Mitochondrial Ca\(^{2+}\) uptake during simulated ischemia does not affect permeability transition pore opening upon simulated reperfusion☆

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Abstract

Objective: Reenergization of ischemic cardiomyocytes may be associated with acute necrotic cell death due in part to cytosolic Ca\(^{2+}\) overload and opening of a permeability transition pore (PTP) in mitochondria. It has been suggested that Ca\(^{2+}\) overload during ischemia primes mitochondria for PTP opening during reperfusion. We investigated the ability of mitochondria to uptake Ca\(^{2+}\) during simulated ischemia (SI) and whether this uptake determines PTP opening and cell death upon simulated reperfusion (SR).

Methods: Rat heart mitochondria were submitted to either hypoxia (anoxic chamber) or to SI (respiratory inhibition, substrate depletion and acidosis) and subsequent SR. Mitochondrial Ca\(^{2+}\) uptake was monitored using Ca\(^{2+}\) microelectrodes after exposure to different [Ca\(^{2+}\)]\(_{\text{u}}\) to 25 μM during SI, and PTP opening was assessed by quantification of mitochondrial swelling (changes in absorbance rate at 540 nm) and calcine release. Mitochondrial Ca\(^{2+}\) uptake (Rhod-2 fluorescence) and cytosolic Ca\(^{2+}\) rise (Fura-2 ratio fluorescence) were further investigated in HL-1 cardiac myocytes submitted to SI/SR, and the effect of reducing mitochondrial Ca\(^{2+}\) load (with 25 μM ruthenium red) or blocking PTP opening (with 0.5 μM cyclosporin A) on the rate of cell death was investigated in adult cardiomyocytes exposed to SI/SR.

Results: SI induced a progressive dissipation of mitochondrial membrane potential (TMRE fluorescence); however, prior to the completion of depolarization, high levels of Ca\(^{2+}\) uptake were observed in mitochondria. SR induced PTP opening but this phenomenon was not influenced by the magnitude of mitochondrial Ca\(^{2+}\) uptake during previous SI. Blockade of the mitochondrial Ca\(^{2+}\) uniporter during SI in cardiomyocytes attenuated mitochondrial Ca\(^{2+}\) uptake but increased cytosolic Ca\(^{2+}\) overload and cell death upon subsequent SR.

Conclusion: Mitochondrial Ca\(^{2+}\) uptake during SI buffers cytosolic Ca\(^{2+}\) overload but its magnitude appears not to be an important determinant of PTP opening upon subsequent SR.

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1. Introduction

Reperfusion of ischemic myocardium may be associated with cardiomyocyte cell death occurring during the initial minutes of restoration of blood flow. Experimental studies have shown that this type of cell death is to a variable extent preventable by interventions applied at the time of reperfusion, indicating that reperfusion itself plays a role in its genesis [1]. The mechanism of reperfusion-induced necrotic cell death is not completely understood, but it is well established that altered cytosolic Ca\(^{2+}\) handling during ischemia may induce structural fragility and excessive contractile activation upon reenergization, ultimately leading to necrotic cell death [2,3]. In recent years, evidence has accumulated indicating that a sudden change in the permeability of the mitochondrial membranes by opening of a high conductance pore (permeability transition pore (PTP) opening) may be another important mechanism for

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reperfusion-induced cell necrotic death [4–8]. Although PTP opening is strongly inhibited by acidosis during ischemia, it is favored by ATP depletion, oxidative stress and high intramitochondrial Ca\(^{2+}\) concentrations, conditions all concurrent during myocardial reperfusion [9].

Among the potential mechanisms responsible for PTP opening during reperfusion, mitochondrial Ca\(^{2+}\) overload has received particular attention. Exposure of normoxic or reperfused mitochondria to concentrations of Ca\(^{2+}\) in the high micromolar range (100–500 μM) consistently results in massive Ca\(^{2+}\) uptake that is eventually followed by PTP opening [10,11]. The total amount of Ca\(^{2+}\) incorporated by mitochondria tends to be reproducible for a given experimental condition and methodology [11], and interventions that mediate a protective effect against reperfusion-induced cell death increase the tolerance of mitochondria to cumulative Ca\(^{2+}\) overload induced by high Ca\(^{2+}\) concentrations [10]. These observations have contributed to the acceptance of a notion that mitochondrial Ca\(^{2+}\) uptake occurring during reperfusion plays a decisive role in PTP opening and cell death. This view assumes that mitochondrial Ca\(^{2+}\) overload occurring during ischemia should bring mitochondria closer to the threshold at which PTP opening takes place, and favor this way the occurrence of PTP opening during reperfusion, a phenomenon described as mitochondrial priming [12]. Additionally, reduced mitochondrial Ca\(^{2+}\) overload during ischemia has been pointed out as a potentially important mechanism of ischemic and pharmacological preconditioning [13]. However, reduced mitochondrial Ca\(^{2+}\) overload could actually be a consequence of a more preserved Ca\(^{2+}\) handling by the sarcoplasmic reticulum in preconditioned myocytes [14] rather than a cause of protection. Moreover, the notion that mitochondrial Ca\(^{2+}\) overload during ischemia plays an important role in reperfusion injury has been challenged by studies proposing that mitochondrial membrane depolarization during ischemia precludes mitochondrial Ca\(^{2+}\) uptake, because Ca\(^{2+}\) influx through the uniporter is driven by the mitochondrial membrane potential (ΔΨ\(_{m}\)) [15]. Definitive experimental evidence demonstrating the contribution of mitochondrial Ca\(^{2+}\) overload occurring during ischemia to PTP opening upon subsequent reperfusion has not been provided.

The purpose of this study was to evaluate the effect of simulated ischemia on Ca\(^{2+}\) uptake by mitochondria exposed to an extramitochondrial [Ca\(^{2+}\)] in the low micromolar range, proven to be incapable of inducing PTP opening in normoxia, and to analyze the consequences of the resulting mitochondrial Ca\(^{2+}\) overload on PTP opening susceptibility occurring upon subsequent reenergization.

2. Methods

This study was performed in isolated rat heart mitochondria, in freshly isolated adult rat cardiomyocytes and in HL-1 cardiac myocytes. The procedure for obtaining cardiac tissue conformed with the Guide for the Care and Use of Laboratory Animals published by the United States National Institute of Health (NIH Publication No. 85–23, revised 1996), and was approved by the Research Commission on Ethics of the Hospital Vall d’Hebron.

2.1. Simulated ischemia and reperfusion in rat heart mitochondria

Rat heart mitochondria were isolated by differential centrifugation [16] and additionally purified by a 20% Percoll gradient. Protein concentration of the mitochondrial suspension was adjusted to 1 mg/ml. Mitochondria were submitted to simulated ischemia (SI) by 60 min incubation in a medium containing (in mM): KCl 150, NaCl 7, HEPES 6, KH\(_2\)PO\(_4\) 2, MgCl\(_2\) 1, sucrose 50, succinate 0.6, ATP 0.1, ADP 0.02, NaCN 2, and a variable concentration of Ca\(^{2+}\) ([Ca\(^{2+}\)] of 0, 15 and 25 μM), at pH 6.4. To achieve Ca\(^{2+}\) free conditions, 1 mM EGTA was added to the medium. Reperfusion (SR) was simulated by removing NaCN, normalizing pH to 7.2 and increasing succinate to 6 mM, ATP to 2.5 mM and ADP to 200 μM. External Ca\(^{2+}\) during SR was maintained at 25 μM. A subset of experiments was performed using an anoxic chamber (Oxygraph Hansatech Instruments, UK) in which O\(_2\) was consumed by mitochondria in a substrate-dependent manner, external pH was maintained at 6.4 to simulate ischemic conditions. Oxygen concentration within the chamber was 300 nmol/ml at the beginning of each experiment, reached 0 nmol/ml after 8–9 min (calibration with sodium hydrosulfite) and recovered to 300 nmol/ml upon SR.

2.1.1. Measurement of mitochondrial Ca\(^{2+}\) uptake

Extramitochondrial free Ca\(^{2+}\) was measured at 25 °C using a Ca\(^{2+}\)-sensitive microelectrode (Oxygraph Hansatech Instruments, UK). Changes in voltage values (mV) were converted into ΔCa\(^{2+}\) using previously calibrated external Ca\(^{2+}\) references.

2.1.2. PTP opening

Opening of PTP was monitored by analyzing both mitochondrial swelling and calcein release. Mitochondrial swelling was assayed spectrophotometrically at 25 °C as a decrease in light absorbance at 540 nm during SI/SR and normoxia under different Ca\(^{2+}\) stimuli, and was expressed as a percentage of swelling with respect to the maximum swelling achieved by exposure to an external Ca\(^{2+}\) concentration of 1 mM. PTP opening was identified as a cyclosporin A (CsA)-sensitive decrease in light absorbance. Calcein release was monitored fluorometrically as changes in fluorescence (Ex:485 nm/Emission:538 nm) of the supernatant of mitochondria previously loaded with 1 μM calcein-AM and submitted to SI/SR and normoxia under different Ca\(^{2+}\) stimuli. In a subset of experiments, total calcein fluorescence was quantified after mitochondrial permeabilization with 0.15% deoxycholate acid sodium salt. PTP opening was...
identified as a CsA-sensitive increase in the fluorescence of the supernatant with respect to control cells.

2.2. Simulated ischemia/reperfusion in cardiac myocytes

HL-1 cardiac myocytes or freshly isolated adult cardiomyocytes obtained as previously described [2] were plated in glass bottom culture dishes and submitted to 45–60 min of SI at 37 °C by incubation with a glucose-free buffer containing (in mM): NaCN 2, 2-DOG 20, NaCl 140, KCl 3.6, MgSO4 1.2, CaCl2 2, HEPES 20, at pH 6.4, and further reoxygenated in a control buffer at pH 7.4 containing (in mM): NaCl 140, KCl 3.6, MgSO4 1.2, CaCl2 2, HEPES 20 and glucose 5 mM.

2.2.1. \( \Delta \psi_m \) during simulated ischemia

Changes in \( \Delta \psi_m \) were measured in intact HL-1 cells loaded for 10 min with 10 nM TMRE, washed and subjected to SI/SR. To investigate whether the inverse operation of the FoF1 ATPase may contribute to maintain \( \Delta \psi_m \), 10 \( \mu M \) oligomycin was present during SI. Mitochondrial membrane potential was recorded every 10 s from a confocal Ar/Kr laser system (Yokogawa CSU10, Nipkow spinning disk) set on an Olympus IX70 (VoxCell Scan, Visitech, UK) and depolarization was detected as a decrease in 590 nm emission from samples excited at 560 nm. Results in fluorescent intensity were expressed as a percentage of change with respect to the value obtained in normoxic polarized mitochondria.

2.2.2. Intracellular pH, and cytosolic and mitochondrial [Ca\(^{2+}\)]

For intracellular ionic measurements, experiments were performed in intact HL-1 cells on the stage of an inverted microscope (Olympus IX70, Japan) either at 40× or 60× immersion oil objective, 1.4 NA (Olympus, Japan). Changes in cytosolic [H\(^+\)] and [Ca\(^{2+}\)] during SI/SR were monitored by ratio fluorescence imaging in cells loaded with 3 \( \mu M \) BCECF or 5 \( \mu M \) Fura-2, respectively (Molecular Probes, USA), as previously described [2,17]. To measure
mitochondrial [Ca\(^{2+}\)], a confocal Ar/Kr laser system (Yokogawa CSU10, Nipkow spinning disk) set on an Olympus IX70 was used. Cells loaded with 4 \(\mu\)M Rhod-2 were excited at 560 nm and changes in single light emission (590 nm) from previously identified as mitochondrial and cytosolic areas were independently monitored throughout time using a commercially available software (VoxCell Scan, Visitech, UK). Results in fluorescent intensity were expressed as a percentage of change with respect to the initial value. To analyze the role of mitochondrial Ca\(^{2+}\) uptake on cytosolic [Ca\(^{2+}\)] and on cell death, the blocker of the mitochondrial Ca\(^{2+}\) uniporter ruthenium red (RuR, 25 \(\mu\)M) was added either during SI or during SR.

2.2.3. Cell death

Lactate dehydrogenase (LDH) release was measured in adult cardiomyocytes. LDH release (expressed as % of enzyme release with respect to the total cell content) was assayed spectrophotometrically (SLT Spectra Vision, Austria) at 340 nm.

2.3. Statistical analysis

Comparisons involving several groups were performed by one-way ANOVA followed, in case of rejection of the homogeneity hypothesis, by post-hoc LSD test. Data are expressed as mean±SEM. Significance level was set at 0.05.

3. Results

3.1. Ca\(^{2+}\) uptake during simulated ischemia in isolated mitochondria

Exposure of mitochondria to 15 \(\mu\)M Ca\(^{2+}\) (Fig. 1, panels A1 and A2) or 25 \(\mu\)M Ca\(^{2+}\) (Fig. 1, panels B1 and B2) induced significant mitochondrial Ca\(^{2+}\) uptake under hypoxic conditions, resulting in total removal of extramitochondrial Ca\(^{2+}\) within the first 5 min. Mitochondrial Ca\(^{2+}\) uptake under normoxia is shown as reference in Fig. A (on-line supplementary material). Under prolonged hypoxia with high extramitochondrial Ca\(^{2+}\) (25 \(\mu\)M) there was a late extrusion of part of the accumulated Ca\(^{2+}\) when pH was 7.2 (Fig. 1, panel B2). However, although Ca\(^{2+}\) was retained under pH 6.4, acidosis resulted in an attenuation of mitochondrial Ca\(^{2+}\) load, both under normoxia and hypoxia (Fig. 2A). Net mitochondrial Ca\(^{2+}\) uptake at the end of 1 h Hx and 1 h Nx under different external Ca\(^{2+}\) concentrations is shown in Fig. 2B.

3.2. Influence of mitochondrial Ca\(^{2+}\) uptake during simulated ischemia on PTP opening upon reperfusion

Normoxic mitochondria did not swell when exposed to Ca\(^{2+}\) concentrations of 15 \(\mu\)M or 25 \(\mu\)M with respect to Ca\(^{2+}\) free conditions. However, the same degree of Ca\(^{2+}\) overload during SI induced significant swelling upon SR. SR induced mitochondrial swelling estimated to be around 40% of the maximal swelling achieved by exposure to 1 mM Ca\(^{2+}\) for all Ca\(^{2+}\) concentrations tested during SI (Fig. 3A). Mitochondrial swelling during SR was prevented by CsA and was not related to the magnitude of Ca\(^{2+}\) overload during the previous SI period, since it did not differ between mitochondria exposed to higher [Ca\(^{2+}\)] during previous SI (25 \(\mu\)M) as compared to mitochondria exposed to lower [Ca\(^{2+}\)] (15 \(\mu\)M) or to Ca\(^{2+}\) free conditions (Fig. 3A and B). Analysis of calcein release also failed to detect any significant influence of increasing Ca\(^{2+}\) overload during SI up to 25 \(\mu\)M on PTP opening upon subsequent SR (Fig. 3C). These results suggest that Ca\(^{2+}\) uptake occurring during SI in the presence of external [Ca\(^{2+}\)] up to 25 \(\mu\)M may not influence PTP opening induced by subsequent SR.
3.3. Effect of inhibition of mitochondrial Ca\(^{2+}\) uptake during simulated ischemia on cell viability

In HL-1 cardiac myocytes, exposure to SI resulted in a progressive but incomplete fall in ΔΨ\(_m\) during the first 20 min of SI. Addition of oligomycin during SI accelerated the rate of mitochondrial depolarization, suggesting that the inverse operation of the FoF1 ATPase contributed to its preservation (Fig. 4A). During the initial 15 min of SR there was a partial recovery of ΔΨ\(_m\), which was significantly improved in the presence of CsA (Fig. 4B). SI resulted in a rapid decrease in intracellular pH that reached its minimum value in 15 min (Fig. 4C). During SR, there was a rapid and almost complete normalization of intracellular pH within the first 15 min (Fig. 4C).

During SI, HL-1 cardiac myocytes showed a marked increase in mitochondrial [Ca\(^{2+}\)] that was significantly attenuated by 25 μM RuR (Fig. 5). Addition of RuR was also accompanied by an increase in cytosolic/nuclei fluorescent signal of Rhod-2 and by a reduction in the fluorescence from areas defined as mitochondria at baseline (Fig. 5B). Mitochondrial depolarization was not significantly modified by the addition of RuR during SI (data not shown). However, inhibition of mitochondrial Ca\(^{2+}\) uptake during SI with RuR was associated with an accelerated rate of cytosolic Ca\(^{2+}\) rise and higher cytosolic Ca\(^{2+}\) levels upon SR (Fig. 6), and with a significant increase in the amount of acute cell death during SR (Fig. 7). PTP opening appeared to contribute to some extent to the increase in cell death associated to RuR, since CsA had a protective although transient effect against it. The importance of the timing of application of RuR was studied in a subset of experiments in which the effect of application of 25 μM RuR during SI (n=4) was compared with its effect when applied during SR (n=3). Cell death was increased by RuR when applied during SI (48.3±3.5 vs. 35.6±1.2 in controls, p<0.05), but was reduced when the drug was applied during SR (28.0±4.1, p<0.05).

4. Discussion

The present study investigated mitochondrial Ca\(^{2+}\) uptake during SI and its effect on PTP opening and cell death occurring upon subsequent SR. The results show that both hypoxia and acidosis attenuate mitochondrial Ca\(^{2+}\) uptake in response to elevated external [Ca\(^{2+}\)] but do not abolish it. Increasing mitochondrial Ca\(^{2+}\) uptake during SI by rising
external \([Ca^{2+}]\) up to 25 \(\mu M\) did not result in higher susceptibility of PTP opening upon subsequent SR. Moreover, inhibition of \(Ca^{2+}\) uptake during SI enhanced cytosolic \(Ca^{2+}\) overload and had a detrimental effect on the tolerance of the cells to subsequent reenergization. Altogether, these results do not support the hypothesis that the magnitude of mitochondrial \(Ca^{2+}\) overload induced by ischemia is an important determinant for PTP opening upon subsequent reperfusion.

4.1. Mitochondrial \(Ca^{2+}\) uptake during ischemia

Under normoxic conditions, mitochondria exhibit a striking ability to accumulate enormous amounts of \(Ca^{2+}\) [18]. However, the occurrence, magnitude and relevance of mitochondrial \(Ca^{2+}\) uptake during ischemia have been the object of debate. While some studies have described that important mitochondrial accumulation of \(Ca^{2+}\) may take place during ischemia [19,20], other studies have described only small increases in mitochondrial \([Ca^{2+}]\) in ischemic cardiomyocytes, always below the cytosolic level [21]. It has also been argued that mitochondria are simply not able to uptake \(Ca^{2+}\) during ischemia due to the loss of \(\Delta\Psi_m\), the driving force for \(Ca^{2+}\) uptake. Our study shows that mitochondria may accumulate \(Ca^{2+}\) during SI. The significant \(Ca^{2+}\) uptake observed under ischemic conditions in the present study is compatible with a relatively modest increase in free mitochondrial \([Ca^{2+}]\) due to the formation of osmotically inactive \(Ca^{2+}\) phosphates complexes within the matrix [11]. Mitochondrial \(Ca^{2+}\) uptake was reduced by RuR, which indicates that it occurs mainly through the \(Ca^{2+}\) uniporter. This mechanism of \(Ca^{2+}\) influx should be expected to be operative in mitochondria not fully depolarized. Conversely, acceleration of mitochondrial depolarization by addition of 10 \(\mu M\) of oligomycin prevented mitochondrial \(Ca^{2+}\) uptake during SI (Fig. B, on-line supplementary material). Previous studies have demonstrated that \(\Delta\Psi_m\) may be preserved during respiratory inhibition through the reverse operation of FoF1 ATP synthase that hydrolyzes ATP and extrudes \(H^+\) from the matrix [22]. In the present study, addition of 10 \(\mu M\) oligomycin accelerated mitochondrial membrane depolarization during SI, supporting the observation that part of the \(\Delta\Psi_m\) is maintained by the reversion of the FoF1 ATPsynthase. It is important to note that the rate of ATP depletion observed in these cells (a drop of about 90% after 15 min of SI, data not shown) is similar to that observed in intact myocardium during normothermic ischemia [23].

The present study demonstrated an attenuation of mitochondrial \(Ca^{2+}\) uptake in acidic conditions as described in a previous study [24]. However, unlike isolated mitochondria, intact cells exposed to SI take several minutes to develop severe intracellular acidosis, following a time-course similar to what has been described in ischemic cardiomyocytes in situ [23]. Thus, incomplete mitochondrial membrane depolarization and moderate acidosis during the decisive initial minutes of ischemia are compatible with net mitochondrial \(Ca^{2+}\) uptake.

4.2. Mitochondrial \(Ca^{2+}\) overload during ischemia and PTP opening

In the present study, modulating mitochondrial \(Ca^{2+}\) accumulation during SI by modifying extramitochondrial \([Ca^{2+}]\) between 0 and 25 \(\mu M\) did not affect PTP opening upon subsequent SR. The extramitochondrial \([Ca^{2+}]\) used during SI (15 \(\mu M\) and 25 \(\mu M\)) was higher than those reached
in the cytosol of ischemic cardiomyocytes, which has been estimated to be less than 10 μM [20], and although SI significantly reduced Ca²⁺ uptake it still induced a significant mitochondrial accumulation of Ca²⁺. This finding argues against a critical role for ischemic Ca²⁺ overload in PTP opening during reperfusion. This determinant role has been suggested by experiments in which progressive Ca²⁺ overload induced by sequential application of pulses of high extracellular [Ca²⁺] induces PTP opening when a certain mitochondrial Ca²⁺ threshold is reached [10,11]. However, the magnitude of Ca²⁺ overload required to induce PTP opening in those experiments is primarily dependent on the experimental conditions (i.e. the presence of adenine nucleotides in the medium [11]), and isolated mitochondria are exposed to extremely high [Ca²⁺], an approach that may be useful to evaluate PTP opening susceptibility in vitro, but that is unlikely to reflect the conditions experienced by mitochondria during ischemia/reperfusion [25]. Modulation of PTP opening during SR by extracellular Ca²⁺ was also readily observable in our model (Fig. C, on-line supplementary material).

The use of molecularly engineered protein-based indicators targeted to different cell compartments has unequivocally established that [Ca²⁺] can dramatically rise in the mitochondria exposed to high [Ca²⁺] microdomains, as those resulting from the anatomical interaction between sarcoplasmic reticulum and mitochondria. The Ca²⁺ sensing function of mitochondria together with their ability to accumulate large [Ca²⁺] loads could partially explain the inability of Ca²⁺ to induce PTP opening. Although several studies have described that preconditioning and other protective interventions are associated with an increased tolerance to Ca²⁺
overload in mitochondria obtained from reperfused myocardium [7,10], this does not necessarily mean that mitochondrial Ca\(^{2+}\) overload is the main trigger of PTP opening during reperfusion. Indeed, some of these interventions increase the in vitro resistance of mitochondria to PTP opening induced by other triggers as oxygen free radicals [26].

The absence of a direct relationship between mitochondrial Ca\(^{2+}\) load and PTP opening had been previously suggested by studies in which both low and high doses of the PTP blocker CsA demonstrated a beneficial effect against SR injury despite having opposing effects on intramitochondrial free Ca\(^{2+}\) [27]. A direct evidence about the correlation between Ca\(^{2+}\) and PTP susceptibility has been provided by the study from Juhaszova et al. [28] in which cardiac myocytes with a pharmacologically disabled sarcoplasmic reticulum were submitted to a controlled Ca\(^{2+}\) overload by an electrical stimulation. ROS-induced PTP threshold was identical to unstimulated cells, although removal of cytoplasm by skinning the cells significantly increased PTP susceptibility to high Ca\(^{2+}\).

4.3. Mitochondrial Ca\(^{2+}\) uptake during simulated ischemia, cytosolic Ca\(^{2+}\) overload and cell death

Previous studies have suggested the importance of mitochondrial Ca\(^{2+}\) uptake in shaping the spatio-temporal pattern of cytosolic Ca\(^{2+}\) rise [18]. This buffering role of mitochondria should be particularly relevant under pathophysiological conditions, e.g. energy deprivation, in which cytosolic Ca\(^{2+}\) homeostasis is disrupted at early time points due to an increase in intracellular Na\(^+\) and the reverse activity of the sarcolemmal Na\(^+\)/Ca\(^{2+}\) exchanger [29]. Under these conditions, Ca\(^{2+}\) uptake by functionally active mitochondria may have indirect beneficial effects by reducing the detrimental consequences of cytosolic Ca\(^{2+}\) overload, specially in muscle cells, in which mitochondria largely contribute to total cell volume. In the present study, inhibition of the mitochondrial Ca\(^{2+}\) uniporter prevented Ca\(^{2+}\) uptake in isolated mitochondria and markedly attenuated the increase in mitochondrial [Ca\(^{2+}\)] in HL-1 cardiomyocytes. This effect was mirrored by an increase in the rate of cytosolic Ca\(^{2+}\) rise, and was associated with increased cell death at the time of SR. The deleterious effect of cytosolic Ca\(^{2+}\) rise during energy deprivation on the rate of cardiomyocyte death upon subsequent reenergization has been extensively documented and related to contractile activation [1,30] and calpain-mediated degradation of membrane cytoskeleton [31]. A recent study has shown that additional Ca\(^{2+}\) influx at the time of reperfusion may not be necessary for induction of hypercontracture [32].

The effect of inhibition of the mitochondrial Ca\(^{2+}\) uniporter on cell death was investigated in freshly isolated rat cardiomyocytes. These cells exhibit full Ca\(^{2+}\)-dependent hypercontracture upon reenergization therefore allowing a better analysis of the role of mitochondria on cytosolic Ca\(^{2+}\) buffering as compared to HL-1 cells. These mouse atrial-derived cells have phenotypic features very similar to those of adult cardiomyocytes [33], and show similar changes in cytosolic ionic composition, including Ca\(^{2+}\) kinetics in response to simulated ischemia, although at a slower rate, probably due in part to reduced contractile activity and more important glycolytic metabolism [34]. However, they show only minimal changes in cell geometry during rigor or hypercontracture, allowing a more reliable quantification of non-ratiometric probes as Rhod-2 and TMRE.
The effect of RuR on cell death was strikingly dependent on the time of its administration. When applied during SI it had a deleterious effect, contrasting with the previously described protective effect when applied at the time of reperfusion [19,35]. This apparent discrepancy could be explained by the fact that during reenergization sarcoplasmic reticulum is a major component of cytosolic Ca\(^{2+}\) homeostasis [1], making inhibition of mitochondrial Ca\(^{2+}\) uptake less determinant for cytosolic Ca\(^{2+}\) control. Additionally, RuR may have a beneficial effect during reperfusion by preventing PTP opening through inhibition of VDAC [36], a molecule recognized to be involved in the mitochondrial permeability multiprotein complex. During SI this mechanism is irrelevant, because acidosis maintains the pore in its closed state.

4.4. Implications

The present results demonstrate ischemic uptake of Ca\(^{2+}\) by mitochondria. Mitochondrial Ca\(^{2+}\) uptake may reduce reperfusion-induced cell death in that it attenuates cytosolic Ca\(^{2+}\) rise during ischemia, and does not increase PTP opening upon subsequent reperfusion. According to these results, the beneficial effect of interventions like mitochondrial K\(_{ATP}\) channel agonists and ischemic preconditioning against reperfusion-induced cell death are not necessarily explained by their effects on mitochondrial Ca\(^{2+}\) overload during ischemia.

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Appendix B. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.cardiores.2006.06.019.

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