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Mitochondrial ATP-Sensitive K^+ Channels Play a Role in Cardioprotection by Na^+-H^+ Exchange Inhibition Against Ischemia/Reperfusion Injury

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OBJECTIVES The possible role of the ATP-sensitive potassium (K_{ATP}) channel in cardioprotection by Na^+-H^+ exchange (NHE) inhibition was examined.

BACKGROUND The K_{ATP} channel is suggested to be involved not only in ischemic preconditioning but also in some pharmacological cardioprotection.

METHODS Infarction was induced by 30-min coronary occlusion in rabbit hearts in situ or by 30-min global ischemia in isolated hearts. Myocardial stunning was induced by five episodes of 5-min ischemia/5-min reperfusion in situ. In these models, the effects of NHE inhibitors (cariporide and ethylisopropyl-amiloride [EIPA]) and the changes caused by K_{ATP} channel blockers were assessed. In another series of experiments, the effects of EIPA on mitochondrial K_{ATP} (mito-K_{ATP}) and sarcolemmal K_{ATP} (sarc-K_{ATP}) channels were examined in isolated cardiomyocytes.

RESULTS Cariporide (0.6 mg/kg) reduced infarct size in situ by 40%, and this effect was abolished by glibenclamide (0.3 mg/kg), a nonselective K_{ATP} channel blocker. In vitro, 1 μM cariporide limited infarct size by 90%, and this effect was blocked by 5-hydroxydecanoate (5-HD), a mito-K_{ATP} channel blocker but not by HMR1098, a sarc-K_{ATP} channel blocker. Infarct size limitation by 1 μM EIPA was also prevented by 5-HD. Cariporide attenuated regional contractile dysfunction by stunning, and this protection was abolished by glibenclamide and 5-HD. Ethylisopropyl amiloride neither activated the mito-K_{ATP} channel nor enhanced activation of this channel by diazoxide, a K_{ATP} channel opener.

CONCLUSIONS Opening of the mito-K_{ATP} channel contributes to cardioprotection by NHE inhibition, though the interaction between NHE and this K_{ATP} channel remains unclear. (J Am Coll Cardiol 2001;37:957–63) © 2001 by the American College of Cardiology

The Na^+-H^+ exchanger type 1 (NHE-1) is an important acid excluder in cardiomyocytes, and its contribution to ischemic myocardial injury has been indicated by consistent observation that administration of NHE-1 inhibitors before ischemia reduces infarct size, myocardial stunning and arrhythmia (1–6). A current hypothesis proposes that inhibition of NHE-1 reduces intracellular Na^+ accumulation in ischemic myocytes, which consequently results in less Ca^{++} overload via the Na^+-Ca^{++} exchanger and, thus, less cellular injury. This hypothesis is supported by the results of studies using nuclear magnetic resonance spectroscopy (4–6), which showed ischemia-induced elevation in cytosolic Na^+ and Ca^{++} levels was significantly delayed by amiloride, ethylisopropyl amiloride (EIPA) and cariporide, a specific NHE-1 inhibitor. However, amiloride derivatives also inhibit the Na^+-Ca^{++} exchanger (7) and Na^+ channel (8). Cytosolic Ca^{++} overload during simulated ischemia was not prevented by hexamethylene amiloride (9) nor by cariporide (10) in isolated cardiomyocytes. Therefore, the suppression of cytosolic Ca^{++} overload may not be an exclusive mechanism of cardioprotection by NHE-1 inhibition.

Recent studies (11–16) have demonstrated that myocardial tolerance against ischemia/reperfusion injury is enhanced by activation of the K_{ATP} channel. This channel has received attention as a trigger as well as a mediator of anti-infarct tolerance in the mechanism of ischemic preconditioning (11–14). Furthermore, two independent studies suggest that K_{ATP} channels may be involved also in pharmacological cardioprotection (15,16). Therefore, in this study, we used inhibitors of the mitochondrial K_{ATP} (mito-K_{ATP}) and sarcolemmal K_{ATP} (sarc-K_{ATP}) channels to examine the role of the K_{ATP} channel and its relevant channel subtype in cardioprotection afforded by NHE inhibition. In addition, interaction of NHE inhibition and the mito-K_{ATP} activity was assessed in isolated cardiomyocytes.

METHODS

Experiment 1: effects of K_{ATP} channel blockers on infarct size-limiting effects of cariporide in vivo. **SURGICAL PREPARATION.** Male albino rabbits (Japanese white) weighing 2.0 to 2.5 kg were prepared to induce myocardial infarction

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Abbreviations and Acronyms

EIPA	= ethylisopropyl amiloride
$I_{K.ATP}$	= sarc-K_{ATP} channel current
LV	= left ventricle or left ventricular
LVDP	= left ventricular developed pressure
LV dP/dt	= the first derivative of left ventricular pressure
mito-K_{ATP}	= mitochondrial K_{ATP} channel
NHE	= Na^+-H^+ exchanger
sarc-K_{ATP}	= sarcolemmal K_{ATP} channel
TTC	= triphenyltetrazolium chloride
5-HD	= 5-hydroxydecanoate

in vivo as in our previous studies (2,17). In brief, a coronary snare was placed around the marginal branch of the left coronary artery after left thoracotomy under pentobarbital anesthesia and mechanical ventilation. Blood pressure monitoring and drug injections were performed by using catheters placed in the carotid artery and the jugular vein, respectively. Precordial electrocardiogram was monitored by bipolar electrodes on the chest.

EXPERIMENTAL PROTOCOLS. Rabbits were divided into four groups and underwent 30-min coronary occlusion and 3-h reperfusion. The cariporide group received 0.6 mg/kg of cariporide 10 min before the coronary occlusion, and the glibenclamide group was given 0.3 mg/kg of glibenclamide 20 min before ischemia. The glibenclamide/cariporide group received the same doses of glibenclamide and cariporide, which were injected at 20 and 10 min before ischemia, respectively. In the control group, only saline was injected. After 3 h of reperfusion, the heart was excised and processed for postmortem analysis of infarct size.

POSTMORTEM ANALYSIS OF THE HEART. As in previous studies (2,17), the excised heart was quickly mounted onto a Langendorff apparatus and perfused with saline. The coronary artery was reoccluded, and Evans Blue dye was injected into the perfusion line to negatively mark the territory of the marginal branch (i.e., area at risk). The heart was sectioned into 2-mm slices, stained by triphenyltetrazolium chloride (TTC), and infarct and risk areas were determined by computer-assisted planimetry.

Experiment 2: effects of K_{ATP} channel subtype selective blockers on infarct size-limiting effects of NHE inhibitors in vitro. SURGICAL PREPARATION. Using rabbits weighing 1.8 to 2.1 kg, isolated heart preparation was prepared as previously reported (13). Isolated rabbit hearts were perfused with oxygenated modified Krebs-Henseleit buffer (NaCl, 118.5 mM; KCl, 4.7 mM; $NaHCO_3$, 24.8 mM; KH_2PO_4, 1.2 mM; $MgSO_4$, 1.2 mM; $CaCl_2$, 2.5 mM; glucose, 10.0 mM). Perfusion pressure and temperature of the perfusate were 75 mm Hg and 37°C, respectively. A 3Fr catheter tip manometer (SPR-524, Millar Instruments Inc., Houston, Texas) was inserted into the left ventricle (LV), and coronary flow was measured by collection of coronary effluent in a graduated cylinder. The heart was excluded from the study if the LV developed pressure (LVDP) was <70 mm Hg or arrhythmias were observed after a 15-min stabilization period.

EXPERIMENTAL PROTOCOLS. Myocardial infarction was induced by 30-min global ischemia and 2-h reperfusion as in a previous study (13). Although this reperfusion period was shorter than in experiment 1, infarcts were clearly identified by TTC staining, presumably because higher coronary flow in vitro facilitated dehydrogenase washout from necrotic tissues. In protocol 1, the hearts received one of the following six pretreatments for 10 min before the 30-min ischemia: no drug (i.e., controls), 1 μM cariporide, 50 μM 5-hydroxydecanoate (5-HD), 1 μM cariporide plus 50 μM 5-HD, 20 μM HMR1098 and 1 μM cariporide plus 20 μM HMR1098. HMR1098 is Na^+ salt of HMR1883, a selective blocker of sarc-K_{ATP} channels (18), and 5-HD is a selective blocker of mito-K_{ATP} channels (19). In protocol 2, the hearts received one of the following four pretreatments: no drug (i.e., controls), 1 μM ethylisopropyl amiloride (EIPA), 100 μM 5-HD and a combination of both agents for 10 min of the preischemic period. The dose of 5-HD was increased to 100 μM in this protocol since the effect of 50 μM 5-HD on EIPA-induced cardioprotection was found to be variable in pilot experiments. At the end of the experiments, each heart was sliced, and infarct size was identified by TTC staining as in experiment 1. The uppermost slices, which contained valves and the perivalvular connective tissues, were excluded from subsequent analysis. The LV area (i.e., area at risk) and infarcts in the LV were measured by computer-assisted planimetry.

Experiment 3: effects of K_{ATP} channel blockers on antistunning effects of cariporide. SURGICAL PREPARATION. Rabbits were instrumented for measuring regional and global ventricular functions in vivo as previously reported (20). A catheter-tipped manometer was placed in the LV, and the first derivative of LV pressure (LV dP/dt) was obtained by electronic differentiation. A coronary snare was placed around the marginal branch of the left coronary artery, and an epicardial Doppler sensor was secured to the epicardium in the region of the marginal branch. This sensor was connected to a Pulsed Doppler Dimension System VF-1 (Crystal Biotec, Hopkinton, Minnesota) to determine regional wall thickening. The onset of systole was determined as the initial rise in LV dP/dt, and the end of systole was considered to be coincident with the peak negative LV dP/dt. The thickening fraction was calculated as the ratio of transmural net systolic thickening to end-diastolic wall thickness multiplied by 100 (21).

EXPERIMENTAL PROTOCOLS. Six groups of rabbits underwent five cycles of 5-min coronary occlusion separated by 5-min reperfusion and reperfusion for 90 min after the fifth coronary occlusion. Saline (vehicle) was injected in the control group, 0.6 mg/kg of cariporide in the cariporide group, 0.3 mg/kg of glibenclamide in the glibenclamide

Table 1. Hemodynamic and Infarct Size Data in Experiment 1

Group	n	Heart Rate (beats/min)		Mean BP (mm Hg)		Area at Risk (cm^3)	Infarct (cm^3)	%IS/AR
		Baseline	Ischemia	Baseline	Ischemia			
Control	8	275 ± 12	271 ± 10	88 ± 6	85 ± 3	0.81 ± 0.10	0.46 ± 0.09	52.6 ± 6.0
Cariporide	8	269 ± 17	263 ± 21	86 ± 3	81 ± 6	0.80 ± 0.05	0.17 ± 0.04*	21.4 ± 3.7*
Glib	5	270 ± 25	276 ± 30	91 ± 3	76 ± 7	0.81 ± 0.08	0.40 ± 0.07	48.0 ± 5.2
Glib/Cariporide	5	288 ± 21	293 ± 18	95 ± 7	88 ± 4	0.89 ± 0.07	0.57 ± 0.08	55.2 ± 4.5

*$p < 0.05$ vs. control. Mean ± SEM.
BP = mean blood pressure; Glib = glibenclamide; %IS/AR = infarct size as a percentage of area at risk.

group, glibenclamide and cariporide in the glibenclamide/cariporide group, 5 mg/kg of 5-HD in the 5-HD group and 5-HD and cariporide in the 5-HD/cariporide group. Cariporide was injected intravenously 10 min before the first coronary occlusion, and glibenclamide and 5-HD were administered 15 min before the ischemia. Although we did not completely randomize all of the rabbits in this protocol, we randomly prepared cariporide-treated and nontreated rabbits for each K_{ATP} channel inhibitor (i.e., no inhibitor, glibenclamide or 5-HD). At the end of the experiments, the area at risk was determined as in experiment 1, and the absence of infarction was confirmed by TTC staining.

Experiment 4: effects of a NHE inhibitor on mito-K_{ATP} and sarc-K_{ATP} channels in cardiomyocytes. The activities of mito-K_{ATP} and sarc-K_{ATP} channels were monitored in isolated rabbit cardiomyocytes as previously reported (12,19). Ventricular myocytes isolated from the rabbit hearts were superfused with an external solution containing 140 mM NaCl, 5 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$ and 10 mM HEPES (pH 7.4 with NaOH). For whole-cell patch recordings, the internal pipette solution included 120 mM potassium glutamate, 25 mM KCl, 0.5 mM $MgCl_2$, 10 mM K-EGTA, 10 mM HEPES and 1 mM MgATP (pH 7.2 with KOH). Whole cell currents were elicited every 6 s from a holding potential of −80 mV by two consecutive steps to −40 mV (for 100 ms) and 0 mV (for 380 ms). Currents at 0 mV were measured 200 ms into the pulse. Endogenous flavoprotein fluorescence was excited with a xenon arc lamp with a band-pass filter centered at 480 nm. Emitted fluorescence was recorded at 530 nm by a photomultiplier tube and digitized (Digidata 1200, Axon Instruments, Foster City, California). Relative fluorescence was averaged during the excitation window and calibrated with the values after 2,4-dinitrophenol (taken as 100% oxidation) and sodium cyanide exposure (taken as 100% reduction). The effects of 100 μM diazoxide on flavoprotein oxidation and sarc-K_{ATP} channel current ($I_{K.ATP}$) were assessed. To investigate whether NHE inhibition modulates the mito-K_{ATP} channel activation by diazoxide, EIPA (1 μM) was added in addition to diazoxide, and flavoprotein fluorescence and $I_{K.ATP}$ were determined.

Chemicals. Glibenclamide and diazoxide were purchased from SIGMA (St. Louis, Missouri), and 5-HD and EIPA were obtained from Research Biochemical Institute (Natick, Massachusetts). Cariporide and HMR1098 were kindly provided by Aventis, Deutschland Gmb, Germany.

Statistical analysis. One-way analysis of variance and two-way repeated measures analysis of variance were used to test intergroup differences in infarct size and those in temporal changes of hemodynamic parameters and contractile functions, respectively. Student-Newman-Keul post-hoc test was used for multiple comparison. The difference was considered significant at $p < 0.05$. SigmaStat (SPSS Inc., Chicago, Illinois) was used to perform the statistical analysis.

This study was conducted in strict accordance with the guidelines for research animal use of Sapporo Medical University and Otsuka America Pharmaceutical, Inc.

RESULTS

Experiment 1. MORTALITY OF RABBITS. A total of 28 rabbits were entered into protocol 1, and two of them (one in the glibenclamide group and one in the glibenclamide/cariporide group) died from irreversible ventricular fibrillation during the coronary occlusion. Thus, 26 rabbits contributed to the following analysis.

HEMODYNAMIC PARAMETERS. Heart rate and mean blood pressure were comparable among all study groups under baseline conditions and during the coronary artery occlusion (Table 1). Blood pressure slightly decreased after reperfusion (data not shown), but there was no significant difference among the groups (p for time-dependent change < 0.001, p for group comparison = 0.878, p for interaction = 0.203).

INFARCT SIZE DATA (TABLE 1). The sizes of risk areas in the study groups were similar. Infarct size expressed as a percentage of area at risk was reduced by cariporide as in our previous study (2). Although glibenclamide alone did not modify infarct size, glibenclamide blocked cariporide-induced protection.

Experiment 2. HEMODYNAMIC DATA (TABLE 2). There were no significant intergroup differences in heart rate, LVDP and coronary flow under baseline conditions in both protocols 1 and 2. In protocol 1, cariporide and 5-HD did not change heart rate, LVDP or coronary flow. HMR1098 reduced coronary flow by 40%, presumably due to blockade of K_{ATP} channels in the coronary vessels (p for interaction in coronary flow comparison was <0.001). Heart rate and coronary flow were reduced after ischemia/reperfusion in all study groups without intergroup differences (data not shown). Left ventricular developed pressure also decreased

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Table 2. Hemodynamic and Infarct Size Data in Experiment 2

Treatment	n	Heart Rate (beats/min)		LVDP (mm Hg)		CF (ml/min)		Area at Risk (cm^3)	Infarct (cm^3)	%IS/AR
		Baseline	After Tx	Baseline	After Tx	Baseline	After Tx			
Protocol 1										
Control	8	240 ± 6	240 ± 6	81 ± 3	81 ± 3	63 ± 4	62 ± 3	2.49 ± 0.05	1.18 ± 0.14	49.2 ± 5.0
Cariporide	6	228 ± 13	219 ± 12	83 ± 5	81 ± 5	67 ± 6	69 ± 5	2.10 ± 0.09	0.12 ± 0.05†	5.5 ± 2.2†
5-HD	8	222 ± 7	216 ± 8	85 ± 3	81 ± 4	71 ± 5	73 ± 5	2.33 ± 0.18	1.47 ± 0.18	61.9 ± 4.1
5-HD/cariporide	5	233 ± 5	222 ± 5	81 ± 2	74 ± 1	70 ± 2	78 ± 4	2.37 ± 0.12	1.59 ± 0.20	66.3 ± 5.8
HMR1098	7	228 ± 6	221 ± 6	78 ± 3	72 ± 2	68 ± 6	41 ± 3*	2.47 ± 0.09	1.38 ± 0.19	55.9 ± 5.8
HMR1098/cariporide	5	224 ± 9	217 ± 7	82 ± 3	75 ± 2	65 ± 3	46 ± 2*	2.44 ± 0.17	0.30 ± 0.10†	11.7 ± 3.9†
Protocol 2										
Control	5	239 ± 6	238 ± 6	81 ± 5	80 ± 3	51 ± 6	52 ± 5	2.46 ± 0.15	1.29 ± 0.17	51.8 ± 4.2
EIPA	4	224 ± 14	220 ± 10	75 ± 2	75 ± 2	63 ± 6	60 ± 8	2.30 ± 0.18	0.14 ± 11†	5.6 ± 4.0†
5-HD	4	239 ± 10	225 ± 14	80 ± 2	80 ± 2	56 ± 12	58 ± 16	2.58 ± 0.13	1.63 ± 0.23	62.6 ± 7.0
5-HD/EIPA	4	219 ± 8	201 ± 5	79 ± 1	79 ± 1	55 ± 3	62 ± 2	2.33 ± 0.16	1.17 ± 0.16	49.9 ± 4.6

*$p < 0.05$ vs. baseline; †$p < 0.05$ vs. control. Mean ± SEM.
CF = coronary flow; EIPA = ethylisopropyl amiloride; LVDP = left ventricular developed pressure; Tx = treatment; 5-HD = 5-hydroxydecanoate; %IS/AR = infarct size as a percentage of area at risk.

after reperfusion, but it was significantly higher in the cariporide-treated and EIPA-treated hearts than it was in the controls (p for interaction <0.001 in both protocol 1 and protocol 2).

INFARCT SIZE DATA (TABLE 2). The sizes of the area at risk (i.e., LV mass) were comparable in study groups in both protocols. The infarct size-limiting effect of cariporide was abolished by 5-HD but not by HMR1098 in protocol 1. Neither 5-HD nor HMR1098 alone significantly changed infarct size. Similar to cariporide, EIPA reduced infarct size, and this protection was also prevented by 5-HD in protocol 2.

Experiment 3. HEMODYNAMIC PARAMETERS (TABLE 3). Under baseline conditions, hemodynamic parameters were comparable among the study groups. Also, the time-courses of the heart rate, arterial pressure, LV pressure and LV dP/dt_{max} during and after ischemia/reperfusion were similar in the study groups. The data during the first to the fourth coronary occlusions are not shown in Table 3 for simplicity.

REGIONAL CONTRACTILE FUNCTION. The regional systolic thickening fractions were similar under baseline conditions in all of the study groups: 22.2 ± 1.4 (SEM) % in the control group, 22.1 ± 1.6% in the cariporide group, 19.6 ± 1.9% in the glibenclamide group, 17.3 ± 1.5% in the glibenclamide/cariporide group, 21.6 ± 1.7% in the 5-HD group and 24.3 ± 2.9% in the 5-HD/cariporide group. The time-courses of thickening fractions normalized as a percentage of baseline level are plotted in Figure 1. Cariporide significantly improved the thickening fraction after reperfusion (Fig. 1A). However, this improvement of regional contractile function by cariporide was not detected in the groups that received glibenclamide or 5-HD (Fig. 1, B and C). The time courses of thickening fractions during ischemia and reperfusion were similar in the control, the glibenclamide and the 5-HD groups. There were no significant differences in the sizes of areas at risk among the six study groups (data not shown).

Experiment 4. As shown in Figure 2, diazoxide increased mitochondrial flavoprotein oxidation to 34.8 ± 2.5%, indicating opening of the mito-K_{ATP} channel. However, EIPA alone did not induce significant flavoprotein oxidation, and it did not enhance the effect of diazoxide. Neither diazoxide nor EIPA activated $I_{K.ATP}$. On the other hand, a 5- to 10-min treatment of 2,4-dinitrophenol activated a sizable $I_{K.ATP}$.

DISCUSSION

Contribution of the K_{ATP} channel to cardioprotection by NHE inhibition. The salient finding in this study was that selective and nonselective blockers of the mito-K_{ATP} channel (i.e., 5-HD and glibenclamide) prevented protective effects of NHE-1 inhibitors (i.e., cariporide and EIPA) on infarct size and myocardial stunning. In contrast, a selective blocker of the sarc-K_{ATP} channel, HMR1098, failed to abolish the cardioprotection afforded by cariporide. The dose of HMR1098 in this study (i.e., 20 μM) was >20-fold higher than the reported IC_{50} to block sarc-K_{ATP} channels in cardiomyocytes (18) and also four-fold higher than the dose that successfully prevented shortening of action potential duration by pinacidil, a K_{ATP} channel opener, in our buffer-perfused rabbit heart preparation (22). Taken together, these findings suggest that activity of the mito-K_{ATP} channel, not that of the sarc-K_{ATP} channel, contributes to the myocardial tolerance, which was afforded by NHE-1 inhibition, against ischemia/reperfusion injury.

In contrast with these results, glibenclamide did not abolish infarct size limitation by NHE inhibitors (EIPA and EMD 85131) in earlier studies using regional ischemia in the rabbit heart in vitro (23) and in the canine heart in situ (24). Although we do not have a clear explanation for this discrepancy, it may be due to the differences in severity of ischemia, reflected by smaller infarcts in untreated con-

Table 3. Hemodynamic Parameters in Experiment 3

Group	n	Baseline	Pre-Occ	5th-Occ	5th-Rep 90 min
Heart rate (beats/min)					
Control	8	259 ± 10	263 ± 8	256 ± 14	264 ± 12
Cariporide	8	276 ± 11	278 ± 12	271 ± 10	265 ± 9
Glib	6	251 ± 6	253 ± 8	257 ± 11	266 ± 14
Glib/cariporide	6	276 ± 9	271 ± 6	267 ± 8	275 ± 6
5-HD	6	261 ± 14	261 ± 15	265 ± 14	254 ± 10
5-HD/cariporide	6	245 ± 6	244 ± 6	241 ± 6	236 ± 6
Systolic blood pressure (mm Hg)					
Control	8	99 ± 4	99 ± 4	89 ± 5	94 ± 5
Cariporide	8	99 ± 2	99 ± 4	85 ± 4*	90 ± 3
Glib	6	98 ± 4	101 ± 4	85 ± 5*	91 ± 2
Glib/cariporide	6	105 ± 7	109 ± 7	97 ± 8	94 ± 5
5-HD	6	97 ± 4	97 ± 4	83 ± 5*	94 ± 4
5-HD/cariporide	6	98 ± 6	97 ± 6	93 ± 6	91 ± 5
Diastolic blood pressure (mm Hg)					
Control	8	77 ± 4	76 ± 4	69 ± 4	71 ± 4
Cariporide	8	79 ± 2	76 ± 4	64 ± 3*	63 ± 3*
Glib	6	74 ± 3	73 ± 2	64 ± 4	67 ± 3
Glib/cariporide	6	79 ± 6	81 ± 6	72 ± 8	66 ± 3*
5-HD	6	79 ± 4	78 ± 3	67 ± 5*	74 ± 4
5-HD/cariporide	6	75 ± 4	76 ± 3	74 ± 4	68 ± 5
LVEDP (mm Hg)					
Control	8	4 ± 1	4 ± 1	11 ± 2*	5 ± 1
Cariporide	8	5 ± 1	6 ± 1	12 ± 1*	6 ± 1
Glib	6	3 ± 1	3 ± 1	12 ± 3*	4 ± 1
Glib/cariporide	6	4 ± 1	5 ± 1	11 ± 1*	5 ± 1
5-HD	6	4 ± 1	4 ± 1	12 ± 2*	4 ± 1
5-HD/cariporide	6	5 ± 1	5 ± 1	11 ± 1*	4 ± 1
LV dP/dt max (mm Hg/s)					
Control	8	4,120 ± 328	4,120 ± 321	2,914 ± 395*	2,995 ± 293*
Cariporide	8	4,488 ± 178	4,500 ± 271	3,163 ± 297*	3,338 ± 224*
Glib	6	4,200 ± 271	4,267 ± 293	2,933 ± 399*	3,083 ± 176*
Glib/cariporide	6	4,517 ± 342	4,667 ± 319	3,667 ± 337*	3,350 ± 301*
5-HD	6	3,900 ± 170	3,900 ± 184	2,940 ± 325*	3,280 ± 227*
5-HD/cariporide	6	4,020 ± 171	4,080 ± 132	3,420 ± 146*	3,320 ± 73*

*$p < 0.05$ vs. baseline value. Mean ± SEM.

Glib = glibenclamide; LV dP/dt max = maximum of first derivative of left ventricular pressure; LVEDP = left ventricular end-disatolic pressure; Pre-Occ = immediately before the first coronary occlusion; 5-HD = 5-hydroxydecanoate; 5th-Occ = 5 min after the fifth coronary occlusion; 5th-Rep 90 min = 90 min after the fifth coronary occlusion.

trols in earlier reports. Thus, the contribution of the mito-K_{ATP} channel to the cardioprotection by NHE inhibition might depend on the experimental conditions and preparations.

How does NHE inhibition modify the mito-K_{ATP} channel activity? It is not clear what role the opening of the mito-K_{ATP} channel plays in the mechanism of cardioprotection by NHE-1 inhibition. However, there are at least two possibilities. First, NHE-1 inhibition may activate the mito-K_{ATP} channel. This possibility was not supported by our findings that EIPA neither directly activated the mito-K_{ATP} channel nor enhanced the activation of this channel by diazoxide in our experimental conditions (Fig. 2). However, the possibility that NHE-1 inhibition indirectly modulated the mito-K_{ATP} channel activity during ischemia cannot be ruled out. Actually, it is a possibility that NHE-1 inhibition may modify the levels of the mito-K_{ATP} channel regulatory factors such as long-chain acyl-CoA during ischemia (25,26). Unfortunately, we cannot assess the interaction of NHE-1 inhibition with the mito-K_{ATP} channel in hypoxic or ischemic cardiomyocytes because flavoprotein fluorescence can only be used to monitor mito-K_{ATP} channel activity under a good oxygenation condition.

Another possibility is that the opening of the mito-K_{ATP} channel may help NHE-1 inhibition to suppress mitochondrial Ca^{++} overload. Ca^{++} overload in mitochondria has been suggested to be one of the mechanisms of myocyte injury, including irreversible injury (27–30). Overloading mitochondria with Ca^{++} impairs ATP generation (31) and may enhance the release of cytotoxic free radicals from the mitochondria (32,33), which play a major role in pathogenesis of myocardial stunning (34). Major determinants of the extent of mitochondrial Ca^{++} overload are the level of cytosolic Ca^{++} and membrane potential across the mitochondrial inner membrane, which is a driving force for Ca^{++} influx into the mitochondrial matrix (28,29,35–37).

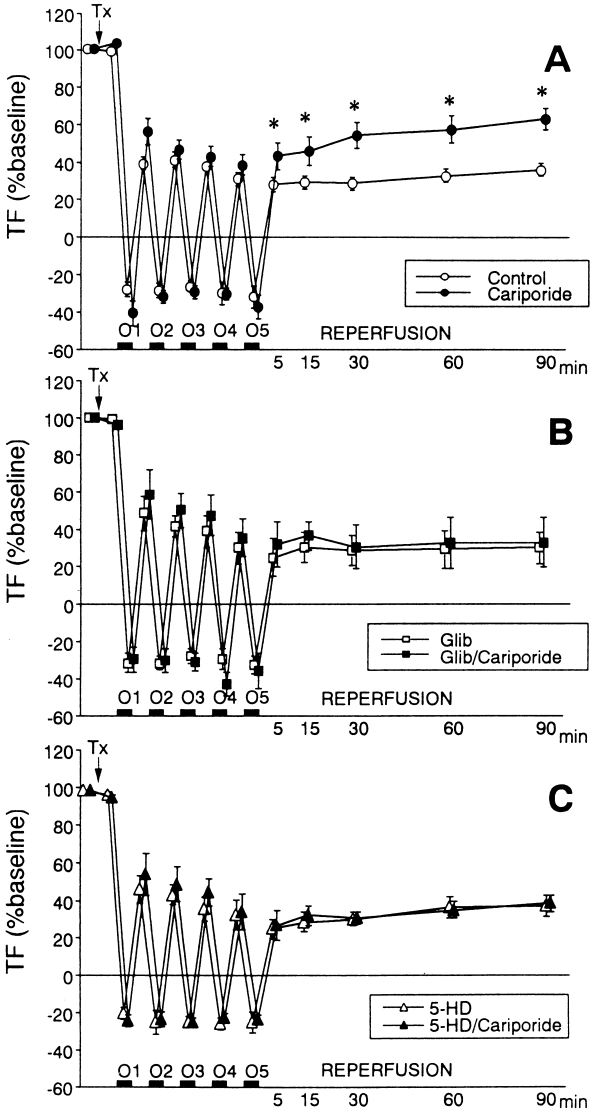

Figure 1. Effects of cariporide on the time-course of the regional thickening fraction during repetitive ischemia and reperfusion. The thickening fraction after reperfusion was higher in the cariporide group than it was in the control group (**Panel A**) ($p = 0.010$ for group comparison and $p < 0.001$ for interaction). *$p < 0.05$ versus data at the corresponding time points. There was no significant difference in the time-courses of thickening fraction between the glib and glib/cariporide groups **(Panel B)** or between the 5-HD and 5-HD/cariporide groups **(Panel C)**. Glib = glibenclamide; O1, O2, O3, O4 and O5 = first, second, third, fourth and fifth coronary occlusions, respectively; TF (% baseline) = the thickening fraction expressed as a percentage of baseline values; 5-HD = 5-hydroxydecanoate; Tx = treatment.

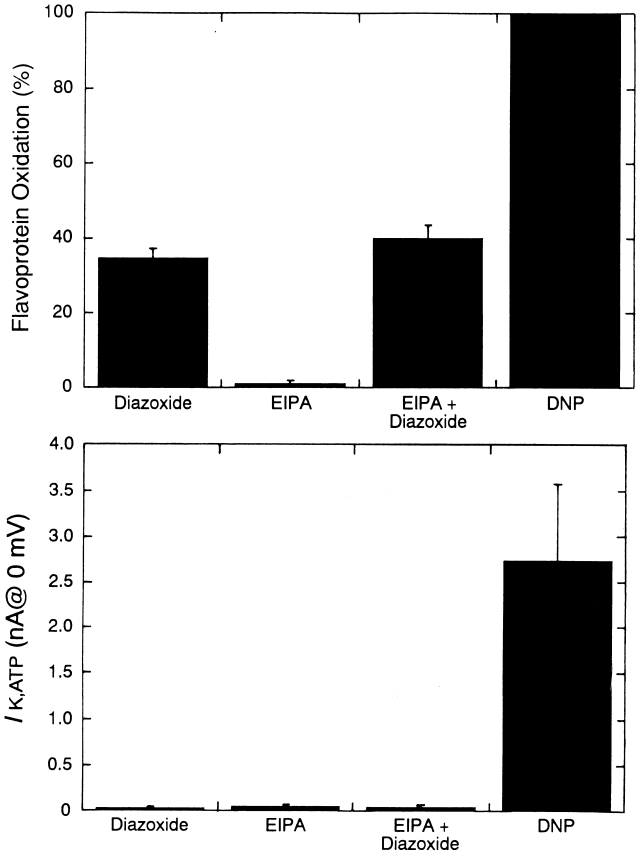

Figure 2. Effects of diazoxide and EIPA on mitochondrial flavoprotein fluorescence and $I_{K.ATP}$. **Upper panel:** although diazoxide (100 μM) induced oxidation of mitochondrial flavoprotein, EIPA (1 μM) did not provoke significant mitochondrial oxidation. Ethylisopropyl amiloride did not enhance mitochondrial oxidation by diazoxide. The flavoprotein fluorescence was calibrated by exposing the cells to 2,4-dinitrophenol (100 μM). **Lower panel:** data for $I_{K.ATP}$ measured at 0 mV. Neither diazoxide nor EIPA increased $I_{K.ATP}$. EIPA = ethylisopropyl amiloride; $I_{K.ATP}$ = sarc-K_{ATP} channel current.

Opening of the mito-K_{ATP} channel would reduce the mitochondrial membrane potential, thus suppressing Ca^{++} influx into the mitochondria. This possibility was supported by results of recent studies by Holmuhamedov et al. (36,37).

Thus, in myocytes with blocked mito-K_{ATP} channels, lowering the cytosolic Ca^{++} level in ischemic myocytes by NHE-1 inhibitors (and other agents) may not sufficiently prevent mitochondrial Ca^{++} overload and cell injury when ischemia is severe or of long duration. Consistent with this speculation are recently reported findings (15,16) that mito-K_{ATP} channel blockers abolish the infarct size–limiting effect of mibefradil, a T-type Ca^{++} channel blocker, which is unlikely to directly interact with the mito-K_{ATP} channel.

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