Regulation of the mitochondrial permeability transition by matrix Ca$^{2+}$ and voltage during anoxia/reoxygenation

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Korge, Paaavo, Henry M. Honda, and James N. Weiss. Regulation of the mitochondrial permeability transition by matrix Ca$^{2+}$ and voltage during anoxia/reoxygenation. Am J Physiol Cell Physiol 280: C517–C526, 2001.—We studied the interplay between matrix Ca$^{2+}$ concentration ([Ca$^{2+}]_m$) and mitochondrial membrane potential ($\Delta \psi$) in regulation of the mitochondrial permeability transition (MPT) during anoxia and reoxygenation. Without Ca$^{2+}$ loading, anoxia caused near-synchronous $\Delta \psi$ dissipation, mitochondrial Ca$^{2+}$ efflux, and matrix volume shrinkage when a critically low $P_{O_2}$ was reached, which was rapidly reversible upon reoxygenation. These changes were related to electron transport inhibition, not MPT. Cyclosporin A-sensitive MPT did occur when extramitochondrial [Ca$^{2+}$] was increased to promote significant Ca$^{2+}$ uptake during anoxia, depending on the Ca$^{2+}$ load size and ability to maintain $\Delta \psi$. However, when [Ca$^{2+}$] was increased after complete $\Delta \psi$ dissipation, MPT did not occur until reoxygenation, at which time reactivation of electron transport led to partial $\Delta \psi$ regeneration. In the setting of elevated extramitochondrial Ca$^{2+}$, this enhanced matrix Ca$^{2+}$ uptake while promoting MPT because of less than full recovery of $\Delta \psi$. The interplay between $\Delta \psi$ and matrix [Ca$^{2+}$] in accelerating or inhibiting MPT during anoxia/reoxygenation has implications for preventing reoxygenation injury associated with MPT.

cardiomyocytes; mitochondrial Ca$^{2+}$ uptake; Ca$^{2+}$ efflux; permeability transition pore

MYOCARDIAL VIABILITY AFTER reperfusion/reoxygenation is critically dependent on the recovery of mitochondrial membrane potential ($\Delta \psi$) to provide the driving force for oxidative phosphorylation. A major factor that could limit recovery of $\Delta \psi$ on reperfusion/reoxygenation is Ca$^{2+}$-dependent opening of large nonselective permeability transition pores (PTP) in the inner mitochondrial membrane, an event called mitochondrial permeability transition (MPT) (6, 19). In addition to triggering apoptotic signaling, MPT occurring near synchronously in the majority of mitochondria in a cell leaves ATP production capability inadequate and facilitates necrotic cell death. Preventing MPT during reperfusion/reoxygenation has obvious therapeutic promise, but developing effective strategies will require better understanding of its regulation under these conditions. For example, cyclosporin A (CSA) is the most potent inhibitor of MPT known, and concentrations <400 nM partially prevented reperfusion/reoxygenation injury. Higher concentrations, however, exacerbated damage (15, 30). As emphasized by Bernardi (2), CSA’s ability to block PTP opening is variable because it is not a completely specific inhibitor and its effect can be antagonized by increased matrix Ca$^{2+}$ concentration ([Ca$^{2+}]_m$), oxidative stress, and other factors known to occur during reperfusion/reoxygenation. Therefore, additional strategies to enhance the efficacy of direct PTP blockers would be highly desirable.

PTP open-closed transitions underlying MPT are highly regulated (2). Matrix Ca$^{2+}$ is the most important regulatory factor, but there are multiple modulators, including $\Delta \psi$ (2). Bernardi et al. (5) have shown that following accumulation of a small Ca$^{2+}$ load by respiring mitochondria, which by itself was insufficient to induce MPT, $\Delta \psi$ dissipation by the protonophore carbonyl cyanide $p$-(trifluoromethoxy)phenylhydrazone (FCCP) rapidly promoted PTP opening. PTP thus behaved as a voltage-dependent channel with higher open probability at depolarized membrane potentials. The idea of a voltage sensor regulating PTP opening is attractive because it could provide a common mechanism to account for the actions of a variety of PTP blockers: activators may shift the threshold voltage to more negative $\Delta \psi$ and cause PTP opening at values close to physiological $\Delta \psi$, with inhibitors doing the converse (3).

However, mitochondrial Ca$^{2+}$ uptake is also regulated by $\Delta \psi$, which provides the driving force for Ca$^{2+}$ uptake into the matrix through the Ca$^{2+}$ uniporter. Well-maintained $\Delta \psi$ promotes Ca$^{2+}$ uptake when cytoplasmic free [Ca$^{2+}$] increases, as occurs during ischemia and hypoxia (6). Therefore, during ischemia/hypoxia, maintenance of $\Delta \psi$ is predicted to have dual effects: it should promote PTP opening by increasing mitochondrial Ca$^{2+}$ uptake into the matrix, but inhibit PTP opening by the voltage-dependent mechanism. Conversely, although $\Delta \psi$ dissipation during ischemia/hypoxia protects the mitochondrial matrix from further Ca$^{2+}$ accumulation, its sudden dissipation...
pation during hypoxia/ischemia may promote MPT if mitochondria are already Ca\textsuperscript{2+} loaded. In this study, we investigated the interplay between these opposing effects of $\Delta \psi$ by examining the effects of anoxia/reoxygenation on PTP opening in suspensions of isolated cardiac mitochondria subject to varying Ca\textsuperscript{2+} loads as well as in permeabilized and intact myocytes.

**MATERIALS AND METHODS**

*Isolation of mitochondria.* Mitochondria were isolated from adult rabbit hearts by enzymatic digestion of finely minced tissue with the bacterial protease nagarse (0.5 mg/ml) for 10 min on ice in homogenization buffer (250 mM sucrose, 1 mM EGTA, and 10 mM MOPS, pH 7.4 with Tris) followed by differential centrifugation, as described previously (25). Mitochondria were resuspended in the EGTA-free homogenization buffer to give about 40 mg mitochondrial protein per milliliter, kept on ice, and used within 6 h after isolation. Respiratory control ratio was regularly determined in mitochondrial incubation buffer [120 mM KCl, 10 mM HEPES (pH 7.2), and 2 mM potassium phosphate] after consecutive addition of 2.5 mM pyruvate, malate, and glutamate and 0.5 mM ADP. Only mitochondrial preparations with ratio >5 were used in these experiments.

*Isolation and permeabilization of myocytes.* Ventricular myocytes were isolated from adult rabbit hearts by conventional enzymatic methods described previously (14). Myocytes were stored in normal Tyrode solution and used within 5–6 h. In some experiments, the sarcolemma was permeabilized by treating cells with digitonin (20 $\mu$M) in a buffer that contained (in mM) 135 KCl, 1 MgCl\textsubscript{2}, 3 ATP, 0.5 EGTA, and 10 HEPES (pH 7.2) for 10 min. Myocytes were pelleted at 50 g, washed, and resuspended in the same buffer as described (1). After this treatment, myocytes retained a rod-shaped morphology, with intact and functional sarcoplasmic reticulum, contractile elements, and mitochondria (1).

*Experimental conditions for anoxia/reoxygenation.* All experiments were carried out with a spectrofluorometer (Ocean Optics) in a closed cuvette at room temperature (22–24°C). Mitochondria or isolated cells were made anoxic by injecting a stream of nitrogen through the hole in the cuvette cover against the surface of the buffer (2 ml) so that the stirred buffer had no contact with the air. Reoxygenation was accomplished by substituting nitrogen with oxygen (95% O\textsubscript{2}–5% CO\textsubscript{2}) in the stream. A fiber-optic oxygen sensor was inserted through the same hole, and partial pressure of oxygen (PO\textsubscript{2}) in the buffer was continuously recorded. The rate of PO\textsubscript{2} decrease (the slope of O\textsubscript{2} trace in Fig. 1) was dependent on the flow rate of the nitrogen stream and was set to achieve critically low PO\textsubscript{2} in ~7–8 min, unless indicated otherwise.

*Determination of mitochondrial matrix volume and $\Delta \psi.* Mitochondria (0.5–1.0 mg) were added to 2 ml of mitochondrial incubation buffer that contained 400 nM tetramethylrhodamine methyl ester (TMRM), pH 7.4, and 2.5 mM Ca\textsuperscript{2+} loaded. In this study, changes in mitochondrial matrix volume were estimated by measuring 90° light scattering using the Ocean Optics spectrophotometer with excitation and emission wavelengths set at 520 nm, similar to the method described by Haworth and Hunter (20). Changes in matrix volume are reported as a percentage of maximum (100%) swelling induced by adding 2.5 $\mu$g of alamethicin at the end of the experiment. $\Delta \psi$ was estimated by transmembrane distribution of TMRM. Changes in $\Delta \psi$ are expressed as percentage of the TMRM fluorescence level at 580 nm in the presence of coupled mitochondrial matrix and substrates (0%), relative to the fluorescence after addition of 0.5 mM FCCP to fully depolarize mitochondria (100%).

*Fig. 1. Effects of anoxia on mitochondrial membrane potential ($\Delta \psi$) in a non-Ca\textsuperscript{2+}-loaded mitochondrial suspension.* A: simultaneous recording of buffer PO\textsubscript{2} and mitochondrial $\Delta \psi$. B: determination of mitochondrial $\Delta \psi$ in isolated intact and permeabilized cells. Isolated intact or permeabilized cells were suspended in a buffer that contained (in mM) 120 KCl, 1 Pi, 10 HEPES, and 0.0004 tetramethylrhodamine methyl ester (TMRM), pH 7.4. Mitochondria were energized with 5 mM pyruvate, malate, and glutamate (A), representative tracing from 6 different prepararations; mitochondria were energized with 5 mM succinate (B), representative tracing from 6 different preparations.

*Mitochondrial Ca\textsuperscript{2+} uptake and efflux determination.* Mitochondria (0.5–1 mg/ml) were incubated in the buffer described above that contained 1 $\mu$M Calcium Green-5N (salt form). The suspension was continuously stirred in the fluorometer cuvette, and changes in extramitochondrial [Ca\textsuperscript{2+}]
were followed by recording of Calcium Green fluorescence (excitation/emission, 475/515 nm). Calibration was achieved by adding known amounts of Ca\(^{2+}\) to the buffer as described previously (23), but in the presence of mitochondria and 5 μM ruthenium red to block Ca\(^{2+}\) uptake.

Other assays and chemicals. Mitochondrial protein was determined by the Lowry method. CSA was a generous gift of Ciba-Geigy. Percoll was purchased from Pharmacia and fluorescent dyes from Molecular Probes. All other chemicals were purchased from Sigma. Mitochondrial substrates were free acids adjusted to buffer pH with Tris.

RESULTS

Effects of anoxia/reoxygenation in mitochondria without Ca\(^{2+}\) loading. Figure 1A illustrates oxygen content and mitochondrial Δψ in a mitochondrial suspension as a nitrogen (N\(_2\)) stream was directed against buffer surface in a closed cuvette. Δψ remained constant until PO\(_2\) reached the threshold of electrode sensitivity, at which point rapid and complete depolarization occurred (n = 6 preparations). On replacing N\(_2\) with O\(_2\), electron transport resumed and restored Δψ rapidly as PO\(_2\) increased (n = 6 preparations). If, instead, the N\(_2\) stream was discontinued to let room air reoxygenate the suspension, electron transport resumed and restored Δψ well before the threshold of the oxygen electrode was reached, consistent with the high-affinity binding of oxygen to cytochrome oxidase (Fig. 1B). Under these conditions, the ability of mitochondria to restore Δψ during reoxygenation depended on the presence of substrates. In Fig. 1A, mitochondria were energized with pyruvate, malate, and glutamate, but each of these substrates individually, as well as succinate, were also effective (e.g., Fig. 1B with succinate alone). Importantly, when no extramitochondrial Ca\(^{2+}\) was added, Δψ dissipation during anoxia was always rapid, occurring synchronously in the whole population of mitochondria (n = 6 preparations). Furthermore, reoxygenation always resulted in almost full recovery of Δψ, irrespective of the substrates used or whether a critical level of PO\(_2\) was achieved rapidly or slowly. Figure 2 summarizes Δψ recovery upon reoxygenation in the presence of different substrates; recovery with succinate or pyruvate, malate, and glutamate was close to 100%, but slightly lower in the presence of pyruvate alone.

Δψ dissipation during anoxia was accompanied by slight matrix shrinkage, which reversed with Δψ recovery upon reoxygenation (n = 3 preparations, Fig. 3A, bottom trace). Δψ dissipation during anoxia also resulted in Ca\(^{2+}\) efflux (n = 5 preparations, Fig. 3B). From the initial part of the tracing, it is evident that freshly isolated energized mitochondria accumulated contaminant Ca\(^{2+}\) from the buffer. During anoxia, mitochondria released this Ca\(^{2+}\) (~1 μM per milligram of protein) when Δψ dissipation occurred. Ca\(^{2+}\) efflux was attributed to the Ca\(^{2+}\) uniporter, since upon reoxygenation, mitochondria rapidly reaccumulated this Ca\(^{2+}\) (Fig. 3B), which would not have been possible had PTP opening occurred. Also, blocking Ca\(^{2+}\) transport by the Ca\(^{2+}\) uniporter with 5 μM ruthenium red completely prevented Ca\(^{2+}\) efflux when Δψ dissipation occurred during anoxia (data not shown).

Effects of Ca\(^{2+}\)-loading mitochondria before Δψ dissipation during anoxia. When extramitochondrial Ca\(^{2+}\) was increased by adding small Ca\(^{2+}\) pulses before Δψ dissipation during anoxia, a different response was observed. With pyruvate as the substrate, only a few 5-μM Ca\(^{2+}\) pulses were required to accelerate Δψ dissipation in a subpopulation of mitochondria before PO\(_2\) reached the critical level inhibiting electron transport, whereupon Δψ rapidly dissipated throughout the full population (n = 6 preparations, Figs. 2 and 4A). In contrast to the matrix shrinkage observed in the absence of added Ca\(^{2+}\) (Fig. 3A), matrix swelling consistent with MPT now occurred in parallel with Δψ dissipation (n = 6 preparations, Fig. 4A). Upon reoxygenation, there was partial, transient recovery of Δψ, followed by Δψ dissipation and further matrix swelling. These changes were due to Ca\(^{2+}\)-induced MPT, since they were prevented by 350 mM CSA (n = 3 preparations, Figs. 2 and 4B). With CSA present, Δψ dissipation and matrix volume changes were similar to those in the absence of added extramitochondrial Ca\(^{2+}\) (Fig. 3A), despite a greater number of Ca\(^{2+}\) pulses.
However, other substrates were more protective against Ca$^{2+}$-induced MPT. When pyruvate was added together with malate and glutamate ($n = 2$), or when succinate was used instead of pyruvate ($n = 4$), a larger number of Ca$^{2+}$ pulses failed to initiate MPT during anoxia (Fig. 5A). With larger Ca$^{2+}$ loads ($n = 4$ preparations), however, matrix swelling that indicated MPT (Fig. 5B) occurred when $\Delta\psi$ dissipated abruptly during anoxia, favoring MPT by way of inner membrane depolarization. The increase in extramitochondrial [Ca$^{2+}$] during anoxia decreased $\Delta\psi$ recovery on reoxygenation in succinate-energized mitochondria ($n = 4$ preparations, Fig. 2). Finally, with more gradual onset of $\Delta\psi$ dissipation during partial anoxia, a similar pattern of slow partial $\Delta\psi$ depolarization associated with matrix swelling, as in pyruvate-energized mitochondria, was observed ($n = 3$ preparations, Fig. 5C, compared with Fig. 4A). The superiority of succinate at delaying MPT during hypoxia in the face of increasing extramitochondrial Ca$^{2+}$ is potentially explained by the proposed role of complex I electron flow in the mechanism of PTP opening (10). The mechanism by which the addition of malate and glutamate to pyruvate protected against Ca$^{2+}$-induced MPT during anoxia is less clear, but could be related to prevention of complex I dysfunction, thought to be responsible for diminished $\Delta\psi$ in kidney cells during hypoxia/reoxygenation (32).

Figure 6 shows that compared with their preanoxic state, mitochondria became sensitized to Ca$^{2+}$-induced MPT during reoxygenation. In Fig. 6A, two Ca$^{2+}$ pulses administered during anoxia led to Ca$^{2+}$ efflux when $\Delta\psi$ dissipated, but Ca$^{2+}$ reuptake occurred upon reoxygenation. This net Ca$^{2+}$ accumulation was maintained at least for >5 min without any indication of Ca$^{2+}$ efflux, until FCCP was added to rapidly dissipate $\Delta\psi$ and release the accumulated Ca$^{2+}$. In Fig. 6B, four Ca$^{2+}$ pulses administered before anoxia were also readily accumulated. During anoxia, Ca$^{2+}$ efflux again occurred coincident with $\Delta\psi$ dissipation. Upon reoxygenation, however, Ca$^{2+}$ reuptake was now only transient and turned into Ca$^{2+}$ efflux within a few minutes. This was due to MPT, since addition of CSA terminated Ca$^{2+}$ efflux and allowed the mitochondria to reaccumulate the released Ca$^{2+}$. Similar findings were obtained in two other preparations. Thus a Ca$^{2+}$ load that was well tolerated by fully polarized mitochondria before anoxia was now capable of triggering MPT during reoxygenation. MPT in this setting was most likely precipitated by rapid Ca$^{2+}$ uptake at the start of reoxygenation before $\Delta\psi$ had recovered fully. As shown in Figs. 4A and 5B, $\Delta\psi$ recovery in this situation was only partial, which lowers the threshold for MPT at a given level of matrix Ca$^{2+}$. Figure 7A shows that pretreatment with CSA (350 nM) also prevented the majority of anoxia-induced Ca$^{2+}$ efflux from Ca$^{2+}$-loaded mitochondria. The small component of Ca$^{2+}$ efflux that remained was blocked by ruthenium red ($n = 2$ preparations), indicating that it originated from reversal of the Ca$^{2+}$ uniporter with $\Delta\psi$ dissipation. However, CSA was often unable to restore $\Delta\psi$ to allow Ca$^{2+}$ reuptake (Fig. 7B) and reverse matrix swelling after.

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This page contains a detailed description of mitochondrial permeability transition during anoxia, including the effects of various substrates and interventions on mitochondrial function. The text discusses the role of Ca$^{2+}$ in the mechanism of the mitochondrial permeability transition (MPT) and the effects of different substrates on mitochondrial swelling and Ca$^{2+}$ uptake. The figures illustrate the changes in mitochondrial membrane potential ($\Delta\psi$) and Ca$^{2+}$ concentration during anoxia and reoxygenation. The supplementary figures and diagrams provide a visual representation of the experimental data and the observed phenomena.
anoxia-induced MPT unless the extramitochondrial Ca\(^{2+}\) was removed with EGTA (Fig. 5, B and C). Thus PTP opening during anoxia/reoxygenation could generally be reversed by CSA and Ca\(^{2+}\) removal.

Effects of Ca\(^{2+}\) loading after \(\Delta\psi\) dissipation during anoxia. In the previous experiments, extramitochondrial Ca\(^{2+}\) was added before \(\Delta\psi\) dissipated during anoxia. During anoxia in intact cardiac myocytes, however, Ca\(^{2+}\) may continue to rise after \(\Delta\psi\) collapses (8), generating a possible situation in which rapid \(\Delta\psi\) recovery during reoxygenation could result in significant Ca\(^{2+}\) uptake and trigger MPT. The resulting \(\Delta\psi\) dissipation and Ca\(^{2+}\) release from the so-affected mitochondria might then enhance the probability of MPT in the

Fig. 4. Ca\(^{2+}\)-loading mitochondria before \(\Delta\psi\) dissipation during anoxia: effects on \(\Delta\psi\) and matrix volume. A: in 5 mM pyruvate-energized mitochondria (0.3 mg/ml), 2 Ca\(^{2+}\) pulses (5 \(\mu\)M, arrows) added during anoxia (N\(_2\) arrow) before \(\Delta\psi\) dissipation caused initially slow \(\Delta\psi\) depolarization and matrix swelling consistent with mitochondrial permeability transition (MPT) in a subpopulation of the mitochondria, followed by near-synchronous rapid \(\Delta\psi\) dissipation and further matrix swelling in the remainder. Reoxygenation (O\(_2\) arrow) led to transient partial \(\Delta\psi\) recovery, followed by \(\Delta\psi\) dissipation and further matrix swelling. Addition of both EGTA and succinate were required for recovery of \(\Delta\psi\) and matrix volume (3 preparations). B: same as A, except in the presence of 350 nM CSA. Addition of 5-\(\mu\)M Ca\(^{2+}\) pulses now failed to induce slow \(\Delta\psi\) depolarization or matrix swelling, although near-synchronous \(\Delta\psi\) dissipation still occurred at critically low Po\(_2\). Changes reversed rapidly with reoxygenation alone, indicating that MPT had been prevented (confirmed in 3 preparations).

Fig. 5. Ca\(^{2+}\)-loading mitochondria before \(\Delta\psi\) dissipation during anoxia: substrate dependence and reversal of MPT by Ca\(^{2+}\) removal + CSA. A: with succinate in place of pyruvate, five 5-\(\mu\)M Ca\(^{2+}\) pulses were still insufficient to trigger slow \(\Delta\psi\) depolarization and matrix swelling indicative of MPT (4 preparations). B: six 10 \(\mu\)M Ca\(^{2+}\) pulses added before and during anoxia did not induce a slow phase of \(\Delta\psi\) depolarization or matrix swelling, although near-synchronous \(\Delta\psi\) dissipation still occurred at critically low Po\(_2\). Changes reversed rapidly with reoxygenation alone, indicating that MPT had been prevented (confirmed in 3 preparations). C: similar experiment in which anoxia was created more slowly by decreasing the flow of the N\(_2\) stream. Five 15 \(\mu\)M Ca\(^{2+}\) pulses induced slow \(\Delta\psi\) dissipation and matrix swelling during anoxia indicating MPT, as in pyruvate-energized mitochondria (Fig. 3A). After reoxygenation, addition of 0.5 mM EGTA alone did not reverse MPT, whereas addition of 350 nM CSA after EGTA led to recovery of \(\Delta\psi\) and reversal of matrix swelling (3 preparations). A–C: mitochondria (0.3 mg/ml) were energized with 5 mM succinate, and \(\Delta\psi\) was recorded with TMRM.
MPT induced by rapid $\Delta \psi$ regeneration in the presence of high extramitochondrial [Ca$^{2+}$] could also be demonstrated by dissipating $\Delta \psi$ with rotenone instead of anoxia before adding Ca$^{2+}$ puls in preparations, Fig. 9A). Mitochondria were then energized with succinate (a substrate that enters distally to the complex 1 inhibition site of rotenone). Succinate regenerated $\Delta \psi$ leading to Ca$^{2+}$ uptake, but this soon turned into net Ca$^{2+}$ efflux (Fig. 9A) unless CSA was present to inhibit MPT (Fig. 9B). In the latter case, the potent chemical MPT inducer phenylarsoxide could still induce MPT.

Effect of anoxia/reoxygenation on mitochondrial $\Delta \psi$ in intact or permeabilized myocytes. To determine whether the responses of in situ mitochondria to anoxia/reoxygenation were similar to those of isolated mitochondria, we performed analogous experiments in intact and permeabilized myocytes, using TMRM to estimate $\Delta \psi$ (Fig. 10). Figure 10A shows that when intact myocytes were added to low-Ca$^{2+}$ KCl buffer (see MATERIALS AND METHODS) containing mitochondrial substrates (succinate + malate + glutamate), there was a significant uptake of TMRM (Fig. 10A). After turning on the $N_2$ stream, buffer $P_{O_2}$ declined progress-

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**Fig. 6. Ca$^{2+}$-loading mitochondria before $\Delta \psi$ dissipation: effects on mitochondrial Ca$^{2+}$ fluxes.**

A: a small Ca$^{2+}$ load of two 15 $\mu$M Ca$^{2+}$ pulses during anoxia insufficient to induce MPT resulted in mitochondrial Ca$^{2+}$ efflux, coinciding with $\Delta \psi$ dissipation that reversed spontaneously upon reoxygenation and was reproduced by $\Delta \psi$ dissipation with FCCP (3 preparations). B: four 10 $\mu$M Ca$^{2+}$ pulses administered before the onset of anoxia ($N_2$) were readily accumulated by mitochondria, which subsequently released the accumulated Ca$^{2+}$ as a result of anoxia-induced $\Delta \psi$ dissipation. On reoxygenation, mitochondria initially reaccumulated Ca$^{2+}$ coincident with $\Delta \psi$ recovery. However, Ca$^{2+}$ uptake soon turned into Ca$^{2+}$ efflux due to MPT, reversely by subsequent application of 350 nM CSA. In both A and B, mitochondria (0.4 mg/ml) were energized with 5 mM succinate, and changes in extramitochondrial [Ca$^{2+}$] recorded with 1 $\mu$M Calcium Green-5N.

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**Fig. 7. Ca$^{2+}$-loading mitochondria before $\Delta \psi$ dissipation during anoxia: effects of CSA on mitochondrial Ca$^{2+}$ fluxes.**

A: in the presence of 350 $\mu$M CSA, three 15 $\mu$M Ca$^{2+}$ pulses administered during anoxia caused modest Ca$^{2+}$ efflux during anoxia, which was blocked by ruthenium red (2 $\mu$M), indicating involvement of the Ca$^{2+}$ uniporter (3 preparations). B: four 15 $\mu$M Ca$^{2+}$ pulses initiated Ca$^{2+}$ efflux during anoxia ($N_2$), which was not reversed by reoxygenation even after 350 nM CSA, FCCP (1 $\mu$M) also had no effect, indicating that mitochondria were fully depolarized (3 preparations). In both A and B, mitochondria (0.4 mg/ml) were energized with 5 mM succinate, and changes in extramitochondrial [Ca$^{2+}$] recorded with 1 $\mu$M Calcium Green-5N.

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**Fig. 8. Ca$^{2+}$-loading mitochondria before $\Delta \psi$ dissipation: effects on mitochondrial Ca$^{2+}$ fluxes.**

A: in the presence of high extramitochondrial [Ca$^{2+}$] could also be demonstrated by dissipating $\Delta \psi$ with rotenone instead of anoxia before adding Ca$^{2+}$ puls in preparations, Fig. 9A). Mitochondria were then energized with succinate (a substrate that enters distally to the complex 1 inhibition site of rotenone). Succinate regenerated $\Delta \psi$ leading to Ca$^{2+}$ uptake, but this soon turned into net Ca$^{2+}$ efflux (Fig. 9A) unless CSA was present to inhibit MPT (Fig. 9B). In the latter case, the potent chemical MPT inducer phenylarsoxide could still induce MPT.

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**Fig. 9. Effect of CSA on mitochondrial Ca$^{2+}$ efflux.**

A: in the presence of 350 $\mu$M CSA, three 15 $\mu$M Ca$^{2+}$ pulses administered during anoxia caused modest Ca$^{2+}$ efflux during anoxia, which was blocked by ruthenium red (2 $\mu$M), indicating involvement of the Ca$^{2+}$ uniporter (3 preparations). B: four 15 $\mu$M Ca$^{2+}$ pulses initiated Ca$^{2+}$ efflux during anoxia ($N_2$), which was not reversed by reoxygenation even after 350 nM CSA, FCCP (1 $\mu$M) also had no effect, indicating that mitochondria were fully depolarized (3 preparations). In both A and B, mitochondria (0.4 mg/ml) were energized with 5 mM succinate, and changes in extramitochondrial [Ca$^{2+}$] recorded with 1 $\mu$M Calcium Green-5N.
sively, and when the threshold of O₂ electrode sensitivity was reached, Δψ rapidly and synchronously dissipated. Turning off the N₂ stream led to slow recovery of Δψ that dissipated rapidly when N₂ was turned back on. Subsequent reoxygenation with an O₂ stream resulted in immediate recovery of Δψ (Fig. 10A). Similar results were obtained in five preparations. These responses were virtually identical to those in isolated mitochondria in the absence of Ca²⁺ load (Fig. 1).

A similar picture was observed in digitonin-permeabilized cells (n = 4 preparations, Fig. 10B). Permeabilized cells added to KCl buffer that contained mitochondrial substrates and 3.5 mM MgATP accumulated TMRM. During anoxia, they maintained Δψ until PO₂ reached a critical level, at which point Δψ dissipated rapidly and synchronously, but to an incomplete extent. The partial Δψ dissipation was due to the presence of MgATP, which allowed ATP synthase (in reverse mode) to pump enough protons to maintain a partial proton gradient, since inhibition of ATP synthase with oligomycin led to rapid and complete collapse of Δψ (n = 3 preparations). Identical findings were obtained in isolated mitochondria when the incubation media contained substrates and MgATP during anoxia/reoxygenation (Fig. 10C, n = 3 preparations).

DISCUSSION

MPT, due to PTP opening, is widely recognized as having an important role in both apoptotic and necrotic cell death (6). PTP opening is regulated by multiple factors, among which matrix [Ca²⁺] and mitochondrial Δψ are two of the most crucial. Elevated mitochondrial matrix [Ca²⁺] is regarded as the most essential requirement, and Δψ dissipation significantly enhances PTP open probability by decreasing the [Ca²⁺] required to induce MPT. During hypoxia/reoxygenation, both occur. Furthermore, cell recovery upon reoxygenation has been shown to depend on recovery of mitochondrial Δψ (7), and recovery is unlikely once critical levels of myoplasmic and mitochondrial [Ca²⁺] are reached during hypoxia (16, 29). These observations are all consistent with the outcome of hypoxia/reoxygenation being determined by whether widespread MPT has occurred. Therefore, it is critically important to understand the interplay between Ca²⁺ and Δψ in facilitating MPT in this setting.

Fig. 8. Effects of Ca²⁺-loading mitochondria after Δψ dissipation during anoxia. A: two 25 μM Ca²⁺ pulses administered after Δψ dissipation during anoxia (N₂) had no further effect on Δψ or matrix volume. Upon reoxygenation, Δψ recovery was partial and transient, followed by Δψ dissipation and matrix swelling indicating MPT that reversed with EGTA (0.5 mM) and CSA (350 nM; 5 preparations). B: eight 10 μM Ca²⁺ pulses administered after Δψ dissipation during anoxia (N₂) led to no Ca²⁺ accumulation until reoxygenation. With reoxygenation, transient Ca²⁺ accumulation was followed by Ca²⁺ efflux partly reversed by CSA (350 nM; 3 preparations). Mitochondria (0.5 mg/ml) were energized with 5 mM succinate; extramitochondrial Ca²⁺ and Δψ recorded with Calcium Green-5N and TMRM, respectively.

Fig. 9. Effects of rapid Δψ recovery on mitochondrial Ca²⁺ uptake and subsequent Ca²⁺ efflux through permeability transition pores. Mitochondria (1 mg) were incubated with 5 μM rotenone (Rot) that rapidly and completely dissipated Δψ (not shown). Ca²⁺ pulses (25 μM) were added as indicated, and then mitochondria were reenergized by adding 5 mM succinate. Transient Ca²⁺ uptake was followed by Ca²⁺ efflux (A), which was prevented by pretreatment with 350 nM CSA (B). In the latter case, 25 μM phenylarsine oxide (PAO) induced Ca²⁺ efflux despite CSA. Changes in extramitochondrial [Ca²⁺] were recorded by Calcium Green-5N (5 preparations).
In the present paper, we have demonstrated that this interplay is very significant. Moreover, our findings suggest ways that MPT might be avoided by appropriate manipulation of these two factors. The two stages, to be considered separately, are MPT occurring during anoxia and MPT occurring during reoxygenation. First, it is important to emphasize that whereas MPT invariably causes mitochondrial swelling and the degree of anoxia, matrix dissipation during anoxia and MPT occurring during reoxygenation (see text for explanation) (3 preparations). N2 out refers to discontinuing the N2 stream, O2 to starting the O2 stream.

MPT occurring during anoxia. Whether MPT occurred during anoxia depended on the extent of matrix Ca2+ loading, which in turn depended on Δψ. When extramitochondrial [Ca2+] was increased before Δψ dissipation during anoxia, mitochondria accumulated Ca2+ via the Ca2+ uniporter. Depending on the respiratory substrate and the degree of anoxia, matrix Ca2+ accumulation could first induce MPT in a subpopulation of mitochondria, producing a slow decrease Δψ in proportion to the size of this subpopulation (Figs. 4A and 6B), followed by MPT in the remainder when Δψ dissipated at critically low PO2 (Figs. 4A and 5B). In the latter case, the remaining mitochondria were not...
yet Ca$^{2+}$ loaded to a level sufficient to induce MPT at normal $\Delta\psi$, but upon $\Delta\psi$ dissipation, the Ca$^{2+}$ threshold for MPT was exceeded. Alternatively, MPT occurred in other cases only at the time of the anoxia-induced rapid dissipation of $\Delta\psi$ (e.g., with succinate, as in Fig. 6C). In this case, mitochondria were insufficiently Ca$^{2+}$ loaded to induce MPT at normal $\Delta\psi$, but upon $\Delta\psi$ dissipation, the voltage-dependent threshold of matrix [Ca$^{2+}$] required to trigger PTP opening was again exceeded, and MPT occurred rapidly throughout the whole population of mitochondria. This scenario is also the likely explanation for the observation (21) that mitoK$_{ATP}$ channel agonists, which depolarize $\Delta\psi$, potentiated PTP opening in normoxic respiring isolated mitochondria subjected to Ca$^{2+}$ loading.

In contrast, when extramitochondrial Ca$^{2+}$ was added after $\Delta\psi$ had already dissipated (Fig. 8A), Ca$^{2+}$ accumulation via the Ca$^{2+}$ uniporter did not occur due to the lack of driving force for Ca$^{2+}$ entry into the matrix, and MPT was avoided during anoxia (but not necessarily after anoxia, see MPT occurring during reoxygenation). Thus in intact cardiac myocytes, the timing of the increase in cytoplasmic free [Ca$^{2+}$] relative to $\Delta\psi$ depolarization during anoxia is likely to be critically important. Because mitochondrial Ca$^{2+}$ uptake accounts for approximately two-thirds of cellular Ca$^{2+}$ uptake, $\Delta\psi$ dissipation before a major increase in extramitochondrial [Ca$^{2+}$] will greatly reduce cellular Ca$^{2+}$ overload (24). In intact myocytes, one approach toward cardioprotection is to decrease the rate at which cytoplasmic Ca$^{2+}$ increases during anoxia by inhibiting transsarcomemmal Ca$^{2+}$ influx, e.g., with Na$^+$/H$^+$ exchange inhibitors (18, 31). A second approach is to prevent mitochondrial Ca$^{2+}$ uptake in polarized mitochondria, despite elevated extramitochondrial Ca$^{2+}$, by inhibiting the mitochondrial Ca$^{2+}$ uniporter, e.g., with ruthenium red, also known to be cardioprotective (9, 28). Finally, a third approach is to minimize mitochondrial Ca$^{2+}$ accumulation by dissipating $\Delta\psi$ more rapidly during anoxia, e.g., with mitoK$_{ATP}$ channel agonists, which is one of the mechanisms proposed to underlie their cardioprotective effects (22, 26). The weight of evidence now suggests that mitochondrial K$_{ATP}$ channels are more important than sarcomemmal K$_{ATP}$ channels in mediating cardioprotection (17). Furthermore, direct protection of mitochondria by mitoK$_{ATP}$ channel agonists during ischemia-reperfusion has recently been demonstrated (11), consistent with prevention of MPT. These findings make sense on general grounds because cardioprotection obviously depends on recovery of mitochondrial function, and transient inhibition of mitochondrial function, when induced before ischemia, could protect them from excessive Ca$^{2+}$ accumulation. The ability of mitochondrial inhibitors, which induce full dissipation of $\Delta\psi$, to prevent hypercontracture and massive enzyme release, and reduce infarct size during reoxygenation/reperfusion (12, 13, 27) may also be an example of the former.

It is also interesting to note that even when MPT has occurred during anoxia, as indicated by CSA-sensitive matrix swelling (e.g., Fig. 4, A and B), we always observed at least partial transient recovery of $\Delta\psi$ (although not matrix volume) upon reoxygenation. This indicates that some mitochondria had still not undergone MPT at the start of reoxygenation. Upon reoxygenation, however, MPT was also induced in this subpopulation, as evidenced by complete $\Delta\psi$ dissipation, and did not reverse spontaneously unless extramitochondrial Ca$^{2+}$ was removed and CSA added (see next section).

**MPT occurring during reoxygenation.** Even if mitochondria have avoided accumulating sufficient Ca$^{2+}$ to induce MPT during anoxia, they are still subject to MPT during reoxygenation (Fig. 8). In the presence of O$_2$, substrate, and elevated extramitochondrial [Ca$^{2+}$], partial $\Delta\psi$ recovery during reoxygenation promotes mitochondrial Ca$^{2+}$ uptake via the Ca$^{2+}$ uniporter. Because the voltage dependence of Ca$^{2+}$ uptake via the Ca$^{2+}$ uniporter saturates at $-110$ mV (6), partial $\Delta\psi$ recovery should not appreciably limit the increase in matrix [Ca$^{2+}$]. However, the lower $\Delta\psi$ is more likely to promote MPT at a given level of matrix Ca$^{2+}$ (Figs. 5B and 8A) due to its voltage sensitivity. In contrast, well-oxygenated and energized mitochondria can tolerate surprisingly high Ca$^{2+}$ loads without PTP opening, provided that mitochondria are able to support normal $\Delta\psi$. In addition, during reoxygenation of intact cells, oxidative stress is considerably elevated and favors PTP opening (6). Thus when extramitochondrial Ca$^{2+}$ remains high during reoxygenation, PTP opening may be triggered even when mitochondria have not become Ca$^{2+}$ loaded during anoxia (Fig. 8). In this setting, lowering of extramitochondrial Ca$^{2+}$ is usually required to reverse MPT, even if CSA is present (Figs. 5B, 7B, and 8A). The three approaches described above for preventing MPT during hypoxia are also predicted to be effective at preventing MPT during reoxygenation by limiting matrix Ca$^{2+}$ accumulation: 1) reducing the extramitochondrial [Ca$^{2+}$] increase during hypoxia/reoxygenation, 2) delaying $\Delta\psi$ recovery upon reoxygenation to keep membrane potential below the value required for Ca$^{2+}$ uniporter activity, or 3) blocking Ca$^{2+}$ uptake by the Ca$^{2+}$ uniporter until after normal cytoplasmic Ca$^{2+}$ levels have been restored by nonmitochondrial Ca$^{2+}$ removal mechanisms.

**Summary and clinical implications.** Mitochondrial $\Delta\psi$ and matrix [Ca$^{2+}$] interact strongly to regulate PTP opening during hypoxia/reoxygenation. Our findings predict that interventions that reduce extramitochondrial Ca$^{2+}$, that prevent mitochondria from accumulating Ca$^{2+}$ by chemically inhibiting the Ca$^{2+}$ uniporter, or that accelerate $\Delta\psi$ dissipation during hypoxia to limit [Ca$^{2+}$] uptake via the Ca$^{2+}$ uniporter are synergistic at preventing MPT during hypoxia/reoxygenation in isolated mitochondria. Furthermore, we have demonstrated that the fundamental responses to anoxia/reoxygenation in isolated mitochondria under non-Ca$^{2+}$-loaded conditions are similar to those of in situ mitochondria in both permeabilized and intact myocytes. Unfortunately, we could not determine whether Ca$^{2+}$-loaded mitochondria in situ behaved identically to isolated mitochondria due to diffi-
culcy in controlling extramitochondrial [Ca^{2+}] accurately under the latter conditions, which is a limitation of this study. Nevertheless, these findings lend strong support to the proposition that a multipronged strategy, combined with direct pharmacological inhibition of MPT with CSA, will be more effective at reducing MPT during hypoxia/reoxygenation and ischemia-reperfusion in cardiac tissue than any of these interventions individually, thereby enhancing cardioprotection.

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