Acidification reduces mitochondrial calcium uptake in rat cardiac mitochondria

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Gursahani, Hema I., and Saul Schaefer. Acidification reduces mitochondrial calcium uptake in rat cardiac mitochondria. Am J Physiol Heart Circ Physiol 287: H2659–H2665, 2004.—Cardiac ischemia-reperfusion (I/R) injury is accompanied by intracellular acidification that can lead to cytosolic and mitochondrial calcium overload. However, the effect of cytosolic acidification on mitochondrial pH (pHm) and mitochondrial Ca2+/Ca2+ handling is not well understood. In the present study, we tested the hypothesis that changes in pHm during cytosolic acidification can modulate Ca2+ handling in cardiac mitochondria. pHm was measured in permeabilized rat ventricular myocytes with the use of confocal microscopy and the pH-sensitive fluorescent probe carboxyseminaphthorhodafluor-1. The contributions of the mitochondrial Na+/H+ exchanger (NHEm) and the K+/H+ exchanger (KHEm) to pHm regulation were evaluated using acidification and recovery protocols to mimic the changes in pH observed during I/R. Ca2+ transport in isolated mitochondria was measured using spectrophotometry and fluorimetry, and the mitochondrial membrane potential was measured using a tetraphenylphosphonium electrode. Cytosolic acidification (pH 6.8) resulted in acidification of mitochondria. The degree of mitochondrial acidification and recovery was found to be largely dependent on the activity of the KHEm. However, the NHEm was observed to contribute to the recovery of pHm following acidification in K+-free solutions as well as the maintenance of pHm during respiratory inhibition. Acidification resulted in mitochondrial depolarization and a decrease in the rate of net Ca2+ uptake, whereas restoration of pH following acidification increased Ca2+ uptake. These findings are consistent with an important role for cytosolic acidification in determining pHm and Ca2+ handling in cardiac mitochondria under conditions of Ca2+ overload. Consequently, interventions that alter pHm can limit Ca2+ overload and injury during I/R.

cardiac ischemia–reperfusion (I/R) injury; Ca2+; pHm; cytosolic acidification; mitochondrial pH; mitochondrial calcium; sodium/hydrogen exchange; potassium/hydrogen exchange; mitochondrial membrane potential

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MATERIALS AND METHODS

Animals. Cardiac myocytes and mitochondria were isolated from the left ventricle of adult male Sprague-Dawley rats. Rats were anesthetized using pentobarbital sodium (100 mg/kg ip) before the heart was excised from the chest cavity. The procedures for isolation were approved by the Institutional Animal Care and Use Committee of the University of California, Davis.

Isolation of rat ventricular myocytes. Left ventricular myocytes were isolated with the use of a standard enzymatic digestion procedure using collagenase and protease (10). Freshly isolated ventricular myocytes were stored at room temperature and used within 8 h of isolation.

Isolation of mitochondria from rat ventricles. Mitochondria were isolated from the left ventricle using mechanical disruption followed by a differential centrifugation procedure as described previously (17). Briefly, left ventricular tissue was minced and homogenized using two 10-s cycles in a polytron followed by a 10-min centrifugation at 2,000 rpm. After resuspension and further centrifugation at 10,500 rpm, mitochondria were resuspended in storage buffer containing (in mmol/l) 118 NaCl, 4.6 KCl, 1.2 MgCl2, 1.2 KH2PO4, 10 HEPES, 1 CaCl2, and 10 dextrose; pH was adjusted to 7.4 using KOH. Mitochondria were used within 3 h of isolation and kept stirred on ice until use. Mitochondrial protein concentration was determined using the Bradford assay and calibrated using bovine serum albumin (BSA).

Measurement of pHm in permeabilized myocytes using carboxy-SNARF-1 and confocal microscopy. Freshly isolated rat ventricular myocytes were loaded with 5 μmol/l 5- (and 6-) carboxyseminaphthorhodafluor-1 (carboxy-SNARF-1) AM acetate for 30 min at room temperature, in an extracellular medium containing (in mmol/l) 118 NaCl, 4.6 KCl, 1.2 MgCl2, 1.2 KH2PO4, 10 HEPES, 1 CaCl2, and 10 dextrose; pH was adjusted to 7.2 with NaOH. For imaging experiments, the loaded myocytes were plated on glass coverslips coated with Cell-tak (BD Biosciences; Bedford, MA). SNARF-1 AM-loaded myocytes were washed in extracellular medium containing 0 Ca2+ and 50 μmol/l EGTA before permeabilization. Myocytes were permeabilized using 0.001% digitonin for 3 min in an intracellular medium containing (in mmol/l) 110 KCl, 10.3-(N-morpholino)propanesulfonic acid (MOPS), 5 MgCl2, 5 Na2ATP, and 0.5 EGTA, plus BSA 2 mg/ml; the pH was adjusted to either 6.8 or 7.2 using Tris base (10). KCl (110 mmol/l) was replaced with 110 mmol/l choline chloride for choline chloride-containing solutions. Oligomycin (4 μg/ml) and KCN (2 mmol/l) were also added to the intracellular medium in experiments involving respiratory inhibition. 5-(N-ethyl-N-isopropyl) amiloride (EIPA) was prepared as a 100 mmol/l stock solution in DMSO, and 0.1% DMSO was used as a vehicle control.

Permeabilized myocytes were imaged using a ×63 oil-immersion objective of an inverted microscope (Carl Zeiss) equipped with laser-scanning confocal unit (Pascal LSM 510). SNARF was excited using the 514-nm line of an argon laser, and the emitted fluorescence at 580 nm and 650 nm was captured using a dichroic mirror at 615 nm. With the use of an optical slice of <0.7 μm, full-frame images (512 × 512 pixel) were acquired, and the mean fluorescence intensities of the mitochondrial regions were analyzed at the two emission wavelengths. pHm was expressed as the SNARF (580 nm/650 nm) emission ratio, and an increase in the SNARF (580 nm/650 nm) ratio represents a decrease in pH.

Ca2+ transport measurements in isolated mitochondria. Ca2+ transport in isolated mitochondria was measured using the metallochromic indicator antipyrylazol III (APIII), which exhibits a strong increase in absorbance at 720 nm when bound to Ca2+ (28). APIII does not enter mitochondria, and hence the changes in APIII absorbance are directly proportional to changes in extramitochondrial Ca2+. For measurement of Ca2+ transport, mitochondria (0.5 mg/ml protein) were suspended in a buffer containing (in mmol/l) 100 KCl, 5 Na2ATP, 10 MOPS, 0.01 EGTA, and 0.1 APIII; pH was adjusted to various values between 6.4 and 7.4 using Tris base. Thapsigargin (0.5 μmol/l) was added to prevent any potential interference from the sarcoplasmic reticulum during Ca2+ measurements. Mitochondria were magnetically stirred, and measurements were made at 25°C using a dual-beam spectrophotometer (Hewlett-Packard). After a period of incubation (3 min), mitochondria were exposed to a Ca2+ bolus (300 nmol/mg protein−1·ml−1), and Ca2+ transport was monitored for 900 s using a 720- to 790-nm wavelength pair. The mitochondrial uncoupler carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone (FCCP, 0.2 μmol/l) was added during the last 60 s of each measurement to induce maximal Ca2+ release.

Net Ca2+ uptake rate was calculated from the linear portion of the net Ca2+ uptake phase and is expressed as arbitrary absorbance units per second (AU/s).

[Ca2+]m was also measured using the fluorescent Ca2+ indicator indo-1 AM. Isolated mitochondria were incubated with 5 μM indo-1 AM in storage buffer for 30 min at room temperature (15). Mitochondria were then centrifuged and resuspended in fresh, indo-1-free storage buffer. Fluorimetric measurements of indo-1 AM fluorescence were made every 2 s using a modified spectrofluorometer (SLM Instruments; Rochester, NY) in a buffer containing (in mmol/l) 100 KCl, 5 Na2ATP, 10 MOPS, and 0.01 EGTA (pH adjusted to 6.8 or 7.4 using Tris base). Indo-1 was excited at 350 nm, and the emitted fluorescence at 385 nm and 465 nm was recorded. Ca2+ is expressed as the indo-1 (385 nm/465 nm) fluorescence ratio, and an increase in the ratio represents an increase in intramitochondrial Ca2+.

Measurements of mitochondrial membrane potential in isolated mitochondria. Quantitative measures of ΔΨm were made as described earlier (8) using tetraphenylphosphonium (TPP+) electrodes that exhibited a linear, Nernstian response connected to a high-impedance pH meter (model 290A, Orion; Boston, MA). Briefly, mitochondria (1 mg protein) were incubated in buffer containing (in mmol/l) 100 KCl, 5 Na2ATP, 10 MOPS, 0.01 EGTA, and 0.003 TPP+Cl (pH adjusted to various values between 6.4 and 7.4 using Tris base). Measurements were made every 5 s, and ΔΨm was calculated using the formula (8):

\[ \Delta \Psi_{m} = 59 \cdot \log(v/V) - 59 \cdot \log\left(10^{6/59} - 1\right) \]

where ΔΨm is measured in millivolts, v is the mitochondrial matrix volume (1.1 μl/mg protein), V is the volume of the incubation medium (1.200 μl), and ΔE is the deflection of the electrode potential from baseline.

Statistics. A two-way ANOVA followed by a Bonferroni post test was used to analyze differences between groups over time.
Data are expressed as means ± SE. Differences were considered significant at P < 0.05.

RESULTS

Measurement of pHm in permeabilized myocytes. Figure 1A is a confocal image of a rat ventricular myocyte loaded with carboxy-SNARF. B: SNARF fluorescence is localized to punctate mitochondrial regions, similar to that seen in an intact myocyte loaded with the selective mitochondrial probe tetramethylrhodamine methyl ester (TMRM). C: calibration of the SNARF (580 nm/650 nm) fluorescence ratios at various pH values. Dotted line, SNARF fluorescence value obtained following treatment of permeabilized myocytes with carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone (FCCP).

Fig. 1. Selective mitochondrial loading of carboxyseminalphorhodafluor-1 (carboxy-SNARF) in permeabilized cardiomyocytes. A: confocal image of a permeabilized rat ventricular myocyte loaded with carboxy-SNARF. B: SNARF fluorescence is localized to punctate mitochondrial regions, similar to that seen in an intact myocyte loaded with the selective mitochondrial probe tetramethylrhodamine methyl ester (TMRM). C: calibration of the SNARF (580 nm/650 nm) fluorescence ratios at various pH values. Dotted line, SNARF fluorescence value obtained following treatment of permeabilized myocytes with carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone (FCCP).

Fig. 2. Mitochondria exhibit increased acidification and delayed recovery in the absence of extramitochondrial potassium. Mitochondrial SNARF fluorescence ratios are plotted for myocytes exposed to an acidic pH (pH 6.8 or pH 5.7) for 5 min followed by recovery to normal extramitochondrial pH (pH 7.2) for 15 min. Experiments were carried out in myocytes incubated in either K+-containing or choline chloride-containing intracellular-like medium. *Mitochondrial SNARF fluorescence was significantly different from myocytes exposed to pH 6.8 medium in KCl. n = 6 myocytes from 3 independent isolations.
both the magnitude of acidification and the rate of recovery from an applied acidification.

**Role of the NHE₉m in mitochondrial acidification and recovery.** The role of the NHE₉m in mitochondrial acidification and recovery was examined using the Na⁺/H⁺ exchange inhibitor EIPA at a concentration of 100 μmol/l. This concentration was chosen based on previous reports in isolated mitochondria, where 100 μmol/l EIPA was shown to inhibit the NHE₉m by ~50% (4). In KCl-containing medium, addition of EIPA did not significantly alter either the magnitude of acidification or recovery compared with vehicle-treated myocytes (Fig. 3A). EIPA also had no significant effect in control experiments, where myocytes were maintained at pH 7.2 throughout the duration of the experiment. These data suggest that the NHE₉m is not a significant contributor to pH₉m regulation either under normal physiological conditions or conditions of cytosolic acidification in KCl medium.

A significant effect of EIPA was observed under conditions where mitochondria were incubated in K⁺-free (choline chloride containing) solutions. As with KCl-containing solutions, EIPA did not significantly alter the magnitude of acidification compared with vehicle-treated controls. However, the recovery of pH₉m in choline chloride medium was nearly abolished in the presence of EIPA (Fig. 3B). In control experiments, where permeabilized myocytes were maintained at pH 7.2 throughout the experiment, EIPA induced a progressive mitochondrial acidification over 20 min of imaging, whereas pH₉m remained relatively stable in vehicle-treated myocytes. These results suggest that in the absence of extramitochondrial K⁺, NHE₉m activity could be responsible for maintaining pH₉m and for recovery of pH₉m following acidification.

**Effect of EIPA during respiratory inhibition.** Because the NHE has been shown to play a critical role under conditions of ischemia and reperfusion (1, 23), we examined the role of the NHE₉m in pH₉m regulation under conditions of respiratory inhibition (Fig. 4). In these experiments, 2 mmol/l KCN and 4 μg/ml oligomycin were included during acidification to inhibit electron transport and to block proton translocation via the F₁F₀ ATPase, respectively. Respiratory inhibition during acidification in a KCl-based medium resulted in mitochondrial acidification that was maintained over the 20 min of monitoring. However, myocytes underwent a significantly greater mitochondrial acidification in the presence of 100 μmol/l EIPA. A similar effect of EIPA was observed at pH 7.2 in the presence of oligomycin and KCN. These experiments suggest that the NHE₉m is responsible for maintenance of pH₉m under conditions of respiratory inhibition when proton translocation out of the mitochondrial matrix is compromised.

**Effect of pH₉m on Ca²⁺ handling and ΔΨ₉m.** To evaluate the effect of mitochondrial acidification on Ca²⁺ handling, Ca²⁺ transport was measured in isolated mitochondria incubated at varying pH levels. Ca²⁺ transport in isolated mitochondria was monitored using the indicator APIII, which measures extramitochondrial Ca²⁺ (28). Isolated cardiac mitochondria were incubated in KCl-based buffer containing ATP, a solution similar to that used in the permeabilized myocyte experiments. Addition of a Ca²⁺ bolus (300 nmol·mg mitochondrial protein⁻¹·m⁻¹) at pH 7.4 resulted in ~50% net uptake of Ca²⁺, followed by net release. With increasing acidification, the rate and the magnitude of maximal uptake of the Ca²⁺ bolus were greatly reduced and essentially no net uptake of Ca²⁺ was observed at pH values of 6.6–6.4 (Fig. 5A). As shown in Fig. 5B, there was a direct linear correlation between extramitochondrial pH and the rate of net Ca²⁺ uptake. Thus extramitochondrial acidification, which also results in acidification of the mitochondrial matrix (Fig. 2), reduces net Ca₉m uptake in isolated mitochondria.

Ca²⁺ uptake via the mitochondrial Ca²⁺ uniporter has been shown to be dependent on ΔΨ₉m (7). To examine whether mitochondrial depolarization secondary to acidification could contribute to reduced Ca²⁺ influx via the uniporter, ΔΨ₉m was measured in isolated mitochondria using a TPP⁺-sensitive electrode. As shown in Fig. 5B, a reduction in extramitochondrial pH resulted in a significant mitochondrial depolarization in mitochondria incubated with ATP. These results suggest that the reduction in ΔΨ₉m during acidification could reduce the driving force for Ca²⁺ influx via the uniporter resulting in a reduction in net Ca₉m uptake.

Because acidification was found to limit Ca₉m uptake, we next investigated whether recovery of pH could restore in-

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Fig. 3. Mitochondrial Na⁺/H⁺ exchanger (NHE₉m) inhibition with 5-(N-ethyl-N-isopropyl) amiloride (EIPA) significantly affects mitochondrial pH (pH₉m) in choline chloride but not KCl medium. Permeabilized myocytes were exposed to either 100 μmol/l EIPA (open symbols) or 0.1% DMSO (control) (closed symbols) in KCl (A) or choline chloride (B), and SNARF fluorescence ratios were measured at the indicated time points. Myocytes were exposed to buffers at pH 6.8 (circles) or pH 7.2 (triangles) during the first 5 min of the protocol. *SNARF fluorescence ratio was significantly different versus the corresponding DMSO control. n = 4–6 myocytes from 3 independent isolations.
increased Ca\(^{2+}\) uptake in mitochondria. These studies were carried out using fluorescence measurements in indo-1 AM-loaded mitochondria, because restoration of pH resulted in an artifact in the APIII absorbance signal. As shown in Fig. 6, [Ca\(^{2+}\)]\(_{\text{m}}\) was significantly lower at pH 6.8 following addition of a Ca\(^{2+}\) bolus, consistent with the results in Fig. 5A. However, normalization of pH to 7.4 resulted in a rapid increase in intramitochondrial Ca\(^{2+}\) to levels similar to that observed in mitochondria incubated at pH 7.4 alone. Measurements of mitochondrial membrane potential using a TPP\(^+\) electrode showed that pH normalization resulted in a reestablishment of V\(_{\text{m}}\) to values similar to that observed at pH 7.4 (data not shown). Furthermore, Ca\(^{2+}\) uptake at pH 7.4 (or following pH normalization to 7.4) was significantly blocked in the presence of ruthenium red (data not shown).

**DISCUSSION**

**Summary of key results.** The aim of the present study was to test the hypothesis that alterations in pH\(_{\text{m}}\) during cytosolic acidification could modulate Ca\(^{2+}\)\(_{\text{m}}\) handling in cardiac mitochondria. With the use of an acidification and recovery protocol in permeabilized myocytes, our results show that 1) pH\(_{\text{m}}\) closely tracks the changes in cytosolic pH during acidification and recovery; 2) the KHE\(_{\text{m}}\), but not the NHE\(_{\text{m}}\), appears to be a major regulator of pH\(_{\text{m}}\) in KCl-containing media; 3) in the absence of extramitochondrial K\(^+\), activity of the NHE\(_{\text{m}}\) appears to be important in bringing about a recovery of pH\(_{\text{m}}\); 4) NHE\(_{\text{m}}\) activity is important in maintaining pH\(_{\text{m}}\) during respiratory inhibition; 5) mitochondrial acidification results in a mitochondrial depolarization and a decrease in net Ca\(^{2+}\)\(_{\text{m}}\) uptake in isolated mitochondria; and 6) recovery of pH following acidification resulted in increased Ca\(^{2+}\)\(_{\text{m}}\) uptake.

**Comparison of our results with previous studies.** The role of mitochondrial proton transporters, particularly the NHE\(_{\text{m}}\), in regulating pH\(_{\text{m}}\) and Ca\(^{2+}\)\(_{\text{m}}\) has been examined in a recent study (27). In an atrial cell line that was subjected to simulated ischemia, inhibition of the NHE\(_{\text{m}}\) with cariporide resulted in reduced mitochondrial acidification. The discrepancy of their findings and those of the present study could possibly be explained by differences in conditions under which NHE\(_{\text{m}}\) activity was measured, i.e., the presence of high concentrations of Ca\(^{2+}\) (10 \(\mu\)mol/l), which was significantly greater than in our studies, and could result in mitochondrial acidification secondary to Ca\(^{2+}\) influx. Under conditions similar to those used by Ruiz-Meana et al. (27), experiments performed in this laboratory using rat ventricular myocytes resulted in irreversible contracture, suggesting important differences between freshly isolated rat ventricular myocytes and the atrial cell line. Interestingly, however, a greater mitochondrial acidification was associated with lower Ca\(^{2+}\)\(_{\text{m}}\), which is in agreement with the results obtained in our study. The exact mechanism for elevated Ca\(^{2+}\)\(_{\text{m}}\) was not addressed in the study by Ruiz-Meana et al. (27), although this effect was not reversed by the mitochondrial mitochondrial Na\(^+\)/Ca\(^{2+}\) exchanger inhibitor clonazepam, suggesting that the Na\(^+\)/Ca\(^{2+}\) exchanger was not involved in Ca\(^{2+}\)\(_{\text{m}}\) loading.

**Role of NHE\(_{\text{m}}\) and KHE\(_{\text{m}}\) in pH\(_{\text{m}}\) regulation.** Incubation of permeabilized myocytes in an acidic medium resulted in an

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Fig. 4. EIPA enhances mitochondrial acidification under conditions of respiratory inhibition. The time course of the changes in pH\(_{\text{m}}\) during incubation of mitochondria in KCl medium containing KCN and oligomycin at pH 6.8 (circles) or pH 7.2 (triangles) is plotted for myocytes treated with 0.1% DMSO (Control) (closed symbols) or 100 \(\mu\)mol/l EIPA (open symbols) throughout the incubation. *SNARF fluorescence ratios are significantly different from the corresponding control. n = 7–8 myocytes from 2 to 3 independent isolations.

Fig. 5. Acidification decreases mitochondrial membrane potential and Ca\(^{2+}\)\(_{\text{m}}\) uptake in isolated cardiac mitochondria. A: representative plot for the changes in the antipyrylazo (APIII) absorbance (extramitochondrial Ca\(^{2+}\)) versus time for mitochondria that were incubated in KCl medium at varying external pH values. A 300 nmol-mg protein \(^{-1}\) Ca\(^{2+}\) bolus and 0.2 \(\mu\)mol/l FCCP were added at the times indicated. AU, arbitrary units. B: mitochondrial membrane potential before addition of Ca\(^{2+}\) (right y-axis) and Ca\(^{2+}\)\(_{\text{m}}\) uptake rates following addition of a Ca\(^{2+}\) bolus (left y-axis) are plotted as a function of extramitochondrial pH. n = 4–6 measurements from 4 independent isolations.
immediate decrease in pHm. The KHEm appeared to play an important role in maintaining pHm under conditions of mitochondrial acidification and recovery from acidification. Previous studies characterizing the KHEm in isolated mitochondrial preparations suggest that the KHEm is latent under normal, physiological conditions (14) and is primarily activated by depletion of matrix Mg2+, by increase in matrix volume, and by alkaline matrix pH (5). However, our studies show that the KHEm could also be active under conditions of mitochondrial acidification and during recovery following mitochondrial acidification.

In contrast to the KHEm, the NHEm did not appear to play a significant role in regulating pHm during acidification or recovery in the presence of a functional KHEm. However, in the absence of extramitochondrial K+, the NHEm appeared to be important in regulating recovery from acidification. Studies in isolated mitochondria have shown that the NHEm is not involved in the maintenance of pHm under normal physiological conditions (14, 19). Our results support and extend this observation by demonstrating that the NHEm did not play an active role in the regulation of pHm under either normal or acidified conditions in the presence of K+ fluxes. However, the NHEm appeared to be important in maintaining pHm in respiratory inhibited mitochondria when proton extrusion by mitochondria was compromised. Under these conditions, inhibition of the NHEm with 100 μmol/l EIPA resulted in a greater mitochondrial acidification, suggesting that the NHEm was functioning to extrude protons from the matrix in exchange for extramitochondrial Na+. Altogether, these results suggest that the NHEm does not regulate pHm in energized mitochondria but can maintain pHm in mitochondria under conditions of respiratory inhibition.

PHm and Ca2+ regulation. The present study shows that extramitochondrial acidification results in mitochondrial depolarization and decreased Ca2+ uptake in cardiac mitochondria. Furthermore, recovery of pHm in previously acidified mitochondria resulted in restoration of increased Ca2+ uptake, suggesting an important role for pHm in determining [Ca2+]m uptake. Because Ca2+ influx via the mitochondrial Ca2+ uniporter depends on ΔΨm (7), it is likely that the reduced Ca2+ uptake on acidification is a consequence of decreased ΔΨm. These findings are in agreement with previous reports where acidosis was shown to reduce Ca2+ accumulation (13) via the ruthenium red-sensitive uniporter (11).

In addition to effects on Ca2+ uptake, mitochondrial acidification has also been shown to affect Ca2+ release via irreversible opening of the MPT. In nonenergized mitochondria, an acidic pHm has been shown to reduce the open probability of the MPT (16) via inhibitory effects of matrix protons on this transporter (3). However, Kristian et al. (24) have shown that acidification promotes MPT opening in energized mitochondria via alterations in mitochondrial phosphate transport. This effect may be relevant under reperfusion conditions when mitochondria are energized under conditions of high cytosolic Ca2+.

Protective effects of NHEm inhibition. The decreased pHm observed with EIPA during respiratory inhibition, coupled with the observation that increased mitochondrial acidification can limit Ca2+ uptake, suggests that inhibition of NHEm may be one mechanism to limit Ca2+m during I/R. This mechanism may also partially account for the protective effects of NHEm blockers observed in several recent studies, where selective inhibition of the NHEm limited the increases in [Ca2+]m and resulted in improvement of cardiac function during I/R (18), (31). Separation of a direct effect of EIPA on Ca2+m loading from that resulting from limitation of cytosolic Ca2+ secondary to plasmalemmal NHE inhibition has not yet been achieved.

Limitations. In the absence of a specific inhibitor of the NHEm, 100 μmol/l EIPA was used as a NHEm inhibitor in our studies. The selective NHE-1 inhibitor cariporide was not an effective inhibitor of the NHEm in our permeabilized rat myocytes, consistent with data that the NHEm may be a different subtype than the sarcolemmal NHE-1 (26) and exhibit a pharmacological profile distinct from the NHE-1 (4, 22). EIPA (100 μmol/l) has been shown to inhibit the NHEm by 50% in previous studies (4). Preliminary studies indicated that lower concentrations of EIPA (10 μmol/l), while effective in inhibiting NHE-1, did not significantly inhibit NHEm. In addition to having known inhibitory effects on the NHE, EIPA has also been shown to be an inhibitor of mitochondrial Na+/Ca2+ exchanger (21). However, inhibition of the mitochondrial Na+/Ca2+ exchanger by EIPA would not be relevant in our study in permeabilized myocytes because the Na2+ was added to the intracellular medium and 0.5 mmol/l EGTA was included to chelate any adventitious Ca2+. EIPA has also been reported to have protonophoric effects in bilayers (9), an effect that could lead to mitochondrial acidification. However, no effect of EIPA on pHm was observed in KCl medium, arguing against a significant role for protonophoric effects in our study.

Our results indicate a key role for mitochondrial acidification in limiting mitochondrial Ca2+ and suggest that recovery of pH may allow for greater Ca2+ overload. However, extrapolation of these results to cardiac I/R may be limited because our experiments involving pH changes in isolated mitochondria under fixed ionic conditions may not accurately model the more complex changes that occur during I/R in the whole heart.
In summary, our studies in rat cardiac mitochondria suggest that increased mitochondrial acidification under simulated ischemic conditions can result in decreased uptake of $\text{Ca}^{2+}/\text{H}^+$ during ischemia and/or reperfusion and that strategies that could enhance the magnitude of mitochondrial acidification during ischemia and/or reperfusion could potentially limit $\text{Ca}^{2+}/\text{H}^+$ loading and enable functional recovery of mitochondria on reperfusion.

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REFERENCES