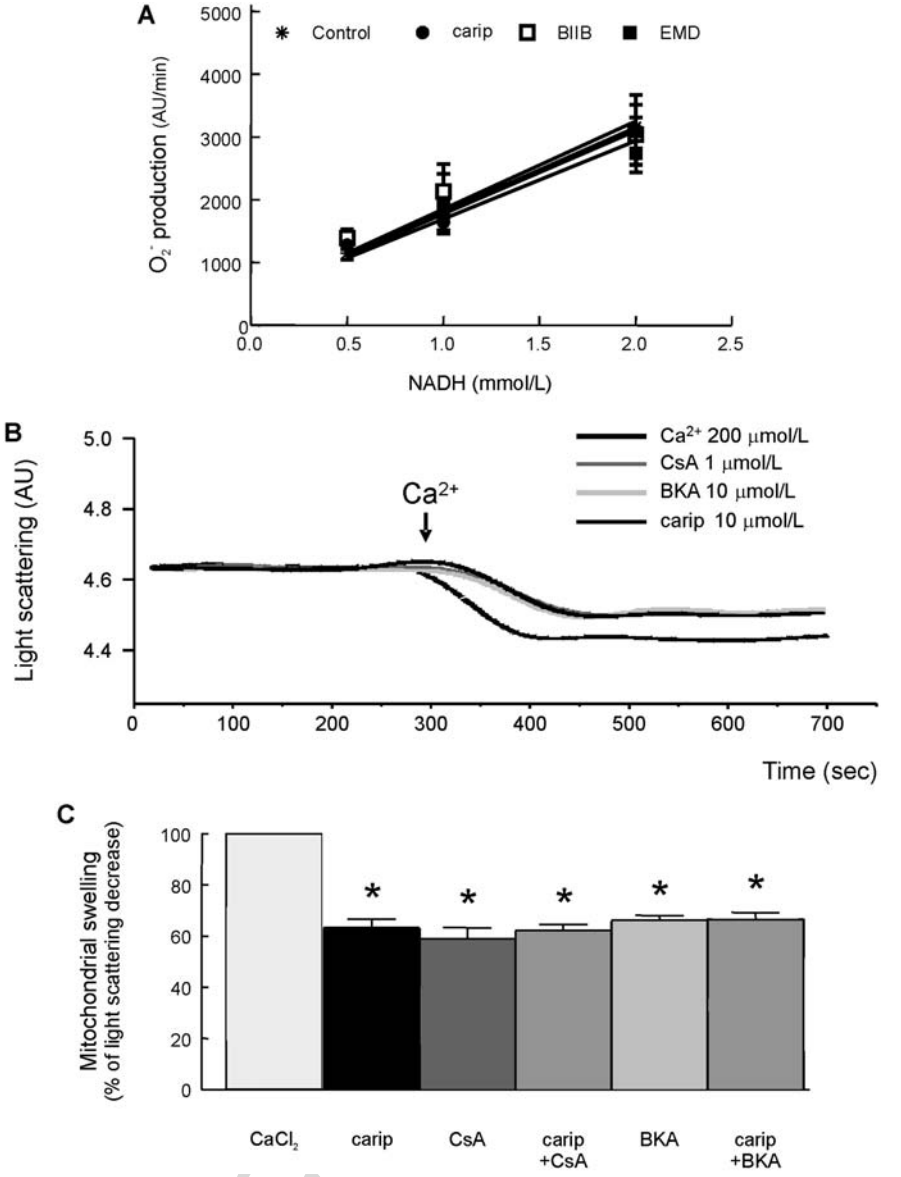

Fig. 13.21 **Panel A**: Lack of ROS scavenger effects of NHE-1 inhibitors. O_2^- production was induced in vitro by PMS and NADH in a range that includes the values of chemiluminescence (in AU/min) obtained with 1–100 nmol/L Ang II. None of the NHE-1 inhibitors (BIIB, carip and EMD) had an effect on the detected levels of $O_2^{-\cdot}$ induced by PMS and NADH ($n = 5$). **Panel B**: Mitochondrial swelling induced by $CaCl_2$. Typical experiment showing that cyclosporine A (CsA) and bongkrekic acid (BKA) significantly attenuated calcium-induced mitochondrial swelling and the decrease in light scattering in mitochondrial suspensions. Cariporide inhibited the decrease in light scattering in a similar magnitude to CsA (1 μmol/L) and BKA (10 μmol/L). **Panel C**: Average results. The combination of both drugs, CsA or BKA with cariporide, did not show any greater effect (n = 7). * $p < 0.05$ vs. $CaCl_2$, ANOVA. Modified from Garciarena et al. (2008) with permission

of the sarcolemmal NHE-1, would prevent the activation of this transporter by ROS. Recent experiments performed with isolated cat ventricular mitochondria allowed us to suggest that the mitochondrial site of action of cariporide could be, directly or indirectly, the mitochondrial permeability transiton pore (MPT), since this drug and the MPT blocker cyclosporine A (CsA) inhibited mitochondrial swelling to the same extent and both effects were not additive (Fig. 13.21B, C) (Garciarena et al., 2008).

It is well known that irreversible opening of the MPT leads to cell death. Mitochondrial ROS burst lower the threshold for MPT opening, triggering the apoptotic cascade (Kim et al., 2003; Shivakumar et al., 2008). However, milder mitochondrial ROS generation after mK_{ATP} opening induces a series of anti-apoptotic events, involving PKC activation, glucogen synthase kinase 3β (GSK3β) phosphorylation and prevention of MPT opening (Juhaszova et al., 2004; Costa and Garlid, 2008; Gomez et al., 2008). We have recently shown that the Ang II- and diazoxide-induced O_2^- production was cancelled after MPT blockade with CsA (Fig. 13.20B, C) (Garciarena et al., 2008). A possible explanation is that MPT opening is necessary to induce the increased production of mitochondrial O_2^-. Supporting this hypothesis, Cheng et al. (Wang et al., 2008) have recently demonstrated that reversible and transient opening of MPT triggers the formation of O_2^- flashes in the mitochondrial matrix.

It is important to note that both, mK_{ATP} activation (which could lead to "protective" mitochondrial ROS production) and inhibition of the NHE-1 by cariporide, have been identified as relevant cardioprotective mechanisms upon ischemia/reperfusion (Karmazyn et al., 1999; Pain et al., 2000; Avkiran and Marber, 2002; Oldenburg et al., 2003, 2004; Kimura et al., 2005b). However, regarding the inhibitory effects of cariporide and CsA on the diazoxide-induced O_2^- production, we could speculate that the protection induced by diazoxide would be lost with cariporide (Fig. 13.22). This speculation, that seems paradoxical, would be and interesting topic for further research.

The intracellular pathways discussed in this section, which involve the participation of the "ROS-induced ROS release mechanism" triggered by the autocrine

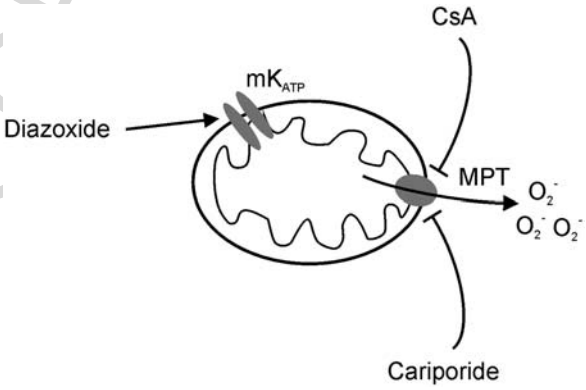

Fig. 13.22 Diazoxide stimulates mK_{ATP} channels leading to an increase in mitochondrial ROS production that might flux across the membrane through the MPT. Either CsA or cariporide inhibited the mitochondrial ROS release, suggesting that they have a common target, the MPT

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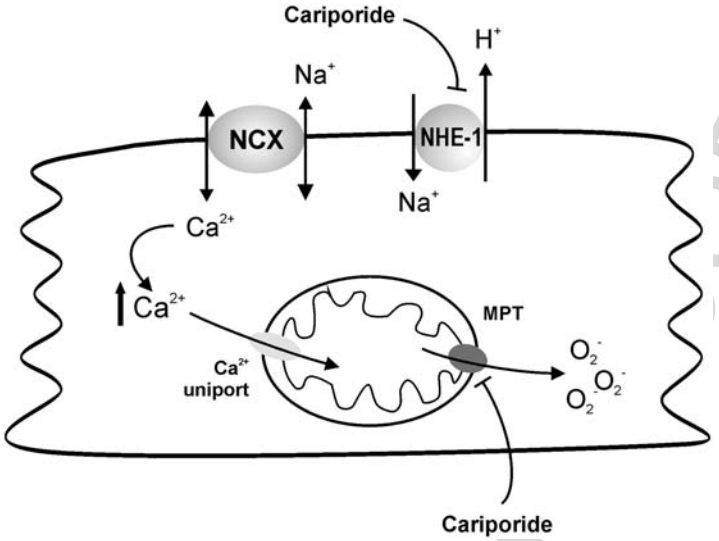

Fig. 13.23 Possible sites of action of cariporide in the cell: the sarcolemma and the mitochondria. The figure shows that cariporide can inhibit NHE-1, leading to a decrease in Na^+_i and Ca^{2+}_i (decrease of NCX reverse mode or increase of NCX forward mode) and therefore also a decrease in mitochondrial calcium. On the other hand, cariporide can inhibit MPT. In both cases, cariporide might attenuate the mitochondrial ROS production

Ang II/ET-1 mechanism are depicted in the cell and mitochondrial schemes of Figs. 13.23 and 13.24, respectively.

13.6 The Mechanical and Hypertrophic Effect of NHE-1 Activation

The possible link between slow force response to stretch and myocardial hypertrophy is supported by the fact that an enhanced activity of the NHE-1 – the cause of the slow force response – is detected in several models of cardiac hypertrophy and, consistent with this, the specific blockade of NHE-1 has been shown to effectively regress cardiac hypertrophy in different models (Hori et al., 1990; Mrkic et al., 1993; Perez et al., 1995; Schussheim and Radda, 1995; Takewaki et al., 1995; Yamazaki et al., 1996, 1998; Schluter et al., 1998; Hayasaki-Kajiwara et al., 1999; Yokoyama et al., 2000; Yoshida and Karmazyn, 2000; Chen et al., 2001; Konstantinou-Tegou et al., 2001; Kusumoto et al., 2001; Camilion de Hurtado et al., 2002b; Engelhardt et al., 2002; Schafer et al., 2002; Bak and Ingwall, 2003; Ennis et al., 2003; Fujisawa et al., 2003; Karmazyn et al., 2003; Rajapurohitam et al., 2003; Saleh et al., 2003; Aker et al., 2004; Chen et al., 2004; Marano et al., 2004; Xu et al., 2004; Baartscheer et al., 2005; Chahine et al., 2005; Javadov et al., 2005; Kilic et al., 2005; Rajapurohitam et al., 2006). The increase in $[Ca^{2+}]_i$ is widely

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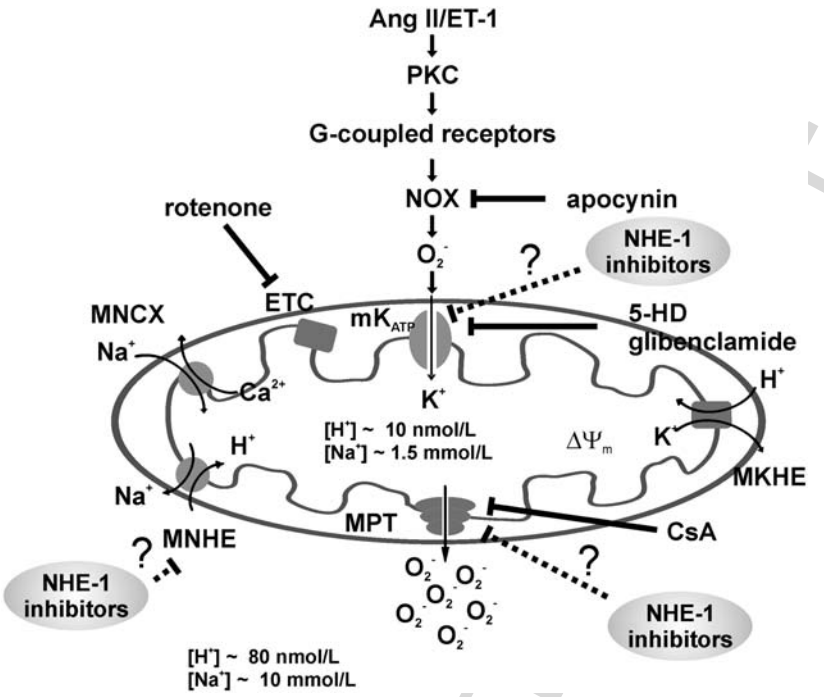

Fig. 13.24 Possible mitochondrial sites of action of NHE-1 inhibitors. The scheme shows the "two step" release of ROS through activation of G-coupled receptors and inhibition of the MPT formation by NHE-1 inhibitors. These inhibitors may act upon different mitochondrial mechanisms, including MNHE. They may act through a decrease in mitochondrial Ca^{2+}, H^+, inner membrane potential affecting the MPT formation or altering the sensitivity to those factors to induce MPT formation. Modified from Garciarena et al. (2008) with permission

recognized as one of the main prohypertrophic intracellular signals. It activates several intracellular pathways like calcineurin/ nuclear factor of activated T cells (NFAT), Ca^{2+}/calmodulin-dependent kinase II (CaMKII), PKC and possibly some others. Nevertheless, we emphasize that $[Ca^{2+}]_i$ may be increased by mechanisms other than that triggered by the hyperactivity of NHE-1. It has been recently suggested that CaMKII is preferentially activated by an increase in a specific subcellular Ca^{2+} pool localized in the perinuclear area after ET-1 stimulation (Wu et al., 2006).

In 1995 an enhanced activity of the NHE-1 was reported in the hypertrophied myocardium of spontaneously hypertensive rats (SHR) (Perez et al., 1995; Schussheim and Radda, 1995). The hyperactivity of NHE-1 has been described in several tissues other than the myocardium in human hypertension (Livne et al., 1987; Rosskopf et al., 1993; Garciandia et al., 1995). Experiments performed in our laboratory showed that the hyperactivity of NHE-1 in the myocardium of the SHR was not accompanied by an increase in pH_i, since there was a simultaneous activation of the acidifying Cl^-–HCO_3^- exchanger (Perez et al., 1995) (see Fig. 13.1). We also reported that the NHE-1 increased activity in this model was the result

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of a PKC-dependent post-translational modification of the exchanger (Ennis et al., 1998). It was further hypothesized that the inhibition of the antiporter activity could regress and/or prevent the development of hypertensive hypertrophy. Kusumoto et al. (2001) proved that NHE-1 was upregulated after myocardial infarction and that the specific inhibition of this exchanger with cariporide decreased hypertrophy and remodeling in these hearts. Experiments from our own laboratory demonstrated that myocardial hypertrophy of SHR regressed after 1-month cariporide treatment (Fig. 13.25) without significantly changing the arterial pressure (Camilion de Hurtado et al., 2002b). In addition, we reported that chronic NHE-1 blockade normalized the enhanced interstitial fibrosis of these hypertrophic hearts, but this effect took longer to occur compared to the regression of myocyte size (Cingolani et al., 2003b) (Fig. 13.26), possibly as a reflection of the lower turn-over rate of collagen (Weber and Brilla, 1991).

The precise mechanism by which NHE-1 inhibition prevents hypertrophy is still unknown, though a number of pathways have been proposed (Fliegel and Karmazyn, 2004) As there is evidence that calcineurin plays a key role in many pathological models of cardiac hypertrophy (Molkentin et al., 1998; Taigen et al., 2000; Haq et al., 2001; Bueno et al., 2002; Nagata et al., 2002; Zou et al., 2002; Wilkins et al.,

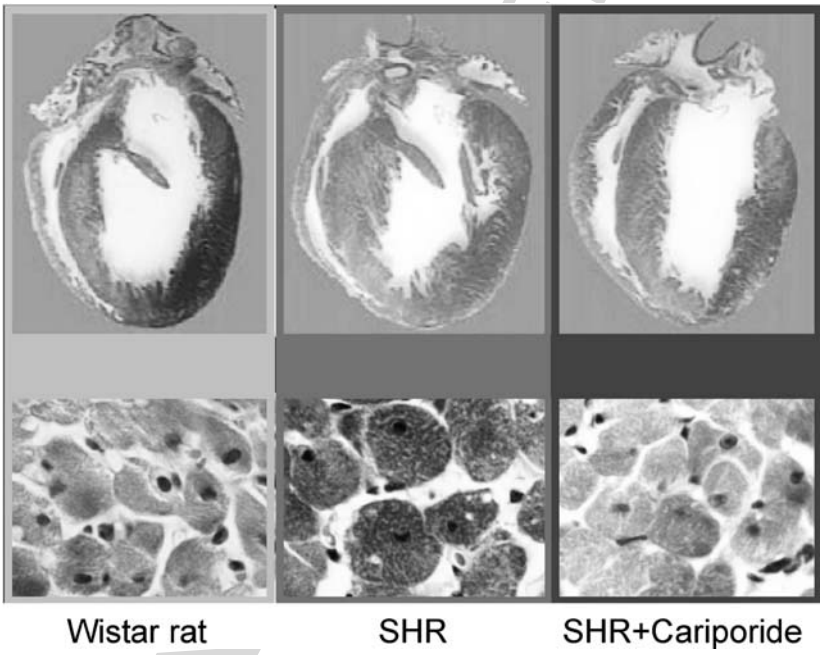

Fig. 13.25 Chronic NHE-1 blockade with cariporide (one-month treatment) regressed myocardial hypertrophy in SHR. *Upper panels* show comparative major axis sections of representative hearts from a Wistar control rat (*left*), a non-treated SHR (*middle*) and a cariporide treated SHR (*right*), and lower panels show representative myocytes cross section micrographs from the three experimental groups. Modified from Camilion de Hurtado et al., (2002b) with permission

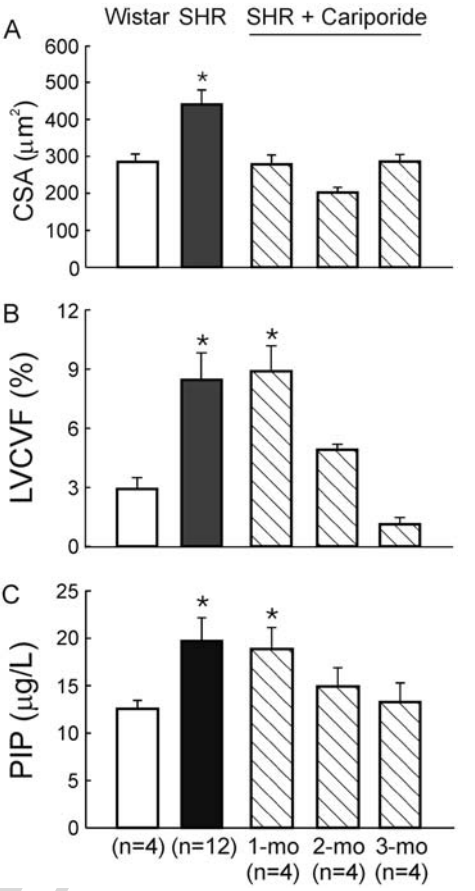

Fig. 13.26 Chronic NHE-1 blockade normalized the enhanced interstitial fibrosis of the hypertrophic SHR hearts, but a longer treatment was necessary to observe this effect. Despite the fact that full regression of myocytes cross sectional area (CSA) was observed as early as after one-month cariporide treatment (**Panel A**), fibrosis indexes like left ventricle collagen volume fraction (LVCVF) (**Panel B**) and serum levels of the carboxyterminal propeptide of procollagen type I (PIP) (**Panel C**) remained elevated. However, when treatment duration was prolonged, normalization of fibrosis was observed (**Panels B** and **C**). Modified from Cingolani et al. (2003b) with permission

2004), we recently investigated its participation in the signaling pathway involved in the regression of cardiac hypertrophy induced by NHE-1 inhibition. We analyzed the expression of the β-isoform of calcineurin A (CnAβ) as an indication of calcineurin activity. The nuclear abundance of NFAT in the left ventricular myocardium of untreated SHR, treated SHR and normotensive rats was measured as a confirmation of calcineurin activation. CnA expression and NFAT nuclear abundance are augmented in the hypertrophied myocardium of untreated SHR, compared with the normotensive rats, and the regression of cardiac hypertrophy induced by NHE-1 inhibition normalizes both parameters (Fig. 13.27) (Ennis et al., 2007) This was the first report showing that the regression of cardiac hypertrophy caused by NHE-1 inhibition, which is independent from any change in blood pressure, is accompanied by normalization of CnAβ expression and NFAT nuclear abundance. Even though we have provided evidence that a decrease in CnA and nuclear NFAT expression takes place during the regression of cardiac hypertrophy induced by NHE-1 inhibition, we cannot rule out the possibility of additional effects of this pharmacological

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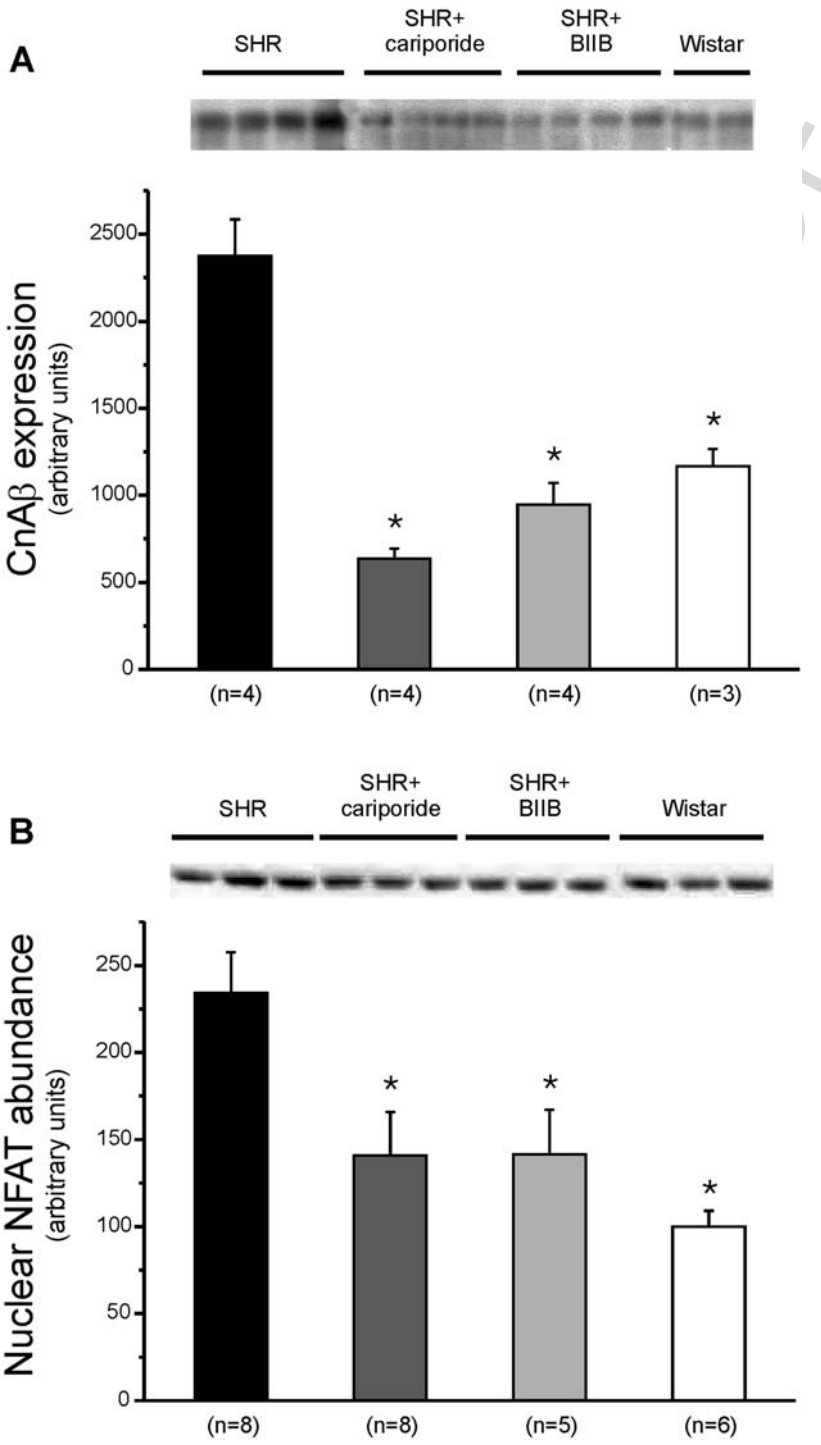

Fig. 13.27 (continued)

Table 13.1 Models of cardiac hypertrophy (CH) where the NHE-1 may play a role

Cardiac hypertrophy model	References
Pressure overload	Arai et al. (1995), Perez et al. (1995), Perez et al. (2003), Marano et al. (2004), and Baartscheer et al. (2005)
Post myocardial infarction	Yoshida and Karmazyn (2000), Camilion de Hurtado et al. (2002b), and Bers et al. (2003)
β–adrenergic stimulation	Weber and Brilla (1991), Schafer et al. (2002), and Cingolani et al. (2003b)
Hyperthyroidism	Li et al. (2002b) and Bak and Ingwall, (2003)
Hyperparathyroidism	Harnett et al. (1988), Azarani et al. (1995), and Saleh et al. (2003)
Mineralocorticoid stimulation	Fujisawa et al. (2003)
Leptin stimulation	Konstantinou-Tegou et al. (2001) and Xu et al. (2004)
Human heart failure	Chen et al. (2001)
Hamster model hereditary cardiomyopathy	Chahine et al. (2005)
α-adrenergic stimulation	Schluter et al. 1998, Xia et al. 2004, Dulce et al. (2006)
ET-1 stimulation	Xu et al. (2004) and Dulce et al. (2006)
Angiotensin II stimulation	Yamazaki et al. (1995) and Hautala et al. (2002)
Stretch	Sadoshima et al. (1993), Yamazaki et al. (1995)
ANP receptor deficient mice	Mrkic et al. (1993)
Carbonic anhydrasa inhibition	Li et al. (2002a)
Combined pressure and volume overload	Baartscheer et al. (2003), Baartscheer et al. (2005) and Baartscheer et al. (2008)
Pacing-induced	Aker et al. (2004)
Monocrotaline-induced	Chen et al. (2001)

intervention. It has been proposed, as we discussed earlier, that cariporide might also exert effects at the mitochondrial level (Miura et al., 2001; Ruiz-Meana et al., 2003; Teshima et al., 2003; Javadov et al., 2005)

Below are summarized several neuro-hormonal models of cardiac hypertrophy in which a link between NHE-1 activity and myocardial growth has been established (Table 13.1):

Fig. 13.27 Panel A: Calcineurin Aβ expression was analyzed in the myocardium of treated (cariporide or BIIB723) and untreated SHR ($n = 4$ each group). Calcineurin Aβ expression was up-regulated in the hypertrophied myocardium of the untreated SHR while a significant decrease in its expression was detected after the regression of cardiac hypertrophy by the NHE-1 inhibitors. For the sake of comparison the results obtained in normotensive rats ($n = 3$) were included in the figure. The calcineurin Aβ expression levels of the cariporide- and BIIB723-treated SHR were not significantly different from those of the NT rats. Panel B: Representative Western blot and average values of NFAT abundance in nuclear extracts from LV of untreated, cariporide- or BIIB723-treated SHR and normotensive (NT) rats ($n = 8$, 8, 5 and 6, respectively). NHE-1 inhibition normalized the nuclear expression of NFAT, previously up-regulated in the hypertrophied myocardium of SHR. * means $p < 0.05$ vs. untreated SHR, ANOVA. Modified from Ennis et al. (2007) with permission

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1. An up-regulation of NHE-1 was reported in a cardiac hypertrophy and failure model of β_1-adrenergic receptor transgenic mice (Engelhardt et al., 2002) The inhibition of this exchanger prevented the development of cardiac hypertrophy and fibrosis, suggesting that NHE-1 was essential for the detrimental cardiac effects of chronic β_1-receptor stimulation in the heart (Engelhardt et al., 2002) Similarly, cardiac hypertrophy induced in rats by chronic isoproterenol administration was prevented by inhibition of NHE-1 (Ennis et al., 2003)
2. Hypertrophied hyperthyroid hearts show enhanced g810 NHE-1 activity and when exposed to acute ischemia, they accumulate more Na^+ than the control non-hypertrophied hearts (Bak and Ingwall, 2003) These changes were prevented by NHE-1 inhibition (Bak and Ingwall, 2003) Furthermore, it has been demonstrated that thyroid hormone, by the interaction of its receptor with the NHE-1 promoter increases the expression of NHE-1 (Li et al., 2002b)
3. In patients with end-stage renal disease and secondary hyperparathyroidism as well as in patients with primary hyperparathyroidism, a strong correlation between cardiac hypertrophy and serum parathyroid hormone levels has been reported (Harnett et al., 1988; Bauwens et al., 1991; Piovesan et al., 1999). This correlation was shown to be even much stronger than that between Ang II and hypertrophy (Bauwens et al., 1991). In addition, a direct evidence that parathyroid hormone improves hypertrophy was also reported (Schluter and Piper, 1992). Though controversial (Mrkic et al., 1993; Azarani et al., 1995), a stimulatory effect of parathyroid hormone on NHE-1 has been described; therefore, it is tempting to speculate about the possible involvement of the antiporter in the signaling pathway evoked by parathyroid hormone in the genesis of cardiac hypertrophy. On the other hand, low sodium plasma levels were detected in patients with NYHA class III–IV heart failure and high levels of parathyroid hormone (Arakelyan et al., 2007). The resulting misbalance of the Na^+/Ca^{2+} may in turn be a factor to consider in the development of cardiac hypertrophy.
4. In rat neonatal ventricular myocytes, aldosterone stimulation induced a hypertrophic response accompanied by NHE-1 up-regulation and increased $[Na^+]_i$. Both, hypertrophy and elevated $[Na^+]_i$, were prevented by the NHE-1-specific inhibitor EMD87580 as well as the aldosterone antagonist spironolactone (Karmazyn et al., 2003). Similar results were obtained in uninephrectomized rats exposed to deoxycorticosterone acetate/salt, in which cariporide treatment completely inhibited hypertrophy and NHE-1 up-regulation (Fujisawa et al., 2003).
5. Cardiac hypertrophy of atrial natriuretic peptide receptor-deficient mice was accompanied by an increased activity of NHE-1, which thereby increased $[Ca^{2+}]_i$ (Kilic et al., 2005). It was shown that these alterations were normalized by chronic treatment with the NHE-1 inhibitor cariporide. These results are in line with the report by Tajima et al. (1998) demonstrating that atrial natriuretic peptide inhibits NHE-1 activity.
6. Emerging evidence indicates that leptin – a protein encoded by the obesity gene- is linked to cardiac hypertrophy (Rajapurohitam et al., 2003, 2006; Xu et al., 2004). Interestingly, leptin has been reported to activate NHE-1 through

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a PKC-dependent pathway (Konstantinou-Tegou et al., 2001). Moreover, it has been reported that leptin elevates ET-1 levels and, though speculative, this may be the pathway involved in NHE-1 stimulation (Xu et al., 2004). Furthermore, a recent report by Karmazyn's group implicated leptin as a mediator of hypertrophic effects of Ang II and ET-1 in cultured neonatal ventricular myocytes (Rajapurohitam et al., 2006).

7. In right ventricular hypertrophy due to monocrotaline-induced pulmonary artery injury, myocardial NHE-1 expression was enhanced. As a consequence, both hypertrophy and NHE-1 up-regulation were abrogated by cariporide treatment (Chen et al., 2001).
8. In rabbits subjected to volume and pressure overload, which induced cardiac hypertrophy and failure, acute inhibition of NHE-1 in isolated left ventricular myocytes reversed ionic remodeling (Baartscheer et al., 2003). In this model, it has also been reported that dietary cariporide treatment, initiated at induction of volume and pressure overload, reduced hypertrophy and prevented the development of heart failure and cellular ionic and electrical remodeling (Baartscheer et al., 2005). Moreover, it has been recently reported by the same group, that in rabbit hearts with established hypertrophy and signs of heart failure (one month after induction of pressure/volume overload), two months of chronic treatment with cariporide caused regression of hypertrophy, heart failure and ionic and electrophysiological remoldeling (Baartscheer et al., 2008).
9. In human hearts with chronic end-stage heart failure exhibiting various degrees of hypertrophy, a significantly greater NHE-1 activity was detected in the human hypertrophied myocytes in comparison to myocytes from normal unused human donor hearts (Yokoyama et al., 2000).

We have also demonstrated that three different antihypertensive pharmacological interventions with different mechanisms of action (nifedipine, a Ca^{2+} channel blocker; enalapril, an inhibitor of angiotensin converting enzyme; and losartan, an AT_1 receptor blocker) caused the normalization of myocardial NHE activity, regression of cardiac hypertrophy (Fig. 13.28), and decrease of arterial pressure in SHR (Alvarez et al., 2002). However, for a similar reduction in systolic blood pressure and NHE-1 activity, losartan induced the largest regression of cardiac hypertrophy. Even though these results give support to the hypothesis that an increased myocardial tension is determining intracellular signals having common end points on the antiporter activity and cellular growth, they also suggest that the eventual recruitment of additional intracellular pathways may be playing a role in the hypertrophic response.

In line with the experiments reported by Kusumoto et al. (2001) showing that NHE-1 inhibition decreased hypertrophy and remodeling after myocardial infarction, we have recently reported that post-myocardial infarction hypertrophy and fibrosis were reduced after phosphodiesterase 5A inhibition by sildenafil, being the phosphodiesterase inhibition accompanied by protein kinase G activation and NHE-1 inhibition (Perez et al., 2007).

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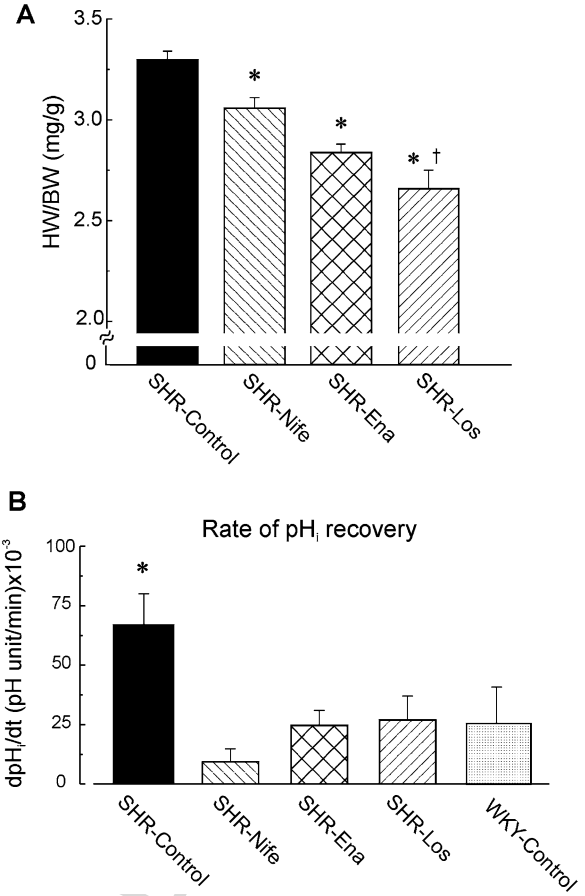

Fig. 13.28 **Panel A**: Effect of nifedipine, enalapril and losartan on cardiac hypertrophy (CH) in SHR. Heart weight to body weight (HW/BW) was used as an index of CH. (*) $P < 0.05$ compared to SHR-control; (†) $P < 0.05$ compared to enalapril-.and nifedipine-treated SHR (ANOVA). Data are means ± SE. **Panel B** shows the values of the rate of pH_i recovery from CO_2-induced intracellular acid load (dpH_i/dt) at a common pH_i value of 6.90 in SHR-Control ($n = 7$); WKY-Control ($n = 6$); SHR-Nife ($n = 5$); SHR-Ena ($n = 8$); and SHR-Los ($n = 5$). (*) $P < 0.05$ compared to all other groups (ANOVA). Data are means ± SE. Modified from Alvarez et al. (2002) with permission

As mentioned before, an enhanced activity of NHE-1 may be the result of an increased expression of the exchanger, an increased turnover of functional units, or a combination of both alternatives. In line with this, the reviewed models clearly exhibited cases of enhanced NHE-1 activity due to up-regulation, post-translational modification, or a combination of both. In either case, the hyperactivity of NHE-1 was linked to cardiac hypertrophy.

Interestingly, whereas chronic NHE-1 inhibition with cariporide in the whole animal induces up-regulation of the exchanger (Camilion de Hurtado et al., 2002a), the normalization of its previously augmented expression has been reported after chronic NHE-1 inhibition (Chen et al., 2001; Engelhardt et al., 2002; Ennis et al., 2003; Kilic et al., 2005). Nevertheless, several aspects deserve further investigation to clarify the precise mechanism by which NHE-1 is involved in the development of cardiac hypertrophy and the possible link with other mechanisms of the intracellular hypertrophic program.

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