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ORIGINAL ARTICLE

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Comparison of the protective effects of ischemic preconditioning and the Na^+/H^+ exchanger blockade

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Abstract The protective effects of ischemic preconditioning (IP) and Na^+/H^+ exchanger blockade (NHE_b) by two blockers [ethylisopropylamiloride (EIPA) and HOE 642] were compared in the isovolumic perfused rat heart. The impairment in systolic and diastolic function detected in control ischemic hearts (C) exposed to 20 min of ischemia and 30 min of reperfusion was diminished in similar extent by IP and by NHE_b with EIPA and HOE 642. At the end of the reperfusion period $+dP/dt_{max}$ values were 57±9% in C hearts and 94±6%, 82±6% and 104±6% after IP and NHE_b with EIPA and HOE 642, respectively. A depletion of ATP levels detected in C hearts after reperfusion (from 20.2±0.8 μmol/g dry weight before ischemia to 6.9±0.7 μmol/g dry weight) was partially prevented by both IP and NHE_b with EIPA (9.2±0.7 μmol/g dry weight and 11.1±0.5 μmol/g dry weight, respectively). The ischemic contracture (IC), assessed by the left ventricular end diastolic pressure (LVEDP), observed in C hearts (35±4 mmHg) was not decreased by IP (40±4 mmHg) but it was prevented by NHE_b (18±4 mmHg and 10±3 mmHg with EIPA and HOE 642, respectively). The ATP levels at the end of the ischemic period were similar in C and IP hearts (4.1±0.2 μmol/g dry wt vs. 3.3±0.4 μmol/g dry wt) but they were significantly higher after NHE_b with HOE 642 (7.0±1.0 μmol/g dry wt). PKC inhibition by chelerythrine abolished the protection induced by IP after reperfusion although not the improvement induced by NHE_b with EIPA.
According to the present results, we can conclude that despite the fact that IP and NHE_b are protecting the postischemic function in a similar magnitude, both interventions are different in terms of modifying IC that develops during the ischemic period. IC was prevented by NHE_b whereas it was not by IP. Furthermore, IP protection and not that obtained by NHE_b is abolished by PKC.

Key words Ischemia · Reperfusion · Contracture · Contractility · Ischemic preconditioning · Na^+/H^+ exchanger · HOE 642 · Protein kinase C · Chelerythrine

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Introduction

When blood flow is restarted after a short ischemic episode, isovolumic rat hearts show a depression of contractility and a decreased diastolic compliance (Braunwald and Kloner 1982; Bolli 1990). This altered ventricular function is the result of changes occurring during both ischemic and reperfusion periods. In some species, like in rat, the contracture (decreased diastolic compliance) develops during the ischemic period and is maintained during reperfusion (Steenbergen et al. 1990; Armstrong and Ganote 1991). Although many reports have correlated the degree of ischemic contracture (IC) with the impairment of postischemic function (Hearse et al. 1977; Ganote 1983; García-Dorado et al. 1992), this relationship remains controversial. An example of the dissociation is observed in the protection induced by one or more brief cycles of ischemia and reperfusion previously applied to a more prolonged ischemia, called ischemic preconditioning (IP). This phenomenon protects the diastolic and systolic function after reperfusion, whereas the contracture during the ischemic period is not modified or even increased (Cave 1995; Kolocassides et al. 1995, 1996).

One way of protection of the myocardium from ischemia/reperfusion seems to be the Na^+/H^+ exchanger blockade (NHE_b; Meng and Pierce 1990; Scholz et al. 1992, 1993, 1995; Bugge et al. 1996). The possibility that NHE can be involved in the mechanism of protection of the IP is controversial (Bugge and Ytrehus 1995; Ramasamy et al. 1995; Shipolini et al. 1997). The NHE can be activated by a protein kinase C (PKC; Fliegel and Fröhlich 1993) and PKC activation also seems to be the necessary

trigger for the protection brought about by IP (Liu et al. 1994; Speechly-Dick et al. 1994; Hu and Nattel 1995).

The objective of the present study was to compare the protection induced by IP with that obtained by blocking NHE.

Materials and methods

Isolated heart preparation. Rats were anesthetized with an intraperitoneal injection of sodium pentobarbital (60 mg/kg body wt). The heart was rapidly excised and perfused by the non-recirculating Langerdorff technique with Ringer's solution containing (in mM): 118 NaCl, 5.9 KCl, 1.2 $MgSO_4$, 1.35 $CaCl_2$, 20 $NaCO_3H$ and 11.1 dextrose. The buffer was saturated with a mixture of 95% O_2/5% CO_2, had a pH of 7.4, and was maintained at 37°C. The conductive tissue in the atrial septum was damaged with a fine needle to achieve atrioventricular block, and the right ventricle was paced at 280±10 beats/min. A latex balloon tied to the end of a polyethylene tube was passed into the left ventricle through the mitral valve; the opposite end of the tube was then connected to a Statham P23XL pressure transducer. The balloon was filled with water to give an end-diastolic pressure (LVEDP) of 8–12 mmHg, and this volume was unchanged for the remainder of the experiment. Coronary perfusion pressure was monitored at the point of cannulation of the aorta and adjusted to approximately 60–70 mmHg. Coronary flow, controlled with a peristaltic pump, was 11±2 ml/min. Left ventricular pressure (P) and its first derivative (dP/dt) were recorded with a direct writing recorder.

Experimental protocols. After 10 min of stabilization, the following experimental protocols were performed (Fig. 1). Control ischemic hearts (C): Hearts were submitted to 20 min of normothermic global ischemia followed by 30 min of reperfusion. Global ischemia was induced by stopping the perfusate inflow line and the heart was placed in a saline bath held at 37°C. Preconditioned hearts (IP): IP was induced by only one cycle of 5 min of ischemia and 10 min of reperfusion followed by the same protocol as in the C group.

For examining the NHE_b effects, alternatively EIPA or HOE 642 was used. Twelve C hearts received 1 µmol/l HOE 642 (gift from Hoechst, Frankfurt/Main, Germany; n=6) 10 min before the 20-min ischemic period or 1 µmol/l ethylisopropylamiloride (EIPA; bought from Research Biochemicals International; n=6). In other C (n=6) and IP (n=7) hearts 25 µg/min chelerythrine (Ch), a PKC inhibitor, was added to perfusion solution through an infusion pump during 10 min.

In five hearts we examined the effects of the combined administration of 1 µmol/l EIPA and 25 µg/min Ch before the long ischemic period.

Four hearts from each of the C, IP and NHE_b (with HOE 642) groups were freeze-clamped with liquid nitrogen-cooled aluminium clamps at the end of the ischemic period and six hearts from each of the same groups (EIPA was used as NHE blocker) were frozen at the end of the reperfusion period while they were being perfused. Another six hearts were frozen after 10 min of stabilization (Pre-I). All the hearts were stored in an ultra-low-temperature freezer (–70°C) until ATP extraction. The hearts were crushed with nitrogen-cooled mortar and pestle, and neutralized perchloric acid extracts were assayed for adenosine triphosphate (ATP) levels by standard enzymatic procedure (Lamprecht et al. 1974).

Systolic function. Myocardial contractility was assessed by the maximal velocity of rise of left ventricular pressure ($+dP/dt_{max}$) values. Data were expressed as percentage of their respective preischemic values.

Ischemic contracture. The contracture during ischemia (IC) was assessed by LVEDP. The time to onset of ischemic contracture (t_0) was defined as the time required to reach an LVEDP value 5 mmHg greater than its preischemic value.

Statistical analysis. Data are given as means ± SEM. The analysis of $+dP/dt_{max}$, LVEDP and ATP levels was performed using repeated measures of one-way analysis of variance (ANOVA) with the Newman-Keul's test for multiple comparisons among groups. Student's t-test was used to analyze the difference of t_0 between C and IP hearts. Values of P<0.05 were considered to be significant.

Results

Effects of 20 min of ischemia and IP

The recovery of systolic function after reperfusion, assessed by $+dP/dt_{max}$, was significantly improved by IP. After 30 min of reperfusion, $+dP/dt_{max}$ values were 57±9%

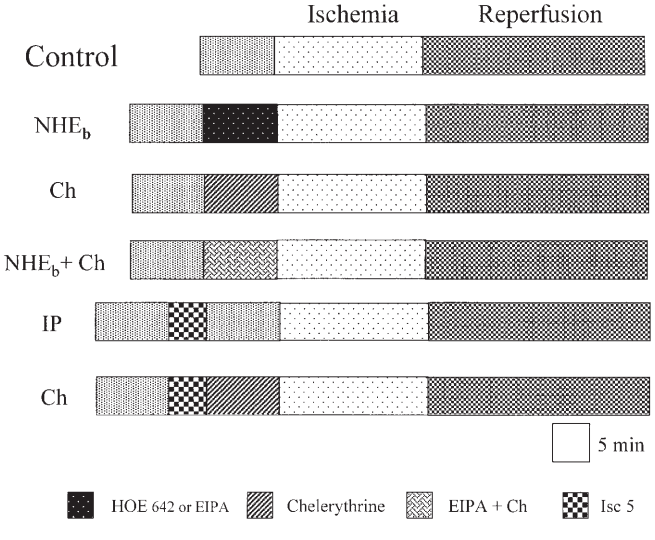

Fig. 1 Experimental protocols used for the different groups

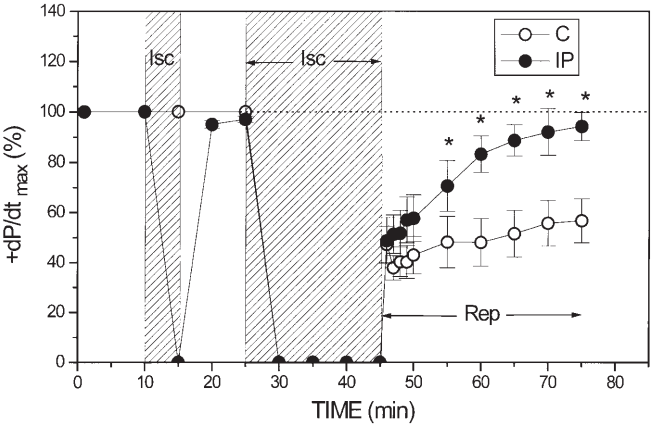

Fig. 2 Changes of $+dP/dt_{max}$ during reperfusion after 20 min of global ischemia in control ischemic (*C*) and preconditioned (*IP*) hearts. IP significantly improved the postischemic recovery obtained in C hearts. *P<0.05

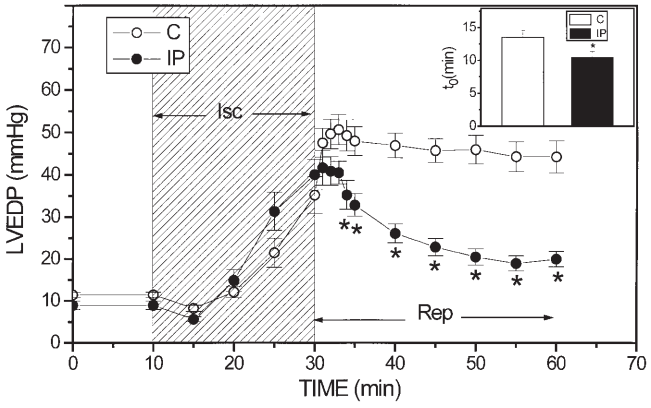

Fig. 3 Effects of 20 min of ischemia and 30 min of reperfusion on left ventricular end diastolic pressure (*LVEDP*) in control ischemic (*C*) and preconditioned (*IP*) hearts. It can be observed that IP significantly attenuated the increment of LVEDP during reperfusion but not the ischemic contracture. *Inset:* The time to onset of ischemic contracture (t_0) was significantly shorter in IP than in C hearts. *P<0.05

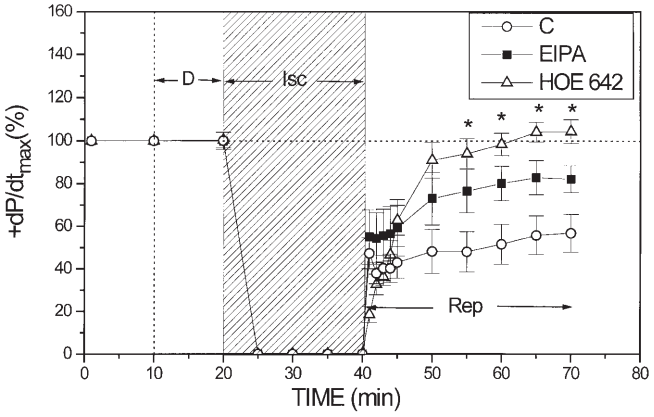

Fig. 4 Effects of NHE_b (with EIPA and HOE 642) on +dP/dt_{max} during reperfusion. Both NHE blockers significantly improved the postischemic recovery. *P<0.05 vs. C

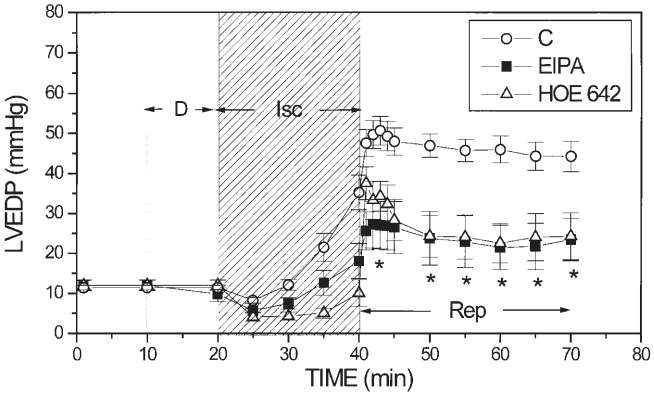

Fig. 5 Effects of NHE_b on left ventricular end diastolic pressure (*LVEDP*) during ischemia and reperfusion. Both NHE blockers [EIPA and HOE 642 (*D*)] significantly diminished the ischemic contracture and the increase of LVEDP during reperfusion. *P<0.05 vs. C

and 94±6% of preischemic levels in C and IP, respectively (Fig. 2).

Since left ventricular balloon volume was held constant during the experiments, an increase in LVEDP reflected an increase in diastolic chamber stiffness or "contracture". Figure 3 shows absolute values of LVEDP during preischemic, ischemic and reperfusion periods in C and IP hearts. Immediately after the interruption of coronary flow LVEDP significantly decreased with respect to preischemic values. This initial decrease in LVEDP could be attributed to the vascular collapse (the so-called garden hose or erectile effect; Vogel et al. 1982). A similar LVEDP increment during ischemia in both C and IP hearts was detected. LVEDP significantly increased during the ischemic period from 11±1 mmHg to 35±4 mmHg (ΔLVEDP=24±4 mmHg) in C hearts and from 9±1 mmHg to 40±4 mmHg (ΔLVEDP=31±3 mmHg) in IP hearts. Although the magnitude of IC was not significantly different between C and IP, the contracture took place faster in IP hearts. This was reflected by the t_0 with values significantly lower in IP (10.5±0.8 min) than in C hearts (13.5±1.5 min; P<0.05). On the other hand, IP significantly attenuated the increase of LVEDP after reperfusion. At the end of this period LVEDP values were 20±2 mmHg and 44±4 mmHg in IP and C hearts, respectively. These results, showing the protection by IP of both systolic and diastolic function after reperfusion, but not a decrease of IC, are in agreement with previously reported results (Cave 1995; Kolocassides et al. 1995).

Effects of NHE blockade

The action of NHE_b on systolic function in C hearts during reperfusion is shown in Fig. 4. At the end of the reperfusion period +dP/dt_{max} values were 82±6% and 104±6% with EIPA and HOE 642, respectively.

The effects of EIPA (1 μmol/l) and HOE 642 (1 μmol/l) on the IC that develops during ischemia are shown in Fig. 5. None of the NHE blockers induced any significant changes in baseline LVEDP values. After the sudden reduction in LVEDP, caused by the interruption of coronary flow, the IC developed.

The magnitude of IC examined during 20 min of ischemia was significantly attenuated by EIPA being the value of LVEDP 18±4 mmHg at the end of the ischemic period. HOE 642 abolished the IC (LVEDP value was 10±3 mmHg after 20 min of ischemia). At the end of reperfusion LVEDP was 23±5 mmHg and 24±6 mmHg after treatment with EIPA and HOE 642, respectively. These values were significantly lower than those obtained in C hearts and they were not significantly different from each other.

The results show that in spite of a similar protection by IP and NHE_b on systolic and diastolic function after reperfusion, their effects on IC are different. Whereas IP does not reduce the level of IC, NHE_b by both blockers does.

At the end of the reperfusion period the ATP levels diminished to 6.9±0.7, 9.2±0.7 and 11.1±0.5 μmol/g dry wt

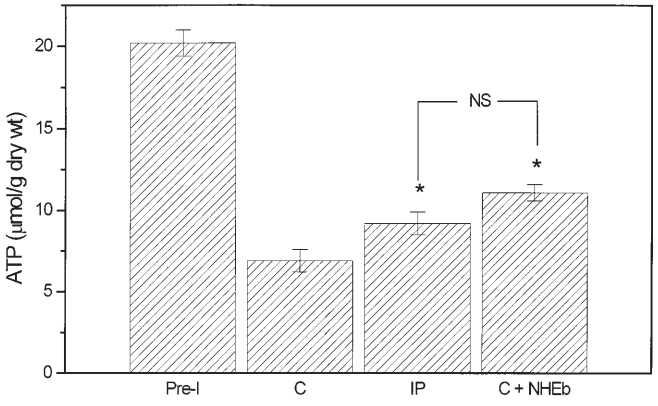

Fig. 6 ATP content measured after stabilization period (*Pre-I*) and after reperfusion in control ischemic (*C*), preconditioned (*IP*) and NHE blockade (NHE_b) hearts with EIPA. ATP values are expressed as µmol/g dry wt. **P*<0.05 vs. C

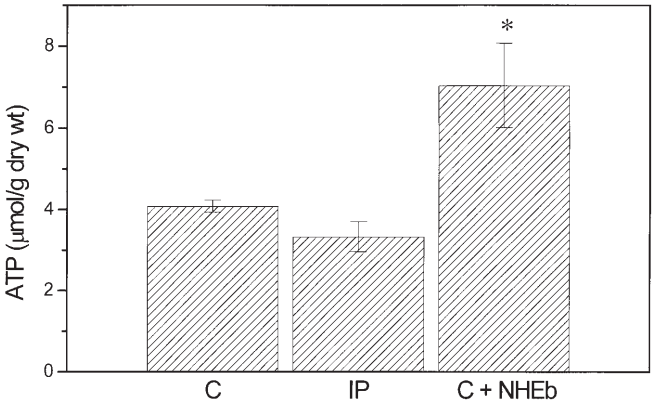

Fig. 7 ATP content measured at the end of ischemic period in control ischemic (*C*), preconditioned (*IP*) and NHE blockade (NHE_b) hearts with HOE 642. ATP values are expressed as µmol/g dry wt. **P*<0.05 vs. C

in C, IP and NHE_b (with EIPA) hearts, respectively, from a preischemic value of 20.2±0.8 µmol/g dry wt. The protection by both interventions, IP and NHE_b, detected after reperfusion was accompanied by preservation of ATP levels to a similar extent (Fig. 6).

The ATP levels decreased similarly at the end of the ischemic period in C and IP hearts, being the values of 4.1±0.2 µmol/g dry wt and 3.3±0.4 µmol/g dry wt, respectively. In the NHE_b group (with HOE 642) the ATP level was significantly higher than in each of the others (7.0±1.0 µmol/g dry wt; Fig. 7).

Effects of PKC inhibition

Since PKC activation seems to be linked to the mechanism of protection induced by IP (Liu et al. 1994; Speechly-Dick et al. 1994; Hu and Nattel 1995), it was interesting to compare the effects of PKC inhibition on the protective effects of IP and NHE_b.

Ch did not alter the basal contractile function. During the last 10 min of reperfusion in C hearts (Fig. 8A) the

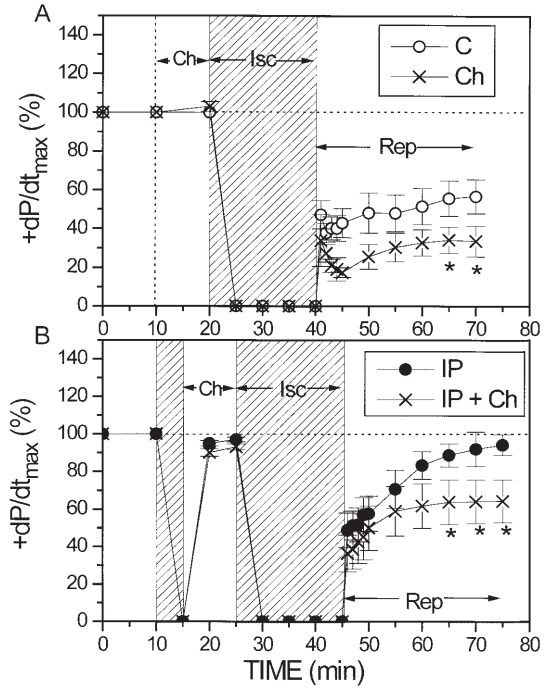

Fig. 8A,B Effects of chelerythryne (*Ch*), a PKC inhibitor, on $+dP/dt_{max}$ during the reperfusion period. It was observed that the systolic function of control ischemic (*C*) hearts was impaired by Ch at the end of reperfusion (**A**) and the improvement of postischemic recovery induced by IP was abolished by PKC inhibition (**B**). **P*<0.05

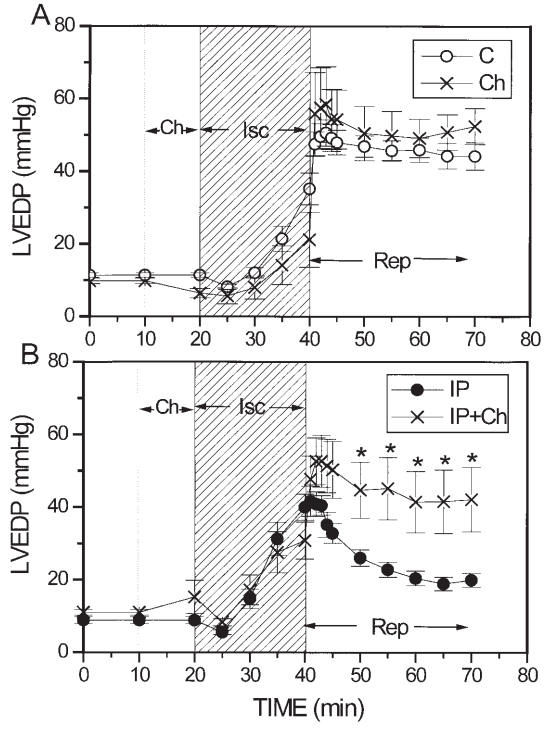

Fig. 9 Changes of left ventricular end diastolic pressure (*LVEDP*) after PKC inhibition with chelerythrine (*Ch*) during ischemia and reperfusion in C (**A**) and IP (**B**) hearts. Ch did not significantly modify the ischemic contracture and abolished the diastolic protection induced by IP. **P*<0.05

diminution of $+dP/dt_{max}$ after Ch treatment was significantly greater compared with the values obtained without PKC inhibition. These results suggest that PKC activation occurs during this ischemic period and provides some protection against reperfusion injury.

In IP hearts the PKC inhibition abolished the systolic (Fig. 8B) and diastolic (Fig. 9B) protection. $+dP/dt_{max}$ recovered only 61±14% after PKC inhibition, whereas recovery by IP without PKC blockade was 94±6% ($P<0.05$). In the other way, Ch did not modify the beneficial action obtained by NHE_b with EIPA. In this experimental group at 30 min of reperfusion $+dP/dt_{max}$ was 82±6% after NHE_b and 87±13% when PKC and NHE were blocked.

The IC observed in C and IP hearts was not altered by Ch (Fig. 9A,B), neither was the decrease in IC induced by NHE_b with EIPA. At the end of the ischemic period the LVEDP values were 21±5 mmHg and 18±4 mmHg (NS) after NHE_b with and without PKC inhibition, respectively.

Discussion

NHE_b and IP were two interventions that provided similar protection against systolic and diastolic dysfunction that occurs after the reperfusion following myocardial ischemia. A similar preservation of ATP levels induced by both interventions was also found after reperfusion. However, the following main differences were detected between both protections: The increase in myocardial stiffness observed during the ischemic period was reduced by NHE_b with both blockers (EIPA and HOE 642) but not by IP, and PKC inhibition abolished the protection afforded by IP but not by NHE_b (EIPA).

The IC observed during the ischemic period was proposed to be the result of Ca^{2+} overload (Barry et al. 1987) and/or ATP depletion (Hearse et al. 1977; Koretsune and Marban 1990; Ventura-Clapier and Veksler 1994). In accordance with previous papers (Kobara et al. 1996; Kolocassides et al. 1996), a similar ATP level at the end of ischemia in IP compared with C hearts was obtained. Our data showing higher ATP values in NHE_b than IP hearts after the ischemic period suggest that NHE_b and not IP is preserving ATP levels during ischemia. However, we should keep in mind that if we accepted that the intracellular acidosis that occurred during myocardial ischemia (Dennis et al. 1991) was exaggerated after NHE_b, the competition between H^+ and Ca^{2+} ions at the level of troponin C (Komukai et al. 1998) could decrease the magnitude of a contracture due to Ca^{2+} overload. Whether or not the NHE_b accentuates the intracellular acidosis induced by ischemia is still uncertain (Hendrikx et al. 1994; Koike et al. 1996; Ruß et al. 1996).

Our results on the IC obtained by NHE_b are in agreement with the investigations of Hendrikx et al. (1994) who demonstrated a delay in the time to onset of IC in the rabbit after treatment with HOE 694. However, these results were not found in experiments performed in isolated rat heart by Shipolini et al. (1997). These authors showed that IP accelerated the beginning of contracture, an effect that was not modified by the addition of NHE_b.

The decrease in the ischemic myocardial contracture induced by intracellular acidosis was described by Bing et al. (1973). Katz and Hecht (1969) and other investigators (Mattiazzi et al. 1979; Ricciardi et al. 1986) contributed to the concept of competition between H^+ and Ca^{2+} ions at the level of the contractile machinery as a factor determining the contractility. More recently, and in agreement with these concepts, a better recovery of contractile function was obtained when reperfusion was started with a low-pH, low-Ca^{2+} perfusate (Kitakaze et al. 1988; Harada et al. 1994; Mosca et al. 1998).

In the experiments described here, it is interesting that no differences in the protection afforded by IP and NHE_b were observed after reperfusion. Systolic and diastolic function as well as ATP levels were preserved to a similar extent by both IP and NHE_b. These data could lead to the conclusion that similar mechanisms are involved in the protection. However, the differences in the IC plus the fact that the PKC inhibition abolished the protection afforded by IP but not by NHE_b argue against a common mechanism of protection.

In a recent publication (Lundmark et al. 1999) the protection by repetitive cycles of acidosis was compared with IP. Both interventions, acidosis and IP, improved postischemic recovery. However, PKC inhibition abolished the protection by IP but not that induced by repeated acidosis.

One important finding of our study to be emphasized is that the protection afforded by NHE_b was obtained with two different NHE blockers. The NHE_b with amiloride derivatives have the potential problems of the pharmacological interventions that not only modify the NHE activity but also other mechanisms. One of the mechanisms proposed to be involved in the protection by NHE_b was the prevention of an increase in intracellular Na^+ leading to Ca^{2+} overload through the Na^+/Ca^{2+} exchanger. However, although it is well known that amiloride derivatives present some effects on the Na^+/Ca^{2+} exchanger (Pierce et al. 1993) and L-channels (García et al. 1990), our results showing the same directional changes with the more specific blocker of the NHE-1 isoform, HOE 642, seem to rule out an action mediated through a different pathway than the NHE.

One question not yet being focussed on in our study is: are we inducing protection from myocardial stunning or necrosis? This is a controversial subject. After 15–20 min of myocardial ischemia in preparations of isolated hearts a significant amount of necrosis does not seem to be detected (Kusuoka et al. 1987; Mosca et al. 1998). Furthermore, the characteristic pattern of a decreased myofilament responsiveness detected in the myocardial stunning after 20 min of ischemia in the rat was reversed by IP (Pérez et al. 1999).

An attractive hypothesis would be that myocardial ischemia induces Ca^{2+} overload. Thus, Ca^{2+} overload induces cytotoxic effects leading to Tn I degradation (Gao et al. 1997). The Ca^{2+} overload can be reduced by IP (Steenbergen et al. 1993). Although the mechanisms in-

volved in the protection by IP are not clear, recent evidence indicates that mitochondrial K_{ATP} channels seem to be phosphorylated by PKC effectors (Wang and Ashraf 1999). NHE_b, on the other hand, would not prevent the Ca^{2+} overload but would blunt its effects, through the competition between Ca^{2+} and H^+ ions. This competition could act at the level of the myofilaments and decrease the magnitude of the IC. The possibility of acidosis acting also at the mitochondrial level and altering the electroneutral K^+/H^+ exchange should also be considered. These alterations may change the intramitochondrial osmotic pressure and mitochondrial volume, important factors in the modulation of metabolic process (Halestrap 1989). In connection with this, Hotta et al. (1998) recently demonstrated that the pretreatment of guinea-pig mitochondrial membranes with EIPA attenuated the Ca^{2+} elevation in this organelle.

In summary: IP and NHE_b protect hearts from ischemia after reperfusion to a similar extent. In spite of this similar protection, whereas IP accelerates the IC, NHE_b decreases its extent. Furthermore, the protection by IP and not that obtained by NHE_b is blunted by PKC inhibition.

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