K⁺-dependent regulation of matrix volume improves mitochondrial function under conditions mimicking ischemia-reperfusion

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Korge, Paavo, Henry M. Honda, and James N. Weiss. K⁺-dependent regulation of matrix volume improves mitochondrial function under conditions mimicking ischemia-reperfusion. Am J Physiol Heart Circ Physiol 289: H66–H77, 2005. First published March 11, 2005; doi:10.1152/ajpheart.01296.2004.—To delineate the role of mitochondrial K⁺ fluxes in cardioprotection, we investigated the effect of extramitochondrial K⁺ on the ability of mitochondria to support membrane potential (ΔΨ), regulate matrix volume, consume oxygen, and phosphorylate ADP under conditions mimicking key elements of ischemia-reperfusion. Isolated energized mitochondria responded to ADP addition with depolarization, increased O₂ consumption, and matrix shrinkage. The time required for full recovery of ΔΨ, signaling the completion of ADP phosphorylation, was used to evaluate the rate of ATP synthesis during repeated ADP pulses. In mitochondria with a decreased ability to support ΔΨ, the rate of ADP phosphorylation was significantly improved by extramitochondrial K⁺ > Na⁺ > Li⁺, especially at higher buffer osmolarity, which promotes matrix shrinkage. K⁺-induced improvement in ΔΨ recovery after ADP pulses was accompanied by more rapid and complete matrix volume recovery and enhanced O₂ consumption. Manipulations expected to affect matrix swelling by regulating K⁺ fluxes or water distribution indicate that matrix volume regulation by external factors becomes increasingly important in mitochondria with decreased ability to support ΔΨ in the face of a high ADP load. Under these conditions, opening of K⁺ influx pathways improved mitochondrial function and delayed failure. This may be an important factor in the mechanism of diazoxide-induced cardioprotection.

mitochondria; matrix swelling; shrinkage; hypoxia; reoxygenation

ISCHEMIC PRECONDITIONING is known to decrease cardiac injury and improve functional recovery after ischemia-reperfusion by activating a powerful endogenous cardioprotective mechanism. Similar cardioprotection can be induced pharmacologically by putative activators of mitochondrial ATP-sensitive K⁺ (mitoKATP) channels. Inoue et al. (18) were the first to demonstrate a mitoKATP channel in fused giant mitoplasts prepared from liver mitochondria. Subsequently, endogenous mitoKATP channels were demonstrated using patch-clamp experiments in one additional study (4). These channels were subsequently characterized by Garlid’s group (1, 13, 14, 19) in intact mitochondria and, using partially purified protein, reconstituted into liposomes. They found that mitoKATP channels are much more sensitive to activation by diazoxide and inhibition by 5-hydroxydecanoate (5-HD) than sarcolemmal KATP channels in the heart (13). Because diazoxide and 5-HD are very effective at inducing and preventing cardioprotection, respectively, the focus turned toward mitoKATP channels rather than sarcolemmal KATP channels as mediators of cardioprotection (11, 35).

Although several hypotheses have been advanced (for reviews, see Refs. 11 and 34), the mechanisms by which mitoKATP channel openers protect mitochondria from ischemia-reperfusion injury remain controversial. At cardioprotective concentrations (≤50 μM), diazoxide has little effect on energized mitochondria (25, 36) except to induce mild matrix swelling, with a corresponding reduction in intermembrane space volume (for a review, see Ref. 12). Through this effect, both diazoxide and ischemic preconditioning have been hypothesized to be protective by preserving a narrow intermembrane space, which facilitates energy transfer from mitochondria to ATP-utilizing sites in the cytosol, reduces permeability of the outer mitochondrial membrane to nucleotides, and slows the rate of ATP hydrolysis (8, 27).

However, diazoxide and 5-HD also have effects on mitochondria that are not related to mitoKATP channels (17, 37). In addition, it is technically demanding to dissect matrix swelling due to diazoxide-induced KATP channel opening from diffusion-mediated nonselective K⁺ uptake or from intrinsic volume-regulatory mechanisms (5, 14, 19, 30). For an in-depth discussion of this current controversy, the reader is referred to recent relevant studies (5, 11, 12). Despite controversy over the existence of mitoKATP channels and the importance of intrinsic versus cation flux-dependent matrix volume regulatory mechanisms, there is little doubt that matrix volume regulation can be influenced by K⁺ fluxes under in vivo conditions (12).

The specific experimental conditions under which mitochondria are studied are likely to be very important. Whereas in situ mitochondria sense profound changes in their cellular environment during ischemia-reperfusion, these changes have not generally been taken into consideration in experiments with isolated mitochondria, which are usually studied under well-energized conditions. However, the ideal cardioprotective agent should disturb mitochondrial function only minimally under normal conditions but exert strong protective effects under pathophysiological conditions. Accordingly, we tested the hypothesis that matrix volume regulation mediated by additional K⁺ influx pathways such as mitoKATP channels has minor effects under well-energized conditions but major effects under conditions relevant to ischemia-reperfusion. We present evidence that under the latter conditions, K⁺ flux-dependent matrix volume regulation becomes critically important to phosphorylating mitochondria.

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METHODS

Isolation of Mitochondria

All animal experiments were conducted in full compliance with state and federal animal care guidelines and were approved by the Office for Protection of Research Subjects of University of California-Los Angeles (ARC protocol 2001-176-11). Mitochondria were isolated from adult rabbit hearts by enzymatic digestion, homogenization, and differential centrifugation as described previously (24). Isolated mitochondria were resuspended in EGTA-free homogenization buffer (250 mM sucrose and 10 mM HEPES, pH 7.4 with Tris) to yield 20–30 mg/ml of mitochondrial protein, kept on ice, and normally used within 5 h after isolation. Freshly isolated mitochondria had coupling ratios of ≥8 in KCl buffer with complex I substrates and 0.4 mM ADP.

Experimental Conditions

All measurements were carried out using a fiber-optic spectrofluorometer (Ocean Optics) in a closed continuously stirred cuvette at room temperature (22–24°C). Mitochondria (regularly about 0.1–0.15 mg/ml) were added to the cuvette in standard buffer consisting of (in mM) 100 KCl and 10 HEPES, pH 7.4 with Tris. In some experiments, buffer contained sucrose or other salts in place of KCl or buffer osmolality was increased. Substrates, Pi, EDTA, various drugs, and fluorescent indicators were added in the concentrations indicated.

To define factors that limit or favor matrix volume recovery during sustained ADP phosphorylation, we used repeated ADP additions to evaluate the extent of matrix volume recovery sequentially over time. To relate changes in matrix volume to mitochondrial function, mitochondrial membrane potential (ΔΨ) and O2 consumption were recorded simultaneously. In most experiments, ADP was added to a relatively low concentration of mitochondria. This high ADP-to-mitochondria ratio was used to mimic reperfusion conditions, where the ADP load per matrix volume was not completely synchronous with the ADP additions. The extent of matrix volume recovery was measured in cuvettes containing tetramethylrhodamine methyl ester (TMRM; 200 nM) in the cuvette solution.ΔΨ was estimated from TMRM fluorescence at 580 nm as described previously (24) and expressed as a percentage of the TMRM fluorescence in the presence of coupled mitochondria and substrates (100%) relative to that after the addition of 0.5 μM FCCP or alamethicin to fully depolarize mitochondria (0%). TMRM fluorescence emission was recorded simultaneously with PO2.

Fig. 1. Fully energized mitochondria respond to ADP additions with a reversible membrane potential (ΔΨ) decrease and matrix shrinkage. A: mitochondria (0.4 mg/ml) were added to KCl buffer (140 mM KCl and 10 mM HEPES, pH 7.4 with Tris). At the arrows, 5 mM Pi, and complex I substrates [pyruvate (Pyr), malate (Mal), and glutamate (Glu), each 1.5 mM] were added, followed by ADP additions at the indicated concentrations. The top tracing shows that ΔΨ dissipated transiently after each ADP addition but fully recovered after a time delay proportional to the amount of added ADP. The middle tracing shows that PO2 consumption (i.e., buffer PO2 decrease) also accelerated transiently during ADP phosphorylation. The bottom tracing shows matrix volume, as assessed by light scattering, with increased light scattering indicating matrix shrinkage. At the end, alamethicin (A; 5 μg/ml) was added to induce complete ΔΨ dissipation and maximum swelling for calibration purposes. B: in the top and middle traces, mitochondria (0.2 mg/ml) were added to 100 mM KCl buffer containing tetramethylrhodamine methyl ester (TMRM), energized with complex I substrates in the presence of Pi, and challenged with 6 ADP pulses (250 μM each). In the bottom trace, TMRM was omitted from the same batch of mitochondria (0.2 mg/ml), and matrix volume changes were continuously recorded from light scattering to show that TMRM was not responsible. The timing of ADP additions was not completely synchronous with the top and middle traces.
Changes in matrix volume. Changes in matrix volume were measured using standard procedures by recording 90° light scattering with excitation and emission wavelengths set at 510 nm. These changes were recorded simultaneously with TMRM fluorescence and O₂ consumption. In separate experiments, ADP-induced changes in light scattering were also determined in the absence of TMRM. These experiments showed similar ADP-induced matrix shrinkage followed by swelling back to baseline, indicating that recorded matrix volume changes were not significantly modified by changes in TMRM redistribution. For statistical analysis, changes in matrix volume are reported as a percentage of maximum (100%) swelling induced by adding 10 μg alamethicin at the end of the experiment.

Mitochondrial ATP synthesis. Mitochondrial ATP synthesis was terminated with the addition of ice-cold perchloric acid at time points and under conditions specified in the figures. ATP concentration was determined in neutralized PCA supernatants, as described previously (23), by continuous monitoring the reduction of NADP. In this assay, an increase in fluorescent NADPH is proportional to [ATP] (2). The assay buffer contained 0.2 mM NADP, 10 mM glucose, 5 U/ml hexokinase, 5 U/ml glucose-6-phosphate dehydrogenase, and 4 mM MgCl₂.

Electron microscopic studies. For electron microscopic studies, mitochondria were processed according to previously described methods (9).

Mitochondrial protein content. Mitochondrial protein content was determined by the Lowry method.

Chemicals and Data Analysis

TMRM was obtained from Molecular Probes, and all other chemicals were from Sigma. Mitochondrial substrates were added as free acids using Tris to buffer pH.

RESULTS

ADP-Induced Changes in ΔΨ and Matrix Volume in Energized Isolated Mitochondria

Figure 1A shows that in isolated mitochondria incubated in 140 mM KCl buffer in the presence of Pᵢ and substrates, the addition of ADP promoted partial ΔΨ dissipation, matrix shrinkage, and an increase in O₂ consumption. After a time period proportional to the amount of ADP added, all changes returned to baseline, signaling the end of ADP phosphorylation. In well-coupled energized mitochondria, ADP-induced changes in matrix volume and ΔΨ recovery were completely reversible, even when ADP was added repeatedly in relatively high concentrations. Pᵢ was required to induce these changes, as expected if due to ATP synthesis, and ADP-induced changes were blocked by atractyloside, an inhibitor of ADP/ATP exchange (not shown). Because ΔΨ recovered fully after the added ADP had been phosphorylated, the time period required for ΔΨ recovery also indirectly indicates the rate of ADP phosphorylation.

Figure 1B demonstrates that the matrix volume changes in response to ADP were not an artifact of TMRM redistribution. In the top trace, mitochondria (0.2 mg/ml) energized with complex I substrates were incubated in 100 mM KCl buffer containing 50 mM KCl (Fig. 2. Importance of extramitochondrial K⁺ for ADP-induced changes in ΔΨ, matrix volume, and O₂ consumption. Mitochondria (0.5 mg/ml) from the same preparation were added to either 300 mM sucrose buffer (A, C, and D) or 200 mM sucrose buffer containing 50 mM KCl (B). Buffer also contained 0.5 mg/ml BSA and 10 mM HEPES, pH 7.4 with Tris. At the arrows, 5 mM Pᵢ (Tris form), 0.75 mM caproic acid (Cap A), 4 or 5 pulses of 150 μM ADP (cyclohexylammonium salt), and Ala were added. A: after the third ADP pulse, ΔΨ and matrix volume failed to recover and O₂ consumption remained low. An additional bolus of 750 μM Cap A (arrow) did not lead to recovery, indicating that these changes were not due to substrate depletion. B: with 50 mM KCl present, however, mitochondria tolerated multiple ADP pulses without adverse consequences. C and D: ADP-induced changes in ΔΨ, matrix shrinkage, and O₂ consumption in sucrose buffer were prevented and reversed by adding 20 mM KCl (arrows). Similar findings were obtained in 3 different preparations.)
(the standard for subsequent experiments) with TMRM present. Mitochondria responded to six ADP additions (250 µM each) with reproducible changes in ΔΨ and O2 consumption. The bottom trace shows matrix volume measurements in a separate aliquot from the same batch of isolated mitochondria but without TMRM in the buffer. ADP-induced matrix shrinkage and recovery were similar as when TMRM was present.

**Importance of Extramitochondrial K+ in ADP-Induced Changes in Fatty Acid-Energized Mitochondria**

Since the initial studies by Hackenbrock (15), energized mitochondria are known to respond to ADP addition with matrix shrinkage, followed by recovery when ADP phosphorylation is complete. These matrix volume shifts occur in nonionic (sucrose or mannitol) as well as ionic buffers, suggesting that matrix volume can be regulated independently of ion fluxes. However, these findings do not exclude the possibility that under stressed conditions, the intrinsic (ion independent) mechanism may be supplemented and enhanced by ionic fluxes, allowing more rapid and complete matrix volume recovery after ADP phosphorylation. Under in vivo conditions, the ability of mitochondria to regulate matrix volume by K+ uptake/efflux is determined by extracellular K+ (12). During reperfusion, regulation is compromised by depression of respiratory chain activity, increased inner membrane leakiness, and a high ADP load, which might be expected to impact matrix volume regulation. To evaluate this possibility, we compared matrix volume changes in response to ADP pulses in isolated mitochondria bathed in either nonionic or ionic buffer. We decreased the mitochondrial ability to support ΔΨ during ADP phosphorylation by replacing pyruvate, malate, and glutamate by caproic acid, a short-chain fatty acid that does not require carnitine to get into the matrix. Fatty acids are important substrates in the heart, and their oxidation is expected to increase during reperfusion due to decreased malonyl-CoA levels (26).

Figure 2A shows that when mitochondria (0.5 mg/ml) were incubated in 300 mM sucrose buffer and energized with 0.75 mM caproic acid, they failed to recover ΔΨ and matrix volume after several 150 µM ADP pulses. In addition, O2 consumption was depressed. These results suggest that the rate of ADP phosphorylation was decreased during each subsequent pulse in nominally K+-free buffer. The failure to recover ΔΨ and matrix volume after several ADP additions was not due to exhaustion of the available caproic acid, because further addition of 0.75 mM caproic acid at this point had no effect.

Figure 2B shows that when the same mitochondrial preparation was challenged with ADP in 200 mM sucrose + 50 mM KCl buffer, ΔΨ recovery was not only much faster initially, but the recovery rate was much more stable during repeated ADP additions. Paralleling ΔΨ recovery, matrix volume recovery was also markedly improved in the presence of K+, and O2 consumption in response to ADP remained robust.

These K+-dependent differences in mitochondrial behavior during ADP pulses are demonstrated even more convincingly in Fig. 2, C and D. In these experiments, 20 mM KCl was added to the sucrose buffer after mitochondria had been challenged with one or several ADP pulses. Figure 2C shows that
the addition of KCl after the first ADP pulse significantly increased the rate of ΔΨ recovery and O₂ consumption in response to subsequent ADP pulses, reflecting an enhanced rate of ADP phosphorylation. Figure 2D shows that improvement was also obtained when KCl was added after ΔΨ and matrix volume recovery has already compromised by several ADP pulses. Because the osmotic effect of the added KCl would have been to cause further matrix shrinkage, these results can only be explained if K⁺ movement into the matrix, followed osmotically by water, allowed the matrix to reexpand, with a consequent improvement in ADP phosphorylation. The slight dissipation of ΔΨ connected with K⁺ uptake is compensated with an increase in ΔpH, leaving the protonmotive force unchanged.

We interpret these findings as indicating that ΔΨ-driven K⁺ (and +P) influx, which is followed by osmotically driven water movement to prevent matrix shrinkage, operates synergistically with respiratory chain power to maintain an increased ADP phosphorylation rate. This K⁺-dependent mechanism, however, only becomes critical for matrix volume regulation when respiratory chain power is reduced. Consistent with these findings, mitochondria energized with full complex I substrates (as in Fig. 1) tolerated repeated ADP additions without adverse consequences. However, if only a limited amount of pyruvate (0.2 mM) was provided as the exogenous substrate, similar results were obtained as with caproic acid (data not shown). Finally, the K⁺-dependent mechanism seems to be less important in hyposmotic sucrose buffer (Fig. 3D), in which mitochondria tolerated repeated ADP additions better, at least initially. Because mitochondria are well-behaved osmometers, the matrix rapidly expands in hyposmotic medium (200 mosM) so that ADP phosphorylation can proceed under more favorable conditions. In this setting, K⁺-dependent differences were more evident when the ADP load per mitochondrion was relatively high (see below). Collectively, these results suggest that, although mitochondria can operate in nominally K⁺ (ion)-free medium under conditions in which their ability to maintain ΔΨ is decreased due to limitations in respiratory chain activity and their matrix volume is contracted, the rate of ADP phosphorylation is greatly enhanced by extramitochondrial K⁺.

Figure 3 shows that other cations were less effective than K⁺ in promoting ΔΨ and matrix volume recovery after ADP additions in mitochondria energized with caproic acid. Assuming that the time period required for ΔΨ recovery indirectly reflects the time required for ADP phosphorylation, ATP production per unit of time decreased in the order of K⁺ > Na⁺ > Li⁺ > sucrose.

Figure 4 illustrates further that the K⁺-dependent volume regulatory mechanism only becomes important when respiratory chain power is reduced. In Fig. 4A, isolated mitochondria in 150 mM sucrose + 25 mM KCl buffer were initially energized with 0.5 mM caproic acid. After the addition of 5 mM Pi and 500 μM Cap A, ΔΨ and matrix volume failed to recover after fewer 50 μM ADP pulses in NaCl buffer than in KCl buffer, although both recovered fully with the addition of complex I substrates (1.5 mM each). Electron micrographs of mitochondria fixed immediately before the addition of complex I substrates showed much less severe matrix condensation in KCl buffer (C) than in NaCl buffer (D). (Dark areas are matrix and white areas are intermembrane spaces between the cristae, which are markedly expanded in D.) Similar findings were obtained in 5 different preparations.
sively delayed. The addition of a full complement of complex I substrates (1.5 mM each of pyruvate, malate, and glutamate) to enhance respiratory chain power, however, led to rapid ΔΨ dissipation and matrix shrinkage were much more dramatic. Figure 4, C and D, illustrates representative electron micrographs of isolated mitochondria obtained immediately before complex I substrates were added in A and B. Note that in KCl-sucrose buffer, the matrix area was significantly larger (Fig. 4C) than in NaCl-sucrose buffer, in which matrix volume was severely contracted but intracristal space expanded (Fig. 4D). These findings establish that the changes in matrix volume recorded from light scattering reflect matrix remodelling and that K⁺ is much more effective than Na⁺ at preventing severe matrix condensation in fatty acid-energized mitochondria.

**Mg²⁺ Further Inhibits Matrix Volume Recovery From ADP-Induced Shrinkage**

During ischemia-reperfusion, cytoplasmic [Mg²⁺] increases by two- to threefold (31, 40), which is expected to inhibit mitochondrial cation fluxes (22, 33). When the experiment shown in Fig. 4A was repeated with the same batch of mitochondria in the presence of 2 mM Mg²⁺, ΔΨ and matrix volume recovery after ADP additions were markedly affected (Fig. 5A). However, full recovery was still achieved when complex I substrates were added. In Fig. 5B, caproic acid-energized mitochondria, incubated in 100 mM KCl buffer in the presence of Mg²⁺ and EGTA (to chelate contaminant Ca²⁺), were challenged with ADP pulses until ΔΨ and matrix volume failure to recover. At this point, the addition of 3 mM EDTA to chelate extramitochondrial Mg²⁺ not only increased ΔΨ but also stabilized it during further ADP additions (Fig. 5B). The improvement of matrix volume and ΔΨ with EDTA was limited, as demonstrated by the further recovery after the addition of complex I substrates (Fig. 5B). Figure 5C summarizes the average ADP-induced matrix volume change in fatty acid-energized mitochondria in the presence of Mg²⁺ (bar a) as well as the complete reversal of these changes with the addition of complex I substrates as in Fig. 5A (bar b) and the partial recovery after EDTA without adding complex I substrates as in Fig. 5B (bar c).

If the inability of mitochondria to recover ΔΨ and matrix volume fully after ADP addition in fatty acid-energized mitochondria is due to impaired osmotic regulation from suppression of K⁺ influx, then appropriate osmotic manipulations should exacerbate or mitigate recovery. Figure 6 compares the effects of low- and high-molecular-weight polyethylene glycols (PEG) on mitochondria incubated in 150 mM sucrose + 25 mM KCl buffer, with 0.5 mM caproic acid as the substrate. In the absence of PEG (Fig. 6A), ΔΨ and matrix volume recovered after four successive additions of 50 μM ADP, although the rate of recovery slightly decreased as in Fig. 4A. Inclusion of 1.25% of 600-molecular weight PEG (PEG600) into the buffer significantly accelerated recovery (Fig. 6B). PEG600 is expected to resist matrix swelling by moving into the intermembrane space and drawing water from the matrix. In contrast, the same concentration of 10,000-molecular-weight PEG6000 further impairs ΔΨ and matrix volume recovery after ADP additions A: in the same batch of mitochondria as in Fig. 3A but with 2 mM MgCl₂ present from the start, ΔΨ and matrix volume recovery after ADP are much poorer in the presence of Mg²⁺. B: mitochondria (0.15 mg/ml) were incubated in 100 mM KCl buffer containing 1 mg/ml BSA, 100 μM EGTA, and 2 mM MgCl₂. After 5 mM Pi and 0.5 mM Cap A were added, 50 μM ADP pulses were added until ΔΨ and matrix volume recovery failed. Chelating Mg with 3 mM EDTA led to partial recovery of ΔΨ and matrix volume, which was further improved by the addition of complex I substrates (Pyr, Mal, and Glu, 1.5 mM each). Ala (5 μg) was added at the end for calibration purposes. C: mean values ± SD (1 preparations, n = 10) for the ADP-induced matrix volume change in fatty acid-energized mitochondria in the presence of 2 mM MgCl₂ (bar a) and recovery after the addition of complex I substrates (bar b) or 3 mM EDTA (bar c). The light scattering level after P, and Cap A addition was taken as the initial zero line. Matrix shrinkage/swelling is expressed as the relative change (in %) from this line, with 100% corresponding to the difference between maximum shrinkage after the final ADP addition and maximum swelling after Ala addition.
Fig. 6. Effects of osmotic interventions on ΔΨ and matrix volume recovery after ADP additions. As described in Fig. 3, mitochondria (0.15 mg/ml) were added to 150 mM sucrose and 25 mM KCl buffer (A) or the same buffer also containing 1.25% 600-molecular weight polyethylene glycol (PEG600; B), 1.25% 10,000-molecular weight PEG (PEG10,000; C), or 1.25% PEG10,000 + 2 mM MgCl2 (D). After 5 mM Pi, and Cap A (500 μM) were added, mitochondria were subjected to successive ADP pulses (50 μM each). ΔΨ and matrix volume recovery after ADP was worsened by PEG600 (B) but enhanced by PEG10,000 (C). Mg2+ abolished the protective effect of PEG10,000 (D). The addition of complex I promoted complete recovery in all cases. Similar findings were obtained in 2 other preparations.

lar weight PEG (PEG10,000) preserved full ΔΨ and matrix volume recovery after even six ADP additions (Fig. 6C). In this case, PEG10,000 (which is impermeant to the outer membrane) is expected to promote matrix volume by decreasing the volume of the intermembrane space, through balancing the osmotic pressure exerted by intermembrane space proteins (16). Similar findings were obtained when PEG was added to 100 mM KCl buffer. When 2 mM Mg2+ was present, however, PEG10,000 was less effective at preventing loss of ΔΨ and matrix volume recovery after ADP (Fig. 6D). These findings substantiate that osmotic factors play a direct role in regulating both ΔΨ and matrix volume recovery after ADP addition.

Opening K+-Selective Influx Pathways Improves ΔΨ and Matrix Volume Recovery From ADP-Induced Changes in the Presence of Mg2+

To determine whether selectively enhancing K+ influx in fatty acid-energized energized mitochondria with Mg2+ present would improve their ability to regulate matrix volume and ΔΨ in response to ADP pulses, we examined the effects of a low concentration of the K+ ionophore valinomycin (20 pM). In Fig. 7A, mitochondria (0.15 mg/ml in 150 mM sucrose buffer containing 25 mM KCl + 2 mM MgCl2) energized with caproic acid (0.5 mM) rapidly lost their ability to recover ΔΨ and matrix volume in response to ADP. However, when the buffer included a low concentration of valinomycin (20 pM), recovery improved significantly (Fig. 7B). When this experiment was repeated with Na+ in place of K+ (Fig. 7C), valinomycin did not improve recovery and, in fact, worsened it (compare with Fig. 4B). The failure to recover ΔΨ and matrix volume after ADP pulses translated directly into reduced ATP synthesizing capacity. Figure 7, D and E, demonstrates that ATP measured after ADP additions was significantly higher in the presence of K+ and valinomycin (Fig. 7D) than in the presence of Na+ and valinomycin (Fig. 7E).

Figure 8, A and B, shows that diazoxide, widely considered to be a mitoKATP channel opener, had similar effects as valinomycin under these conditions. Inclusion of the mitoKATP channel blocker 5-HD (300 μM) abolished diazoxide’s protective effect (Fig. 8C). Figure 8D summarizes the average values for ΔΨ and matrix volume recovery after five ADP additions.

Effect of Anoxia-Reoxygenation on Mitochondrial ADP Phosphorylation

In hearts subjected to anoxia/reoxygenation, mitochondria lose electron transport power and face elevated intracellular levels of Mg2+ and ADP. To examine the effects on ΔΨ and matrix volume regulation, we subjected isolated mitochondria (incubated in 100 mM KCl buffer containing 5 mM Pi and 1%...
PEG	extsubscript{600} to simulate increased osmolarity) to anoxia/reoxygenation (Fig. 9A). Mitochondria were fully energized with complex I substrates from the outset. Anoxia caused $\Delta \Psi$ dissipation when $P_{O2}$ reached a critical level. $\Delta \Psi$ dissipation was followed by matrix volume shrinkage, as expected from the loss of driving force for K\textsuperscript{+} influx into the matrix. Both $\Delta \Psi$ and matrix volume recovered nearly completely with reoxygenation, and these mitochondria subsequently responded to ADP-induced changes with only slightly impaired recovery of $\Delta \Psi$ and matrix volume. In contrast, when the same protocol was performed in the presence of 2 mM Mg\textsuperscript{2+}, matrix volume recovered only partially after reoxygenation. More importantly, after ADP additions, the matrix contracted further with very little recovery from extensive contracture, despite the presence of complex I substrates. At the same time, $\Delta \Psi$ failed to recover from ADP-induced dissipation (Fig. 9B). Under these conditions, mitochondrial ATP production was significantly decreased to ~60% of that in the absence of Mg\textsuperscript{2+} (Fig. 9D, compare traces a and b). Diazoide (50 $\mu$M) improved both $\Delta \Psi$ and matrix volume recovery (Fig. 9, C and E) and preserved ATP synthesis (Fig. 9D, trace c). Recovery of $\Delta \Psi$ and matrix volume after the final ADP addition is summarized in Fig. 9E for the three cases: anoxia/reoxygenation, anoxia/reoxygenation with 2 mM Mg\textsuperscript{2+} present, and anoxia/reoxygenation with Mg\textsuperscript{2+} + 50 $\mu$M diazoide present. Although K\textsuperscript{+} fluxes were not directly recorded, these findings, combined with the results presented above, strongly suggest that K\textsuperscript{+} fluxes are important for maintaining matrix volume, $\Delta \Psi$, and ADP phosphorylation in the setting of anoxia/reoxygenation when extramitochondrial Mg\textsuperscript{2+} is elevated, even when a full complement of exogenous substrates is present.

**DISCUSSION**

Recovery of cardiac function after ischemia-reperfusion is critically dependent on rapid recovery of mitochondrial function to restore ATP production and to avoid activation of cell death pathways by release of proapoptotic signaling molecules. Prolonged ischemia is characterized by progressive increases in [ADP], [P\textsubscript{i}], [Mg\textsuperscript{2+}], and osmolality. Upon reperfusion, the ability of mitochondria to recover and support ATP synthesis, when extramitochondrial Mg\textsuperscript{2+} is elevated, even when a full complement of exogenous substrates is present.

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K⁺-Dependent Regulation of Matrix Volume

Fig. 8. Diazoxide (Diaz) improves ΔΨ and matrix volume recovery after ADP additions. A-C: mitochondria (0.15 mg/ml) were added to 150 mM sucrose buffer containing 25 mM KC1, 2 mM MgCl₂, HEPES, BSA, and EGTA (A) and either 50 μM Diaz (B) or 50 μM Diaz + 300 μM 5-hydroxydecanoate (5-HD; C). After 5 mM Pi, and 500 μM Cap A were added, ΔΨ and matrix volume were recorded during successive 50 μM ADP pulses. Compared with control or Diaz + 5-HD, Diaz improved ΔΨ and matrix volume recovery after ADP. The inclusion of 300 μM 5-HD into buffer A had no significant effect on the mitochondrial response. In all cases, ΔΨ and matrix volume recovered completely after full energization with complex I substrates (1.5 mM each). D: mean recovery (±SD) of ΔΨ and matrix volume after 5 ADP pulses (50 μM) for the 4 cases.

Nonionic Versus Ionic Matrix Volume Regulation

We used light scattering to track matrix volume changes, based on previous observations that light scattering correlates with matrix volume shifts with no obvious effect on total mitochondrial volume (15, 28). In the original studies, a single 250 μM ADP pulse was added to a relatively large amount of mitochondria (>0.5 mg/ml), and ADP-induced changes were determined in parallel by electron microscopy and correlated with light scattering. Although alternative explanations for light scattering changes, such as adenine nucleotide translocator conformational changes (5), have been proposed, they remain controversial (12). Our electron micrographs shown in Fig. 4 confirm directly that light scattering changes were associated with dramatic matrix volume shifts without affecting total mitochondrial volume.

On the basis of the observation that similar light scattering changes in response to ADP occurred in sucrose or mannitol as well as ionic buffer, an independent intrinsic mechanism for matrix volume regulation was initially proposed (15). Although the basis of this intrinsic mechanism is not clearly understood, other studies showed that the ionic environment can have a strong impact on these volume changes, and a relatively low K⁺ concentration (0.4 mM) was effective at significantly accelerating matrix volume recovery after ADP, provided that valinomycin was added to open an additional K⁺ influx pathway (28). Our data suggest that, although mitochondria were able to recover from ADP-induced matrix shrinkage in nominally ion-free medium, this intrinsic ability was limited when respiratory chain power is reduced. We demonstrated that in the presence of cations, the ability of mitochondria to phosphorylate ADP increases in the order of Li⁺ < Na⁺ < K⁺. Furthermore, this K⁺-dependent increase seemed to be especially important in mitochondria with a high ADP load and decreased ability to support ΔΨ. The matrix volume changes that we observed consistently paralleled changes in ΔΨ, as expected if related to ΔΨ-driven K⁺ (and Pi) uptake followed by H₂O uptake and matrix swelling. O₂ consumption was also impaired when matrix volume was excessively reduced by ADP pulses. Increased osmolarity outside mitochondria has been known for a long time to shrink matrix volume, inhibit swelling, and impair substrate oxidation (32), especially with fatty acids as substrates (16). Matrix condensation has been shown to impair diffusion of metabolite-sized fluorescent...
K⁺-DEPENDENT REGULATION OF MATRIX VOLUME

In the presence of Mg²⁺, ΔΨ and matrix volume recovery after ADP pulses were significantly worse in fatty acid-energized mitochondria. This Mg²⁺ effect was rapidly reversible with EDTA. Mg²⁺ is known to decrease inner membrane permeability to cations like Li⁺, Na⁺, and K⁺ (22, 33) and is also endogenous inhibitor of mitoK<sub>ATP</sub> channels. Both Mg²⁺ and ATP are required to close mitoK<sub>ATP</sub> channels, and only then is diazoxide effective at opening channels (19). Mg²⁺ is not expected to rapidly enter the matrix, as demonstrated using Furaptra-loaded mitochondria (20), and P<sub>i</sub>-stimulated electrophoretic Mg²⁺ uptake had little effect on matrix-free [Mg<sup>2+</sup>] (21). Furthermore, increases in matrix-free [Mg²⁺] would be expected to inhibit the K⁺/H⁺ antiporter (12) and, as a result, favor matrix swelling. All these findings, together with the ability of EDTA to rapidly abolish effects of Mg²⁺-induced matrix shrinkage, suggest that this cation acts on cytoplasmic side of the inner membrane.

Implications for Mitochondrial Protection in the Setting of Ischemia-Reperfusion

To demonstrate the importance of K⁺-mediated matrix volume regulation in ADP phosphorylation, we performed most studies using a relatively low concentration of caproic acid as the sole exogenous substrate to limit respiratory chain power. In state 3, respiratory rates are expected to be lower in the presence of short-chain fatty acids compared with pyruvate, malate, and glutamate given together. In addition, octanoate (C<sub>8</sub>) has been shown to inhibit pyruvate oxidation by isolated mitochondria in state 3, an effect abolished with malate (6). Whatever the reason for the decreased ability to support ΔΨ...
during ADP phosphorylation, under those conditions matrix volume regulation becomes compromised and requires K+ flux into the matrix to prevent excessive shrinkage during sustained phosphorylation. In mitochondria energized with caproic acid or low concentration of pyruvate (0.2 mM), which failed to recover after repeated ADP pulses, provision of a full complement of complex I substrates (1.5 mM each of pyruvate, malate, and glutamate) to enhance respiratory chain power restored both ΔΨ and matrix volume. More directly relevant to ischemia-reperfusion, we showed in Fig. 9 that mitochondria subjected to anoxia/reoxygenation in the presence of elevated Mg2+ exhibited similar K+-mediated improvements in matrix volume regulation and ADP phosphorylation, even when a full complement of complex I substrates was present. In this setting, the ability of mitochondria to recover and support ΔΨ during ADP phosphorylation is decreased due to inhibition of respiratory chain complexes, loss of cytochrome c, and increased inner membrane leakiness (29). We show that, similar to mitochondria in which respiratory power is limited by substrate availability, K+-dependent volume regulation becomes critical for efficient ADP phosphorylation in this setting. Our findings are consistent with the study by Ozcan et al. (38), who reported that diazoxide attenuated the anoxia-induced decrease in ATP production by isolated heart mitochondria, energized with saturating concentrations of pyruvate and malate. Although this study was not designed to solve the controversy of whether the inner membrane contains the mitochondrial ATP-sensitive K+ channel, opening the mitochondrial ATP-sensitive K+ channel protects the ischemic heart. Am J Physiol Heart Circ Physiol 283: H284–H295, 2002.


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REFERENCES


