Cariporide preserves mitochondrial proton gradient and delays ATP depletion in cardiomyocytes during ischemic conditions

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Ruíz-Meana, Marisol, David García-Dorado, Pilar Pina, Javier Inserte, Luis Agulló, and Jordi Soler-Soler. Cariporide preserves mitochondrial proton gradient and delays ATP depletion in cardiomyocytes during ischemic conditions. Am J Physiol Heart Circ Physiol 285: H999–H1006, 2003; 10.1152/ajpheart.00035.2003.—The mechanism by which inhibition of Na+/H+ exchanger (NHE) reduces cell death in ischemia-reperfused myocardium remains controversial. This study investigated whether cariporide could inhibit mitochondrial NHE during ischemia, delaying H+ gradient dissipation and ATP exhaustion. Mouse cardiac myocytes (HL-1) were submitted to 1 h of simulated ischemia (SI) with NaCN/deoxyglucose (pH 6.4), with or without 7 μM cariporide, and mitochondrial concentration of Ca2+ (Rhod-2), 2’,7’-bis(2-carboxyethyl)-5(6)carboxyfluorescein (BCECF) and the charge difference across the mitochondrial membrane potential (ΔΨm, JC-1) were assessed. ATP content was measured by bioluminescence and mitochondrial swelling by spectrophotometry in isolated mitochondria. Cariporide significantly attenuated the acidification of the mitochondrial matrix induced by SI without modifying ΔΨm decay, and this effect was associated to a delayed ATP exhaustion and increased mitochondrial Ca2+ load. These effects were reproduced in sarclemma-permeabilized cells exposed to SI. In these cells, cariporide markedly attenuated the fall in mitochondrial pH induced by removal of Na+ from the medium. In isolated mitochondria, cariporide significantly reduced the rate and magnitude of passive matrix swelling induced by Na+ acetate. In isolated rat hearts submitted to 40-min ischemia at different temperatures (35.5°, 37°, or 38.5°C) pretreatment with cariporide limited ATP depletion during the first 10 min of ischemia and cell death (lactate dehydrogenase release) during reperfusion. These effects were mimicked when a similar ATP preservation was achieved by hypothermia and were abolished when the sparing effect of cariporide on ATP was suppressed by hyperthermia. We conclude that cariporide acts at the mitochondrial level, delaying mitochondrial matrix acidification and delaying ATP exhaustion during ischemia. These effects can contribute to reduce cell death secondary to ischemia-reperfusion.

Inhibition of the Na+/H+ exchanger (NHE) has a strong protective effect against cardiomyocyte death secondary to ischemia-reperfusion (1, 10, 15, 28), but the exact mechanism of this protection is, however, far from being fully elucidated. The initial hypothesis that NHE inhibition prevented cell death by attenuating cytosolic Ca2+ overload during reperfusion, associated to correction of intracellular acidosis, has not been confirmed by experimental results (26, 27). It has been suggested that the protective effect of NHE inhibition is mediated by a slower normalization of intracellular pH during reperfusion (30). However, the observed delay in pH recovery is very small, and other studies (27) have shown that the NaHCO3 cotransporter may compensate for NHE inhibition, allowing a rapid normalization of intracellular pH during reperfusion. Although with some discrepancies (10), studies in different models have shown that to be effective, NHE inhibition must act during coronary occlusion (6, 16) or cardioplegia (28), whereas its application at the time of reperfusion affords little, if any, protection against cell death. Recent reports (6, 18, 25) indicate that NHE inhibition delays the progression of ischemic injury as manifested by ATP depletion or development of rigor contracture. It has been suggested that NHE1 is markedly inhibited during severe ischemia (23).

More recently, it was proposed that NHE inhibitors could exert their anti-ischemic effect at the mitochondrial level by favoring the opening of mitochondrial ATP-sensitive K+ channels (20). However, the mechanism by which cariporide could open these channels, and the link between the ATP-sensitive K+ channel opening and reduced cell death remain elusive. NHE is not exclusive of plasmatic cell membranes, but it also exists in the inner mitochondrial membrane (22). H+ extrusion from the mitochondrial matrix is coupled to electron transport during cell respiration and results in a large H+ gradient between both sides of the inner mitochondrial membrane. This chemical gradient, together with the electrical charge difference across the inner mitochondrial membrane (ΔΨm) generates the protonotive force that moves H+ into the mitochondrial matrix through the H+ channel of the F0F1 complex (ATP synthase). H+ influx into the mitochondrial matrix through the mitochondrial NHE (MNHE) constitutes a form of H+ leakage not coupled to ATP synthesis that tends to dissipate the energy stored as transmembrane proton H+ gradient. During anoxia or ischemia, the normal mechanism of H+ extrusion associated to electron transport stops, mito-

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INHIBITION of the Na+/H+ exchanger (NHE) has a strong protective effect against cardiomyocyte death secondary to ischemia-reperfusion (1, 10, 15, 28), but the exact mechanism of this protection is, however, far from being fully elucidated. The initial hypothesis that NHE inhibition prevented cell death by attenuating...
Mitochondrial matrix is flooded with H+, H+ gradient collapses, and ATP synthesis ceases (5) and may be eventually replaced by ATP hydrolysis coupled to H+ extrusion (4, 5). In the present study, we investigated the hypothesis that inhibition of MNHE during ischemia slows both acidification of the mitochondrial matrix and ATP hydrolysis and that the resulting delay in energy depletion significantly contributes to the slowed progression of ischemic injury and reduced cell death.

METHODS

This study was performed in HL-1 cells, a culture of atrial-derived mouse cardiac myocytes (3) plated at 20,000 cells/cm² density in glass-bottom culture dishes until a 70–80% confluence was achieved. Additional studies were performed in isolated, perfused rat hearts from male Sprague-Dawley rats (300–350 g body wt) and in isolated rat heart mitochondria. The experimental procedures conformed to the Institutional Guide for the Care and Use of Laboratory Animals (NIH Publication No. 85-23, Revised 1996) and were approved by the Research Commission on Ethics of the Hospital Vall d’Hebron.

Simulated ischemia in intact and permeabilized HL-1 cells. Experiments were performed on the stage of an inverted microscope (model IX70, Olympus) at ×20 magnification (UplFL, Olympus). For JC-1 fluorescence measurement a real-time laser confocal microscope was used (Nipkow system, QCL100, Visitech). Cells were submitted to 1 h of simulated ischemia at 37°C by incubation with a glucose-free buffer in the presence or in the absence of 7 μM cariporide. The buffer was composed of the following (in mM): 2 NaCl, 20 2-deoxyglucose (DOG), 140 NaCl, 3.6 KCl, 1.2 MgSO₄, 2 CaCl₂, 20 HEPES, or, when so stated, 24 NaHCO₃ at pH 6.4. Experiments were performed in HEPES buffer for comparison with studies in membrane-permeabilized cells and replicated in bicarbonate-containing buffer to rule out the possible influence of the effects of cariporide on cytosolic pH in the absence of bicarbonate. These protocols were well tolerated, and, after reexposure to control containing glucose at pH 7.4 for 30 min (simulated reperfusion), the rate of cell death, assessed by propidium iodide, was <10% in all groups.

A subset of experiments was performed in myocytes permeabilized by 10-min incubation in an “intracellular-like” medium containing the following (in mM): 135 KCl, 10 NaCl, 0.5 KH₂PO₄, 0.5 MgCl₂, and 20 HEPES (pH 7.2) as well as 10 μM digitonin. In these cells, ischemia was simulated by addition to the “intracellular-like” medium of NaN₂ (2 mM) and CaCl₂ (10 μM) at pH 6.4.

Intramitochondrial [H⁺], [Ca²⁺], and ΔΨₘ. Changes in mitochondrial [H⁺] ([H⁺]ₘ) mitochondrial [Ca²⁺] ([Ca²⁺]ₘ) and ΔΨₘ were monitored by a fluorescence-imaging system in cells subjected to simulated ischemia either in the presence or in the absence of cariporide. [H⁺]ₘ was measured in cells loaded with 3 μM 2’,7’-bis-(2-carboxyethyl)-5(6)carboxyfluorescein (BCECF; Molecular Probes) (450/490 nm) for 1 h in medium 199, and the cells were then washed and postincubated. To eliminate the cytosolic component in the overall signal, the sarcolemma was permeabilized after the loading procedure. Permeabilized cells were then submitted to 1 h of simulated ischemia, and alternating excitation of the BCECF dye at wavelengths of 450 and 505 nm was performed with the use of a fast-speed monochromator (Visitech). Emitted light was collected with an air-cooled intensified digital camera, and 450-to-505-nm ratios were calculated for each pixel at 10-s intervals from background-subtracted signal intensities in pairs of images consecutively obtained at the two wavelengths. Color-coded 450-to-505-nm ratio images were automatically generated and the average ratio was calculated for regions of interest defined within the cells. Changes in these average ratio values throughout time were analyzed. To measure [Ca²⁺]ₘ and ΔΨₘ cells underwent a loading protocol with 4 μM Rhod-2 and 10 μM JC-1 for 25 and 10 min, respectively, and were subjected to the same experimental protocol described above except that excitation was performed at 552 nm for Rhod-2 probe and at 488 nm for JC-1 probe (emission fluorescence was recorded at 530 nm and at 590 nm). Results in fluorescence intensity were expressed as the percentage of change respect to the initial value and for JC-1 probe as a percentage of change respect to initial 590-530 nm emission ratio.

Na⁺-dependent changes in [H⁺]ₘ. To confirm that 7 μM cariporide may inhibit NHE at the mitochondrial level, changes in [H⁺]ₘ were monitored in permeabilized cells exposed to an abrupt removal of Na⁺ from the intracellular-like buffer in the presence and in the absence of the drug. Complete washout (50 μM) was achieved throughout the Na⁺ removal protocol to block mitochondrial Na⁺/Ca²⁺ exchange, thus preventing Na⁺-dependent changes in [Ca²⁺]ₘ.

Matrix swelling in isolated mitochondria. In a separate series of experiments, the potential effect of different concentrations of cariporide on mitochondrial matrix swelling was assessed spectrophotometrically in a suspension of isolated mitochondria. Rat heart mitochondria were isolated by differential centrifugation, according to the method described by Holmuhamedov et al. (12). Hearts were rapidly excised from pentobarbital-anesthetized rats (100 mg/kg) and placed in an ice-cold isolation buffer containing the following (in mM): 50 sucrose, 200 mannitol, 5 KH₂PO₄, 1 EGTA and 5 MOPS and 0.1% BSA (at pH 7.15) adjusted with KOH. After both atria were removed, ventricular myocardium was homogenized in three 20-s cycles using a Polytron (PT2100). Homogenized tissue was centrifuged 10 min at 750 g. The resulting supernatant containing the mitochondrial fraction was further centrifuged 20 min at 7,000 g, and the pellet was resuspended in the isolation buffer with no EGTA. Protein content of the mitochondrial suspension was determined by Bradford assay before the experiments.

Increases in mitochondrial matrix volume were determined as changes in the light scattering parameter of mitochondrial suspensions (8) with the use of a spectrophotometer (Lambda20, Perkin-Elmer). Light absorbance of the mitochondrial suspension (at 0.5 mg/ml protein) was measured at 546 nm. Passive swelling was induced with Na⁺ acetate solution at pH 7.2 (2). K⁺-acetate was added to mitochondria from a control group in which matrix swelling was absent. The effect of cariporide on MNHE at 2.7, or 20 μM and the effect of 10-min preincubation with cariporide (7 μM) were measured as a reduction of mitochondrial swelling induced by Na⁺ acetate.

Cellular ATP content. ATP content was measured in cells frozen in liquid N₂ by means of the Bioluminescent Somatic Cell Assay (Sigma Aldrich) after different periods of simulated ischemia.

Studies in isolated perfused rat heart. The relationship between the effect of cariporide on ATP depletion during ischemia and its protective effect against cell death induced by ischemia-reperfusion was studied in isolated, perfused hearts (n = 56). After intraperitoneal injection of pentobarbital sodium (100 mg/kg), the hearts were excised and perfused with a modified Krebs-Henseleit bicarbonate buffer composed of (in mM) 140 NaCl, 24 NaHCO₃, 2.7 KCl, 0.4...
reached its maximum after 10 min, and 7/H9262 are compared. A critical curve functions rather than isolated numerical observations.

Functional ANOVA test for functional data was used in which the activity in the coronary effluent ischemia. Lactate dehydrogenase (LDH) activity was determined as described for cell cultures. Myocardial ATP content was measured as described (7). The homogeneity between groups was tested by ANOVA test; the effect of treatments on changes in ATP content, time of onset of rigor were studied by means of the MANOVA test. Blockade of NHE during simulated ischemia with cariporide was associated to a more sustained [Ca\(^{2+}\)]\(_m\) overload. At the end of simulated ischemia, Rhod-2 fluorescence was 199 ± 29% of the preischemic value in control cells and 238 ± 30% in cariporide-treated cells (functional ANOVA test, P = 0.004; Fig. 2). In permeabilized cells, the initial rise in [Ca\(^{2+}\)]\(_m\) was followed by a plateau and a subsequent decay, probably reflecting Rhod-2 leakage in deenergized mitochondria and/or a net Ca\(^{2+}\) extrusion in the absence of further influx. In these cells, the relative increase in Rhod-2 fluorescence was attenuated respect to what was obtained in intact cells, probably because part of the fluorescent signal in intact cells comes from cytosolic compartment. As in intact cells, cariporide increased the overall amount of [Ca\(^{2+}\)]\(_m\) during simulated ischemia in permeabilized cells, and Rhod-2 fluorescence at the end of ischemia was 86 ± 10% of preischemic value in control cells and 127 ± 17% in cariporide-treated cells, P = 0.004 (Fig. 2). To assess whether the increase in [Ca\(^{2+}\)]\(_m\) was mediated by reverse operation of the mitochondrial Na\(^+\)/Ca\(^{2+}\) exchanger, additional experiments were performed, in which this exchanger was inhibited by addition of clonazepam (50 μM) during simulated ischemia. This intervention did not result in a prevention of mitochondrial Ca\(^{2+}\) influx, but rather in an increase of the total mitochondrial Ca\(^{2+}\) load.

RESULTS

**Effect of cariporide on [H\(^{+}\)]\(_m\) during simulated ischemia.** Simulated ischemia at pH 6.4 induced a progressive increase in [H\(^{+}\)]\(_m\) (reflecting a pH decline) that reached its maximum after 10 min, and 7 μM cariporide markedly attenuated this increase (Fig. 1). This effect reflected a blockade of NHE at the mitochondrial membrane because results were obtained in permeabilized cells in which cytosolic composition was kept constant. To provide an idea of the magnitude of the effect of cariporide on mitochondrial pH, the BCECF ratio was calibrated by the exposure of additional membrane-permeabilized cells to media with different and known pHs in the presence of 30 μM of the mitochondrial membrane protonophore dinitrophenol (DNP) under identical acquisition conditions. The average BCEF ratio observed after 10 min of simulated ischemia corresponded to a mitochondrial pH of 6.7 in control cells and of 7.0 in the cariporide group.

**Contribution of NHE to [Ca\(^{2+}\)]\(_m\) overload during simulated ischemia.** [Ca\(^{2+}\)]\(_m\) started to rise very early after the initiation of simulated ischemia, a kinetics that clearly differs from that obtained in cytosolic compartment, in which Ca\(^{2+}\) starts to rise after a more prolonged time gap. Blockade of NHE during simulated ischemia with cariporide was associated to a more sustained [Ca\(^{2+}\)]\(_m\) overload. At the end of simulated ischemia, Rhod-2 fluorescence was 199 ± 29% of the preischemic value in control cells and 238 ± 30% in cariporide-treated cells (functional ANOVA test, P = 0.004; Fig. 2). In permeabilized cells, the initial rise in [Ca\(^{2+}\)]\(_m\) was followed by a plateau and a subsequent decay, probably reflecting Rhod-2 leakage in deenergized mitochondria and/or a net Ca\(^{2+}\) extrusion in the absence of further influx. In these cells, the relative increase in Rhod-2 fluorescence was attenuated respect to what was obtained in intact cells, probably because part of the fluorescent signal in intact cells comes from cytosolic compartment. As in intact cells, cariporide increased the overall amount of [Ca\(^{2+}\)]\(_m\) during simulated ischemia in permeabilized cells, and Rhod-2 fluorescence at the end of ischemia was 86 ± 10% of preischemic value in control cells and 127 ± 17% in cariporide-treated cells, P = 0.004 (Fig. 2). To assess whether the increase in [Ca\(^{2+}\)]\(_m\) was mediated by reverse operation of the mitochondrial Na\(^+\)/Ca\(^{2+}\) exchanger, additional experiments were performed, in which this exchanger was inhibited by addition of clonazepam (50 μM) during simulated ischemia. This intervention did not result in a prevention of mitochondrial Ca\(^{2+}\) influx, but rather in an increase of the total mitochondrial Ca\(^{2+}\) load.

![BCECF Ratio vs Time](image)

**Fig. 1.** Effect of 7 μM cariporide on mitochondrial matrix acidification as assessed by ratiofluorescence induced by simulated ischemia (SI) in membrane-permeabilized cardiomyocytes loaded with 2,7’-bis(2-carboxyethyl)-5(6)-carboxyfluorescein (BCECF). An increase in the 450-to-490-nm ratio indicates an increase in [H\(^{+}\)].

![Graph](image)

**Fig. 2.** Changes in mitochondrial [Ca\(^{2+}\)] as assessed by fluorescence imaging induced by SI in membrane-permeabilized and intact cardiomyocytes loaded with Rhod-2 (n = no. of cells) are shown.
Mitochondrial depolarization during energy deprivation. The cells exposed to simulated ischemia experienced a rapid fall in the $\Delta \Psi_m$, as measured by the decline in the 590/530 JC-1 emitted fluorescence. Mitochondrial depolarization reached its minimum level at 10–15 min, a time coincident with ATP exhaustion in this model. The addition of cariporide during simulated ischemia did not preserve $\Delta \Psi_m$ or significantly delayed its decline (Fig. 3).

Effect of cariporide on intracellular ATP depletion during simulated ischemia. Intracellular ATP content started to decline immediately after the onset of sim-
ulated ischemia. In NaHCO₃ buffer, the ATP fall reached 0.6 ± 0.1% of its initial level after 15 min, whereas in HEPES buffer, the ATP fall was significantly slower (data not shown). The presence of cariporide significantly delayed the rate of ATP exhaustion (Fig. 3).

Na⁺/H⁺-dependent changes in [H⁺]m. Removal of Na⁺ from the intracellular-like buffer induced an abrupt increase in [H⁺]m in permeabilized cells. This increase was markedly attenuated by 7 μM cariporide (Fig. 4).

Effect of cariporide on mitochondrial swelling. The addition of Na⁺-acetate solution to a suspension of isolated rat heart mitochondria was immediately followed by passive matrix swelling, as determined spectrophotometrically by a decrease in the 546-nm absorbance. Cariporide (7 and 20 μM) significantly decreased the degree of mitochondrial matrix swelling induced by Na⁺-acetate and the rate at which it occurred, measured as the slope of the curve, whereas at 2 μM did not have any effect. Preincubation with 7 μM cariporide 10 min before the addition of Na⁺-acetate did not prevent matrix swelling development (Fig. 5).

Contribution of ATP preservation to protective effect of NHE inhibition against ischemic-reperfusion cell death. In isolated rat hearts exposed to nonflow ischemia at 37°C, pretreatment with cariporide significantly increased the degree of mitochondrial matrix swelling induced by Na⁺-acetate and the rate at which it occurred, measured as the slope of the curve, whereas at 2 μM did not have any effect. Preincubation with 7 μM cariporide 10 min before the addition of Na⁺-acetate did not prevent matrix swelling development (Fig. 5).

Fig. 4. Effect of cariporide on mitochondrial matrix acidification induced by acute withdrawal of Na⁺ from intracellular-like medium in membrane-permeabilized cardiomyocytes loaded with BCECF. [Na⁺]o, extracellular Na⁺ concentration. A: time course of changes in 450-to-490-nm ratio. An increase in ratio indicates an increase in [H⁺]. B: representative ratiofluorescence images obtained before (A1 and B1) and after acute removal of Na⁺ in the absence (A2) and in the presence (B2) of 7 μM cariporide.

Fig. 5. Effect of cariporide on the prevention of mitochondrial matrix swelling in isolated rat heart mitochondria. A: changes in light absorbance (arbitrary units) in isolated rat heart mitochondria submitted to passive matrix swelling in Na⁺-acetate-based medium. A decrease in light absorbance reflects mitochondrial matrix swelling. Each curve represents the average of five experiments performed in the absence (control) or in the presence of 7 μM cariporide in the Na⁺-acetate medium. B: changes in the slope of the absorbance curves in isolated rat heart mitochondria during the initial 5 s of the addition of different treatments as a parameter of the rate of mitochondrial swelling development. Prevention of mitochondrial swelling in a Na⁺-acetate medium was observed with cariporide at 7 and 20 μM, but not at 2 μM. Preincubation with cariporide at 7 μM during 10 min before the addition of Na⁺-acetate did not attenuate mitochondrial swelling. K⁺ acetate was used as a control group, in which mitochondrial swelling was absent. a.u., Arbitrary units of light absorbance.
delayed the onset of rigor contracture by ~3 min, increased myocardial ATP content observed after 10 min of ischemia by 41% ($P < 0.01$), and reduced by >50% ($P < 0.01$) the extent of reperfusion-induced hypercontracture, as assessed by peak left ventricular diastolic pressure, and cell death, as assessed by LDH release. Reducing myocardial temperature during ischemia had per se a strong effect on myocardial ATP content, and did mimic the protection afforded by cariporide. Increasing myocardial temperature accelerated ATP consumption during ischemia and attenuated the effects of the drug. In the whole series of hearts, a close relationship was observed between the rate of ATP depletion during the first 10 min of ischemia and the time of onset of ischemic rigor ($r = 0.85$). Both variables were closely correlated with LDH release during reperfusion ($r = 0.99$) (Fig. 6). Nevertheless, these relationships were not modified by cariporide. Multiple regression analysis showed that time of rigor onset accurately predicted the extent of LDH release during reperfusion, and that inclusion of treatment allocation in the regression model did not significantly improve its predictive value.

**DISCUSSION**

This study demonstrates that NHE inhibition with cariporide exerts a protective effect at the mitochondrial level, delaying acidification of the mitochondrial matrix in cultured cardiomyocytes during simulated ischemia without modifying $\Delta \Psi_m$. This effect is associated to slowed ATP depletion and is not a mere reflection of changes in cytosolic cation concentration or pH, because it is observable in permeabilized cells. The protective effect of cariporide against ATP depletion is also observable in isolated rat hearts submitted to nonflow ischemia, and regression analysis indicates that it fully accounts for the reduction in cardiomyocyte death during subsequent reperfusion. These results are consistent with the hypothesis that NHE inhibitors may protect myocardium against cell death secondary to ischemia-reperfusion by acting on mitochondrial membrane, an effect that results in less H$^+$ entry into the matrix, and preservation of transmembrane H$^+$ gradient and ATP concentration.

**Effect of cariporide on mitochondrial matrix acidification.** Cariporide is an amiloride-related compound with a potent and highly selective inhibitory effect on NHE, in particular on the NHE1 isof orm. The hypothesis that cariporide inhibits MNHE implies that it can cross cell membranes and reach mitochondria and that the mitochondrial exchanger can be inhibited by the drug. To our knowledge, no published study has measured the permeability of cell membranes to cariporide or other NHE inhibitors. However, calculation of the logarithmic number of partition coefficient for cariporide yields a value of 0.1 at a pH higher than its pKa (pH > 4.42). This predicts that cariporide can penetrate cell membranes and reach MNHE. The observation that cariporide markedly attenuates both the decrease in mitochondrial pH induced by removal of Na$^+$ in permeabilized cells and passive matrix swelling induced by Na$^+$ acetate in isolated mitochondria clearly indicates that the drug can inhibit MNHE in cardiomyocytes.

The importance of NHE at the inner mitochondrial membrane has been known for almost three decades. In higher eukaryotes, studies have found that MNHE is encoded by the NHE6 gene and has a molecular structure similar but not identical to sarcolemmal NHE (22), although recent reports question the unequivocal identification of NHE6 to the MNHE (21). MNHE can be effectively inhibited by sulfonyleurea derivatives, including amiloride and more recently developed inhibitors of sarcolemmal NHE (2, 13, 14).

Under normal conditions, a gradient of H$^+$ across the inner mitochondrial membrane, with the mitochondrial matrix more alkaline than the intermembrane or cytosolic spaces, is maintained by the active extrusion of H$^+$ coupled with electron transport along the respiratory chain, as was first postulated by Mitchell in his chemiosmotic theory (19). During ischemia H$^+$ extrusion stops, leading to matrix acidification and a progressive dissipation of H$^+$ gradient. The results of the present study support the hypothesis that H$^+$ influx into the mitochondrial matrix via MNHE significantly contributes to matrix acidification during ischemia. The attenuated matrix acidification observed in the presence of cariporide cannot be the indirect consequence of changes in cytosolic pH because it is observed for a constant pH in permeabilized cells. Moreover, previous studies have demonstrated a lack of effect of

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**Fig. 6.** Correlation between lactate dehydrogenase (LDH) release during reperfusion and time of onset of ischemic rigor contracture ($A$) or ATP content after 10 min of ischemia ($B$) in isolated rat hearts submitted to 40 min of nonflow ischemia at different temperatures. Both correlations are very high and not influenced by treatment with cariporide.
cariporide on cytosolic pH when other mechanisms of pH correction, like NaHCO₃ cotransporter, are active. The effect of cariporide should be an enhancement of cytosolic acidosis that would not explain the reduced acidification of mitochondrial matrix. Because the H⁺ gradient across the inner mitochondrial membrane is one of the two components of the protonotive force responsible for H⁺ flow through the F₀ channel of the ATP synthase, and its reduction may determine ATP hydrolysis by the F₁ unit of ATP coupled to H⁺ extraction, delayed matrix acidification during simulated ischemia should be expected to result in attenuated rate of ATP depletion, as observed.

To our knowledge, no previous study has analyzed the effect of NHE inhibition on the increase in [Ca²⁺]ₘ induced by ischemia. In a recent study (31), pretreatment with the selective NHE inhibitor SM-20550 was associated with attenuated Ca²⁺ overload in mitochondria obtained from rat hearts submitted to 40 min of ischemia and 20 min of reperfusion, a result in agreement with the protective effect of NHE inhibition against cell necrosis secondary to ischemia-reperfusion, but [Ca²⁺]ₘ during ischemia was not measured. Our observation that cariporide, at concentrations that have been consistently found cardioprotective in isolated cells (25, 27) and intact myocardium in vivo (6, 16), enhanced mitochondrial Ca²⁺ accumulation during simulated ischemia may appear surprising. Although increased [Ca²⁺]ₘ has been shown to have detrimental effects (4), there is a lack of information on the consequences of mitochondrial Ca²⁺ overload during ischemia. It has been previously shown that mitochondria with severe Ca²⁺ overload secondary to hypoxia or simulated ischemia may rapidly recover metabolic competence upon reoxygenation (9). Also, Ca²⁺ sequestration into mitochondria may attenuate cytosolic Ca²⁺ overload during ischemia and reperfusion. Moreover, extracellular acidification, a condition known to be strongly protective against hypoxic injury, is associated to enhanced increase of [Ca²⁺]ₘ induced by hypoxia (see below).

Several recent reports have described the ability of mitochondria to accumulate Ca²⁺, acting as a local spatial buffering system often in close association with sarcoplasmic reticulum (24). The presence of 50 µM clonazepam during simulated ischemia did not result in a prevention of mitochondrial Ca²⁺ load suggesting that reverse-mode operation of the mitochondrial Na⁺/Ca²⁺ exchanger is not responsible for mitochondrial Ca²⁺ influx during prolonged ischemia and may in fact represent a route of Ca²⁺ efflux.

**Study limitations**. Because the present study was focused on the potential role of mitochondria in the protective effect of cariporide during ischemia, a cell system most adequate to this purpose was used. HL1 cardiomyocyte cells accurately reproduce the changes in cytosolic composition induced by simulated ischemia in freshly isolated cardiomyocytes, but do not develop the changes in cell shape associated to ischemic rigor contracture and reenergization-induced hypercontracture. These changes in cell shape create important artifacts in nonratiometric fluorescent signals, including those from several important mitochondrial probes. On the other hand, these cells present the disadvantage of being more tolerant than freshly isolated cardiomyocytes to energy deprivation. This precluded any attempt to establish a direct correlation between the mitochondrial effects of cariporide during simulated ischemia and its protective effect against cell death, which occurs during reenergization. However, the direct correlation observed in intact rat hearts between the magnitude of the effect on ATP and the reduction of cell death suggests that the mitochondrial effects observed in cell cultures participate in the protection.

In conclusion, this study demonstrates that NHE inhibitors may have effects at the mitochondrial level, delaying mitochondrial matrix acidification and slowing down ATP depletion during ischemia. These results provide a new mechanism for the beneficial effect of NHE inhibitors against cell death secondary to ischemia-reperfusion and stresses the importance of mitochondria as targets for novel therapeutic approaches.
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DISCLOSURES
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