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Mechanisms by which opening the mitochondrial ATP-sensitive K^+ channel protects the ischemic heart

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Dos Santos, Pierre, Alicia J. Kowaltowski, Muriel N. Laclau, Subramanian Seetharaman, Petr Paucek, Sihem Boudina, Jean-Benoit Thambo, Liliane Tariosse, and Keith D. Garlid. Mechanisms by which opening the mitochondrial ATP-sensitive K^+ channel protects the ischemic heart. *Am J Physiol Heart Circ Physiol* 283: H284–H295, 2002. First published February 14, 2002; 10.1152/ajpheart.00034.2002.—Diazoxide opening of the mitochondrial ATP-sensitive K^+ (mitoK_{ATP}) channel protects the heart against ischemia-reperfusion injury by unknown mechanisms. We investigated the mechanisms by which mitoK_{ATP} channel opening may act as an end effector of cardioprotection in the perfused rat heart model, in permeabilized fibers, and in rat heart mitochondria. We show that diazoxide pretreatment preserves the normal low outer membrane permeability to nucleotides and cytochrome *c* and that these beneficial effects are abolished by the mitoK_{ATP} channel inhibitor 5-hydroxydecanoate. We hypothesize that an open mitoK_{ATP} channel during ischemia maintains the tight structure of the intermembrane space that is required to preserve the normal low outer membrane permeability to ADP and ATP. This hypothesis is supported by findings in mitochondria showing that small decreases in intermembrane space volume, induced by either osmotic swelling or diazoxide, increased the half-saturation constant for ADP stimulation of respiration and sharply reduced ATP hydrolysis. These effects are proposed to lead to preservation of adenine nucleotides during ischemia and efficient energy transfer upon reperfusion.

mitochondria; metabolism; creatine kinase; membrane transport; cytochrome *c*; ischemic preconditioning

THERE IS NOW GENERAL AGREEMENT that the mitochondrial ATP-sensitive K^+ (mitoK_{ATP}) channel plays a pivotal role in cardioprotection against ischemia-reperfusion injury (16, 17, 20, 21, 36, 64); however, little is known about the mechanism of this protection. It has been proposed that mitoK_{ATP} channel opening triggers protection by increasing the generation of reactive oxygen species (ROS) (13, 47), and this effect has now been demonstrated by several laboratories (10, 13, 44, 57). We proposed that the mitoK_{ATP} channel is also an end effector of protection (16), and many studies have confirmed that the mitoK_{ATP} channel is required to be open during the ischemic phase (11, 46, 58, 59, 64). Thus the mitoK_{ATP} channel is both a trigger and an end effector of cardioprotection, and these two roles are temporally and mechanistically distinct.

We have previously suggested that cardioprotection by mitoK_{ATP} channel opening is due in part to volume regulation, which serves to preserve the structure-function of the intermembrane space (IMS) and the low permeability of the outer membrane to nucleotides (31). Nucleotide transport across the outer membrane occurs primarily through the voltage-dependent anion channel (VDAC) (2, 34, 49, 50). We hypothesize that VDAC permeability to nucleotides is regulated in part by IMS volume, which in turn is regulated by K^+ flux across the inner membrane.

This study focuses on the end effector mechanisms by which an open mitoK_{ATP} channel protects the heart during ischemia and reperfusion. We show, in saponin-skinned rat heart fibers, that diazoxide, like ischemic preconditioning (IPC) (31), prevents ischemia-induced alterations in mitochondrial function, which include increased outer membrane permeability to nucleotides and cytochrome *c* (cytC). Studies on isolated mitochondria and saponin-skinned fibers strongly support the hypothesis that increasing matrix volume, due to mitoK_{ATP} channel opening, reduces the permeability of VDAC to nucleotides. The consequences of this effect are *1*) to reduce the rate of ATP hydrolysis during ischemia, thereby preserving total adenine nucleotide content, and *2*) to permit efficient energy transformation upon reperfusion, thereby preventing mitochondrial ROS production and irreversible damage to mitochondria.

MATERIALS AND METHODS

Langendorff perfusion. Male Sprague-Dawley rats weighing 350–375 g were anesthetized with 40 mg pentobarbital sodium injected intraperitoneally. The thorax was opened,

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and hearts were rapidly excised, immediately cooled in iced Krebs buffer, and perfused by an aortic canula delivering 37°C buffer at a constant pressure of 100 mmHg. Hearts were perfused with a modified phosphate-free Krebs-Henseleit solution containing (in mM) 118 NaCl, 5.9 KCl, 1.75 $CaCl_2$, 1.2 $MgSO_4$, 0.5 EDTA, 25 $NaHCO_3$, and 16.7 glucose. The high glucose concentration was employed to overcome possible limitations of glucose uptake by the cardiomyocytes. The perfusate was gassed with 95% O_2-5% CO_2, which resulted in a Po_2 above 600 mmHg at the level of the aortic canula and a buffer pH of 7.4. The pulmonary artery was transected to facilitate coronary venous drainage, and a left ventricular polyethylene apical drain was inserted through a left atrial incision to allow Thebesien venous drainage. Left ventricular pressure was monitored from a water-filled latex balloon placed through the left atrial appendage and connected to a pressure transducer. The volume of the balloon was adjusted to obtain a left ventricular diastolic pressure of 7 mmHg and kept constant throughout the entire experiment. Hearts were not paced and mechanical performance was evaluated as the product of heart rate and developed pressure (RPP).

Perfusion protocols. Four groups of hearts were studied ($n = 6$ in each group). The control group was perfused under well-oxygenated conditions for 85 min. The ischemia-reperfusion group was perfused under well-oxygenated conditions for 40 min, subjected to 30-min zero-flow ischemia, and then reperfused for 15 min. For the diazoxide group, 100 μM diazoxide was added to the perfusate 20 min before ischemia. For the diazoxide + 5 hydroxydecanoate (5-HD) group, 100 μM diazoxide and 300 μM 5-HD were added to the perfusate 20 min before ischemia. Diazoxide and 5-HD were not added to the perfusate during reperfusion.

Permeabilized cardiac fibers. Mitochondrial function was assessed on permeabilized skinned fibers of the left ventricle obtained immediately at the end of the perfusion protocols. An additional group of hearts was studied immediately after excision of the organ without prior perfusion (in situ group, $n = 6$). Preparation of permeabilized cardiac fibers has been extensively described (28, 30, 54, 61). Briefly, small pieces of cardiac muscle were taken from the left ventricle and put into cold *solution A* (see below). They were rapidly dissected into bundles of fibers and incubated for 30 min with shaking in 1.8 ml *solution A* in the presence of 50 μg/ml saponin to selectively permeabilize the sarcolemma. The bundles were subsequently put in *solution B* twice for 10 min to wash out adenine nucleotides, phosphocreatine (PCr), and saponin. Respiration of skinned fibers (0.5–0.75 mg dry wt) was measured at 25°C using a Clark electrode in an oxygraphic cell containing either 2 ml *solution B* supplemented with 10 mM pyruvate, 5 mM malate, and 1 mg/ml BSA or 2 ml KCl solution. The solubility of oxygen was assumed to be 215 nmol O_2/ml. Additional experiments were performed on control fibers before and after addition of 8 nM nigericin, a K^+/H^+ antiporter. For these experiments, oxygraphic measurements were performed in *solution B* without BSA.

Solution A contained (in mM) 2.77 CaK_2EGTA, 7.23 K_2EGTA (pCa = 7), 6.56 $MgCl_2$, 0.5 dithiothreitol (DTT), 50 K-MES, 20 imidazole, 20 taurine, 5.3 Na_2ATP, and 15 PCr. pH = 7.1 and was adjusted at 25°C.

Solution B contained (in mM) 2.77 CaK_2EGTA, 7.23 K_2EGTA (pCa =7), 1.38 $MgCl_2$, 0.5 DTT, 50 K-MES, 20 imidazole, 20 taurine, and 3 KH_2PO_4. pH = 7.1 and was adjusted at 25°C.

KCl solution contained (in mM) 125 KCl, 20 HEPES, 10 pyruvate, 5 malate, 3 Mg acetate, 5 KH_2PO_4, 0.4 EGTA, and 0.3 DTT. pH = 7.1 and was adjusted at 25°C, and 2 mg/ml BSA was added.

Assessment of outer membrane permeability to cytC. State 2 respiration of skinned cardiac fibers was measured in KCl solution, and respiration was then stimulated by the addition of 1 mM ADP, which induced a maximum activation of respiration (state 3). cytC was added at a final concentration of 8 μM. In KCl medium, endogenous cytC dissociates from the outer surface of the inner mitochondrial membrane, but continues to support maximal respiration as long as the outer membrane retains its impermeability to cytC (6, 24). In this condition, addition of exogenous cytC will have no effect on respiration. If the outer membrane has become permeable to cytC, some cytC will be lost from mitochondria, and addition of cytC will increase the respiratory rate (6, 28, 31).

Determination of the half-saturation constant of ADP. Respiration of skinned cardiac fibers was measured in *solution B* containing pyruvate and malate. Increasing amounts of ADP ranging from 0.0125 to 1 mM were successively added. The stimulatory effect of ADP was calculated from respiration rates measured in the presence of ADP minus the value in the absence of ADP (state 2). The half-saturation constant for ADP [$K_{1/2}$ (ADP)] was calculated from double-reciprocal plots of respiration versus ADP concentration in the presence and absence of 20 mM creatine (Cr).

Bioenergetic studies on isolated rat heart mitochondria. Rat heart mitochondria were isolated by differential centrifugation from the hearts of three to four Wistar strain rats, as described by Kay et al. (27). Unless specified, respiration experiments were conducted at 30°C in *solution B*. K-MES concentrations were varied to obtain different osmolalities. $K_{1/2}$ (ADP) was determined as described for skinned fibers. Hexokinase (0.01 U/ml) and 1 mM glucose were present in all incubations to consume ATP generated by mitochondria (54).

ATP hydrolysis was determined using a luciferin/luciferase photodynamic system (35). Mitochondrial suspensions (0.5 mg/ml) were incubated for 90 s in *solution B* supplemented with 2 μM antimycin A and 200 μM ATP, after which ATP hydrolysis was stopped by the addition of oligomycin (2 μg/ml) and samples were frozen in liquid nitrogen. Mitochondria were thawed, the membranes were pelleted at 10,000 *g* for 2 min, and the ATP remaining in the supernatant was determined. Light emission was integrated for 60 s, 15 s after the addition of 187.5 μg/ml luciferin/luciferase (Sigma). Total ATP was determined in control preparations pretreated with oligomycin, and the results are expressed as the percent ATP remaining.

Mitochondrial membrane potentials ($\Delta\Psi$) were determined from the fluorescence changes of safranin O at excitation and emission wavelengths of 495 and 586 nm, respectively. Calibration was performed through K^+ distribution in a K^+-free, tetraethylammonium ion-based media, as previously described (1).

Measurements of mitochondrial volume. Matrix volume was manipulated by varying osmolality or by adding diazoxide. IMS volume was further manipulated by adding polyethylene glycol (PEG)-8000 (10% wt/vol), to which the outer membrane is impermeable (18). Medium osmolalities were determined from freezing point depression. Changes in matrix volume, which accompany net salt transport across the inner membrane, were followed using a quantitative light-scattering technique calibrated to mitochondrial matrix water content. This technique is based on the principle that reciprocal absorbance of the mitochondrial suspension, when corrected for the extrapolated absorbance at infinite protein concentration, is linearly related to matrix volume within well-defined regions, as described in detail by Beavis et al. (5). The osmolality at which the outer membrane begins to break was also determined from light-scattering measure-

ments as previously described (5). Matrix water content (in μlH_2O/mg protein) was determined in parallel experiments as sucrose-free pellet water, as previously described (5).

Statistical analysis of experimental data. Data are expressed as means ± SE. A two-way ANOVA for repeated measurements was performed to analyze hemodynamic parameters at different time points and under different experimental conditions. Single-factor ANOVA followed by an unpaired Student's *t*-test of the means was used to investigate respiration parameters. A value of $P < 0.05$ was considered statistically significant.

RESULTS

Hemodynamic data. The results in Fig. 1 show that the control hearts were stable, with constant diastolic pressure and <15% decrease in RPP over the 85-min perfusion period. Ischemia resulted in cardiac arrest. Ischemic contracture was observed after 10 min of ischemia with a maximum of 63 ± 4 mmHg ($P < 0.001$) measured after 25 min of ischemia. After 15 min of reperfusion, only 4% recovery of systolic function was observed, with a maximum RPP of 1,400 ± 800 mmHg·beats·min^{-1} ($P < 0.001$). These data show the early occurrence of contracture during ischemia and a poor early recovery of systolic function after reperfusion.

Pretreatment with 100 μM diazoxide induced a slight, but insignificant, decrease in RPP. Ischemia resulted in cardiac arrest, but diazoxide treatment prevented the occurrence of contracture. After 15 min of reperfusion, 57% recovery of systolic function was observed, with a mean RPP value of 17,000 ± 2,700 mmHg·beats·min^{-1} ($P < 0.01$ vs. the ischemic group). Despite the absence of ischemic contracture, a significant increase of left ventricular diastolic pressure was observed upon reperfusion. Overall, these data show that diazoxide completely prevented ischemic contracture and significantly improved postischemic recovery of systolic function. These cardioprotective effects were abolished when 300 μM 5-HD was included with the diazoxide. Ischemic contracture was similar to that observed in the ischemia-reperfusion group, and recovery of RPP upon reperfusion was significantly decreased compared with the diazoxide group (9,000 ± 2,000 mmHg·beats·min^{-1}, $P < 0.05$).

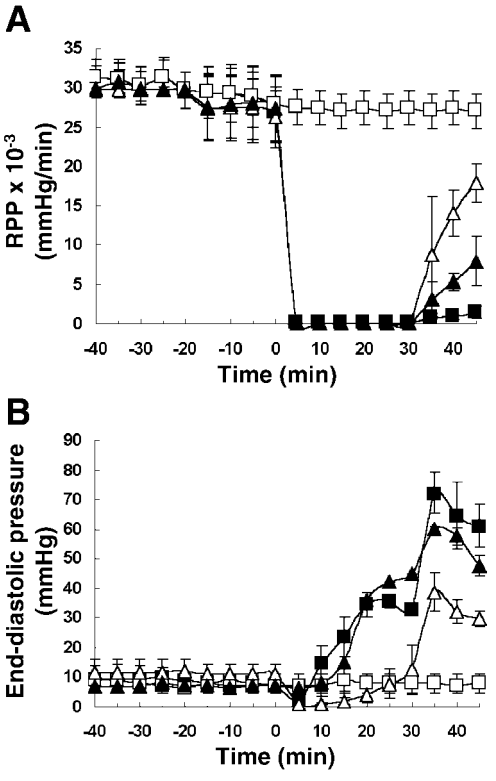

Fig. 1. Rat heart perfusion: hemodynamic data. *A*: left ventricular rate-pressure product (RPP). RPP was measured in control (□), ischemic (■), diazoxide-treated (△), and diazoxide + 5-hyroxydecanoate (5-HD)-treated (▲) Langendorff-perfused rat hearts. *B*: end-diastolic pressure. Symbols are the same as in *A*.

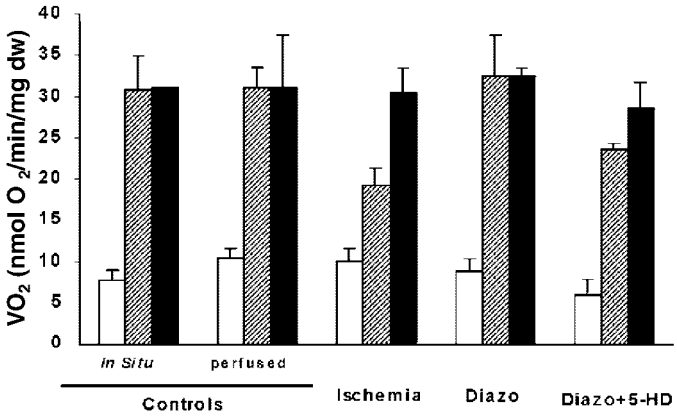

Fig. 2. Permeability of the outer mitochondrial membrane to cytochrome *c* (cytC). Shown are respiration values [V_{O_2}; in nmol O_2·min^{-1}·mg dry wt (dw)$^{-1}$] in KCl medium in the absence of ADP (open bars), in the presence of 1 mM ADP (hatched bars), and in the presence of 1 mM ADP + 8 μM cytC (solid bars). Respiration was evaluated in permeabilized fibers obtained immediately after heart excision (in situ control), after 90 min of normoxic Langendorff perfusion (perfused control), after 30 min of ischemia followed by 15 min of reperfusion (ischemia), after diazoxide perfusion before ischemia-reperfusion (Diazo), and after diazoxide + 5-HD perfusion before ischemia-reperfusion (Diazo + 5-HD).

Permeability of the outer mitochondrial membrane to cytC. We measured the effect of 8 μM exogenous cytC on the maximal rate of respiration determined at 1 mM ADP, as described in MATERIALS AND METHODS, with the results shown in Fig. 2. Maximal respiration in situ and in perfused control groups was 31 ± 4 and 31 ± 3 nmol O_2·min^{-1}·mg dry wt^{-1}, respectively, and neither group was stimulated by cytC. There was a small increase in state 2 respiration in perfused versus in situ controls hearts (10.4 and 7.7 nmol O_2·min^{-1}·mg dry wt^{-1}, respectively, $P = 0.15$); however, respiration after inhibition of the ADP-stimulated respiration by oligomycin (15 μM) and atractyloside (13 μM) were the same in both control groups (data not shown). Thus the perfusion protocol per se did not significantly modify respiratory function or outer membrane permeability to cytC.

After 30 min of ischemia followed by 15 min of reperfusion, maximal respiration was significantly decreased by 38% compared with the control perfused group ($P < 0.01$). In these fibers, addition of exogenous

cytC stimulated respiration back to control values. It is noteworthy that ischemia followed by reperfusion had no effect on state 2 respiration, indicating that the mechanism of cytC permeabilization does not involve opening the permeability transition with gross rupture of the outer membrane.

In the diazoxide group, maximal respiration was the same as that observed in the control perfused group and was significantly higher than in the ischemia group without diazoxide. Moreover, exogenous cytC did not stimulate respiration in these fibers, showing that perfusion with diazoxide protects mitochondria against ischemia-induced outer membrane permeabilization to cytC. In the diazoxide + 5-HD group, maximal respiration was decreased by 35% compared with the control and diazoxide groups and was similar to that obtained in the ischemic group. Exogenous cytC induced a significant 16% stimulation of the maximal respiration rate in the presence of ADP ($P < 0.01$). These data show that 5-HD almost completely abolished the protective effects of diazoxide on outer membrane permeability.

Regulation of respiration of permeabilized cardiac fibers by ADP and Cr. The data shown in Fig. 3 contain $K_{1/2}$ (ADP) values determined from double reciprocal plots in the presence or absence of Cr, as described in MATERIALS AND METHODS. In in situ and perfused control hearts, $K_{1/2}$ (ADP) values were 395 ± 75 and 327 ± 45 μM, respectively [not significant (NS)]. In the presence of Cr, these values decreased to 65 ± 23 and 85 ± 24 μM, respectively (NS). This is due to the presence in the IMS of the mitochondrial isoform of creatine kinase (Mi-CK), which rapidly phosphorylates Cr with freshly synthesized ATP. The ADP formed returns to the matrix, and the creatine phosphate is exported to the cytosol.

In fibers from ischemic hearts, $K_{1/2}$ (ADP) decreased dramatically to 92 ± 7 μM. In the presence of Cr, $K_{1/2}$ (ADP) decreased to 64 ± 7 μM, a value similar to those obtained in both control groups. In the diazoxide-treated hearts, $K_{1/2}$ (ADP) remained high, with a value of 266 ± 66 μM. This value is statistically the same as the value measured in fibers from control perfused hearts and reflects the low permeability of the outer mitochondrial membrane for ADP. Addition of Cr resulted in a decrease in $K_{1/2}$ (ADP) to 51 ± 14 μM. Fibers treated with diazoxide + 5-HD reproduced the data obtained in the ischemia-reperfusion group, with a decrease in $K_{1/2}$ (ADP) to 42 ± 6 μM and with no further decrease in the presence of Cr. These data show that perfusion of diazoxide before ischemia-reperfusion preserves adenine nucleotide compartmentation and functional coupling. Abolition of these effects by 5-HD indicates that they are linked to the opening of mitoK$_{ATP}$ channels.

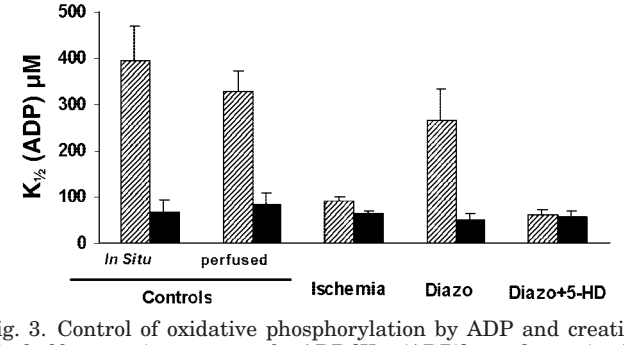

Fig. 3. Control of oxidative phosphorylation by ADP and creatine. The half-saturation constant for ADP [$K_{1/2}$ (ADP)] was determined in the presence (solid bars) or absence (hatched bars) of 20 mM creatine. Respiration was evaluated in permeabilized fibers, and the experimental groups are the same as those defined in Fig. 2.

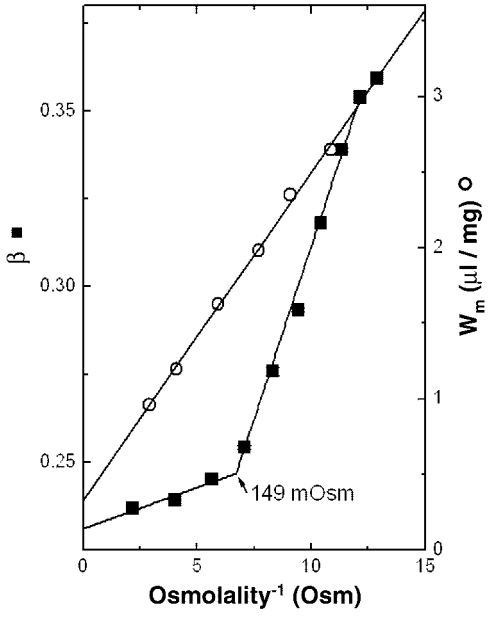

Fig. 4. Osmotic behavior of rat heart mitochondria. Rat heart mitochondria (0.1 mg/ml) were incubated in media containing 5 mM HEPES (pH 7.2), 0.1 mM EGTA, 0.2 μg/ml rotenone, and concentrations of KCl to obtain the osmolalities indicated. Total mitochondrial volume from light scattering (β) and matrix water content (W_m) were measured as described in MATERIALS AND METHODS. β is defined as $[A^{-1} - A(\infty)^{-1}]$. The β-values shown represent the means of 3 independent measurements. W_m values are the means of 2 separate experiments.

Osmotic behavior of mitochondria. The mitochondrial inner membrane is highly permeable to water, and mitochondria therefore behave as osmometers, responding instantly to a change in the osmotic strength of the medium (14). The osmotic curves shown in Fig. 4 were obtained from rat heart mitochondria using two different techniques: radioisotope distribution to measure matrix volume and light scattering to estimate total particle volume (5). The data shown in Fig. 4 are qualitatively the same as those obtained previously for rat liver mitochondria (5), and we interpret the results in the same way. The osmotic curve for matrix volume is linear over the entire range, and it is also reversible (data not shown). In contrast, the osmotic curve for total volume is segmentally linear and exhibits a sharp transition at 149 ± 2 mosM ($n = 3$), or 1.9 μl matrix H_2O/mg mitochondrial protein. At osmolalities higher than 149 mosM, this osmotic curve is also reversible in both liver (5) and heart (data not shown) mitochondria.

Stoner and Sirak (56) showed that matrix swelling-contraction in this isosmotic range occurs at the expense of the IMS. The transition at 149 mosM occurs when matrix volume exceeds that which can be contained within the outer mitochondrial membrane and reflects the volume at which the outer membrane begins to rupture due to excessive matrix swelling (5). As expected, the light scattering osmotic curve is no longer reversible after the outer membrane breaks (5).

It should be noted that the osmolality at which the transition point occurs depends on conditions of isolation and storage. For example, when K^+ efflux is retarded by including 0.5 mM quinine in the isolation and storage buffer media to inhibit the K^+/H^+ antiporter (41), a higher matrix volume is maintained, and the transition shifts to higher osmolalities (data not shown).

It is known that the mitochondrial matrix is fully expanded in vivo, with a narrow IMS, whereas the matrix is highly contracted in vitro (4). This is reflected in the data shown in Fig. 4, in which the outer membrane does not break until the medium is made considerably hypotonic. Indeed, perhaps the most important and least appreciated isolation artifact of mitochondria is the matrix contraction caused by loss of potassium (via K^+/H^+ exchange) and anions during isolation in K^+-free medium (12). As shown diagrammatically in Fig. 5, the matrix contraction of isolated mitochondria can readily be reversed in vitro by two independent means: changing the osmotic strength of the medium or through respiration-driven uptake of K^+ salts. These two methods, which will be applied in subsequent sections, are additive and entirely equivalent.

Effects of mitoK_{ATP} channel openers and blockers on matrix volume. Figure 6*A* shows the changes in steady-state matrix volume due to opening and closing mitoK_{ATP} channels in isolated rat heart mitochondria.

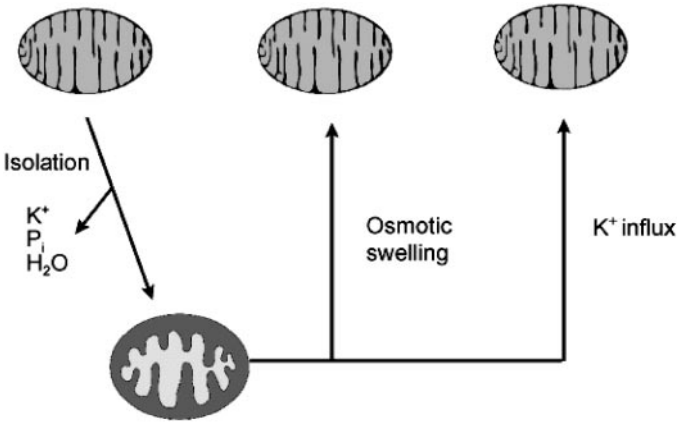

Fig. 5. Restoration of matrix and intermembrane space (IMS) volumes after mitochondrial isolation. The diagram illustrates the loss and restoration of matrix volume after isolation. K^+ salts and water are lost from the matrix during isolation in K^+-free sucrose (12). This results in matrix contraction and reciprocal expansion of the IMS. This isolation artifact can be reversed by 2 independent and equivalent methods: decreasing the osmolality of the system or allowing respiration to drive reuptake of K^+. In either case, excessive matrix swelling will rupture the outer membrane.

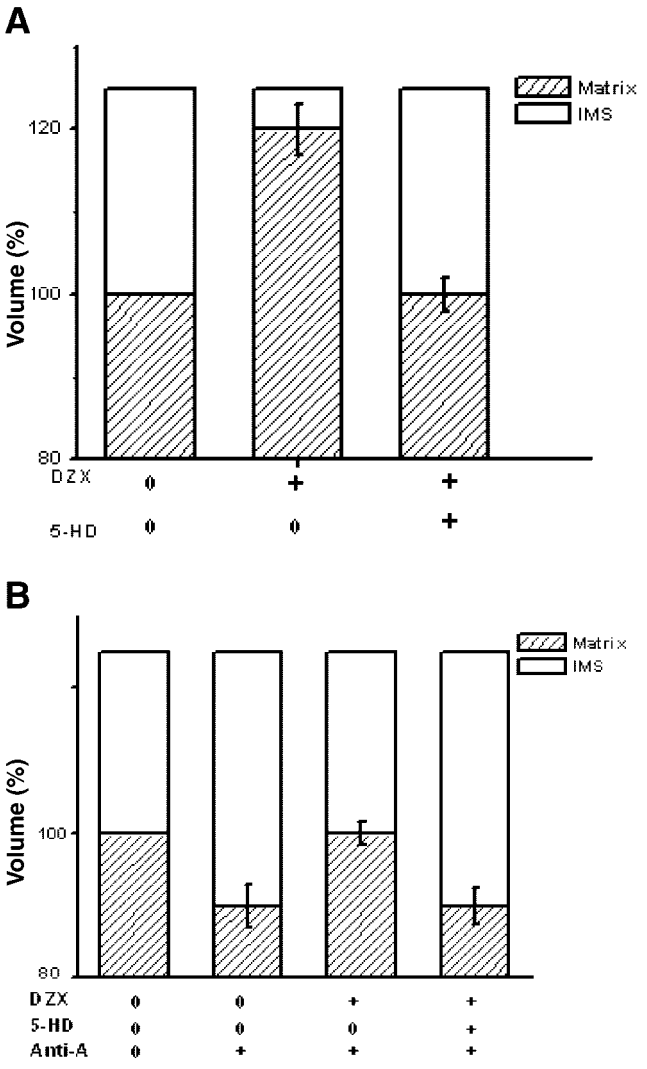

Fig. 6. The effect of diazoxide (DZX) on matrix volume. *A*: during state 2 respiration. Shown are relative steady-state matrix volumes of rat heart mitochondria (0.1 mg/ml) incubated for 4 min in 0.5 mM $MgCl_2$, 0.5 μg/ml oligomycin, 0.2 μg/ml rotenone, and K^+ salts of 135 mM Cl^-, 5 mM succinate, 2.5 mM phosphate, 200 μM ATP, and 100 μM EGTA; pH 7.2. Where indicated, 30 μM diazoxide and 300 μM 5-HD were added. The results shown represent the means ± SE of at least 3 independent experiments. *B*: during simulated ischemia. Shown are relative steady-state matrix volumes of rat heart mitochondria (0.1 mg/ml) that were preincubated for 5 min in *solution B* (see MATERIALS AND METHODS) supplemented with 2 mM K^+ malate and 5 mM K^+ pyruvate. Where indicated, 2 μM antimycin A (Anti-A), 30 μM diazoxide, and 300 μM 5-HD were added. Steady-state values were attained within 4 min. The results shown represent the means ± SE of at least 3 independent experiments.

It can be seen that diazoxide caused a 15–20% increase in steady-state volume, and this effect was blocked by 5-HD. We have found that diazoxide has little effect on respiration, membrane potential, or Ca^{2+} uptake in isolated mitochondria due to the relatively low K^+ flux catalyzed by mitoK_{ATP} channels (29); however, Marban and co-workers (36, 39) propose that mitoK_{ATP} channel opening in vivo causes sufficient K^+ cycling to uncouple mitochondria, leading to reduced Ca^{2+} uptake and cardioprotection. While this issue remains unresolved,

our conclusion that the increased K^+ cycling caused by mitoK_{ATP} channel opening is insufficient to cause uncoupling (29) is supported by a variety of in vivo and in situ studies: Standen and co-workers (33) observed diazoxide cardioprotection in cardiomyocytes with no detectable change in FAD fluorescence or in mitochondrial $\Delta\Psi$. Carroll et al. (7) observed diazoxide-induced mitochondrial swelling without effects on $\Delta\Psi$ in human atrial cells. Grover et al. (22, 23) showed that cardioprotective concentrations of K_{ATP} channel openers have no effect on the cardiac efficiency of oxygen utilization in the intact heart, which appears to exclude significant uncoupling. Ventura-Clapier and co-workers (45) showed that moderate doses of diazoxide had no detectable bioenergetic effects in permeabilized fibers.

The conditions shown in Fig. 6*B* are intended to simulate the ischemic state. Antimycin A was used to block mitochondrial respiration, and membrane potential was supported solely by ATP hydrolysis. Antimycin A caused a contraction in matrix volume that was reversed by diazoxide. 5-HD blocked the diazoxide effect. These data reflect a fundamental difference in the effects of opening mitoK_{ATP} channels in the resting heart versus the ischemic heart. In the former case, there is a net uptake of K^+ and water. In the latter case, opening mitoK_{ATP} channels prevents matrix contraction and maintains matrix and IMS volumes at near-normal levels.

Effects of matrix and IMS volumes on oxidative phosphorylation in heart mitochondria. The $K_{1/2}$ (ADP) is strongly influenced by outer membrane permeability to ADP. Saks et al. (52, 54) have shown that isolated mitochondria have a high affinity for ADP, with $K_{1/2}$ (ADP) ~20 μM, whereas the value in situ is much higher, ~250 μM. We undertook to determine whether this difference may arise in part from the artifactual contraction of the matrix (and expansion of the IMS) caused by isolation (Fig. 5). Indeed, the $K_{1/2}$ (ADP), determined as illustrated in Fig. 7, was found to increase significantly as a result of a small change in medium osmolality. Decreasing medium osmolality from 243 to 212 mosM caused $K_{1/2}$ (ADP) to increase from 17.1 to 71.3 μM (Fig. 8*A*, open bars). When osmolality was decreased below the point of outer membrane rupture, $K_{1/2}$ (ADP) returned to the level observed with contracted mitochondria. After outer membrane rupture, the $K_{1/2}$ (ADP) was no longer affected by changes in osmolality in the 145–243 mosM range (data not shown). In these experiments, mitochondria are taking up K^+ salts and water rapidly during the 1–2 min of incubation before the first addition of ADP, and therefore the outer membrane will rupture at higher osmolalities than those observed in the equilibrium experiments shown in Fig. 4. We believe that this accounts for the finding that the volume effect shown in Fig. 8*A* reached a maximum at 212 mosM. Diazoxide also caused an increase in $K_{1/2}$ (ADP) (Fig. 8*A*, hatched bars), and this effect was additive with hypotonic swelling until a maximum was achieved at 85 ± 3 μM. These results demonstrate that restoration

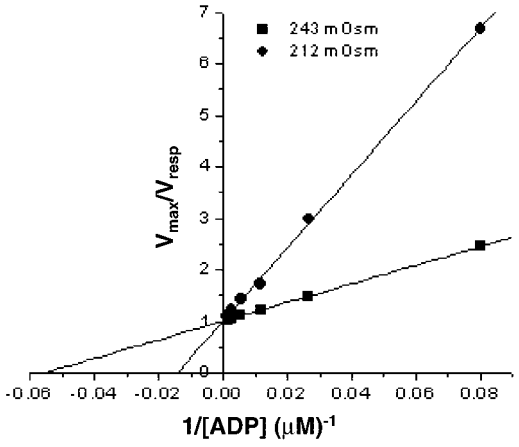

Fig. 7. Increasing the matrix volume decreases the mitochondrial affinity for ADP. Rat heart mitochondria (0.5 mg/ml) were incubated in *solution B* (see MATERIALS AND METHODS) supplemented with 0.01 U/ml hexokinase and 1 mM glucose at the osmolalities shown. ADP-stimulated respiration (V_{resp}) was determined at different ADP concentrations and normalized for the maximum ADP-stimulated respiration (V_{max}), and values of $K_{1/2}$ (ADP) were determined from double-reciprocal plots such as those illustrated. In this experiment, lowering osmolality from 243 to 212 mosM caused $K_{1/2}$ (ADP) to increase from 17.8 to 71.5 μM. V_{max} increased slightly, due to volume-activation of electron transport, from 162 to 176 ng atom $O \cdot min^{-1} \cdot mg^{-1}$ (data not shown).

of matrix and IMS volumes results in lower affinity for ADP, reflecting a restoration of the low nucleotide permeability of the outer membrane.

Although the data shown in Fig. 8*A* demonstrate a fivefold increase in $K_{1/2}$ (ADP) as a result of restoring matrix volume, the values remain lower than those observed in situ (Fig. 3). Macromolecules are lacking in the studies shown in Fig. 8*A*, and Gellerich et al. (19) reported that macromolecules could cause selective contraction of the IMS and maintenance of intermembrane contact sites similar to those observed in vivo. Accordingly, we repeated the experiments in media containing 12.5 mM PEG and obtained the results shown in Fig. 8*B*. In 243 mosM medium, $K_{1/2}$ (ADP) increased from 17 to 127 μM, confirming previous findings of the effect of macromolecules on ADP affinity (18, 32, 66). Moreover, the $K_{1/2}$ (ADP) increased further by incubation in solutions of lower osmolality, achieving a value of 250 μM, which is similar to values obtained in situ by Saks et al. (52, 54). As was observed in the absence of PEG, diazoxide-induced K^+ uptake increased the $K_{1/2}$ (ADP) at higher osmolalities, $K_{1/2}$ (ADP) returned to low values after outer membrane rupture, and $K_{1/2}$ (ADP) was no longer sensitive to changes in matrix volume after outer membrane rupture.

These results appear to suggest that mitochondrial affinity for ADP is decreased by IMS contraction caused by incubation in high-molecular-weight solutes (66); however, we were unable to confirm this suggestion. We found that PEG increased $K_{1/2}$ (ADP) from 17 μM (Fig. 8*A*) to 100 μM (Fig. 8*B*) even after the outer membrane was osmotically broken in 145 mosM media. Moreover, digitonin-treated mitochondria incubated in 212 mosM media with PEG exhibited a $K_{1/2}$

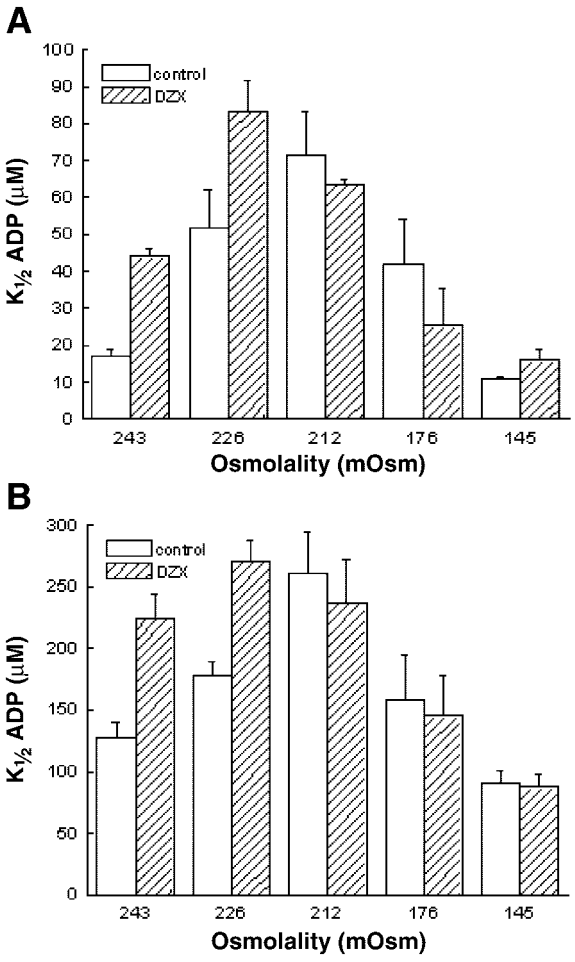

Fig. 8. In vitro regulation of mitochondrial affinity for ADP by matrix volume. The $K_{1/2}$ (ADP) was measured at the osmolalities shown, as described in Fig. 7. The hatched bars represent experiments conducted in the presence of 30 μM diazoxide. *A*: experiments were performed in the absence of polyethylene glycol (PEG)-8000 (nominal MW 8000). *B*: experiments were performed in the presence of 12.5 mM PEG-8000. In both media, mild osmotic swelling caused progressive increases in $K_{1/2}$ (ADP), and diazoxide-mediated K^+ influx provided an additional effect at each osmolality. At 145 mosM, when the outer membrane was broken, $K_{1/2}$ (ADP) returned to control values. The results shown are the means ± SE of at least 3 repetitions at each osmolality.

(ADP) of 92 ± 13 μM (n = 3), a value similar to that obtained in osmotically swollen mitochondria incubated in PEG but higher than that seen in media devoid of PEG. These results indicate that the PEG-induced increase in $K_{1/2}$ (ADP) may not be caused by changes in IMS volume but rather are due to a macromolecule effect, such as binding of ADP to PEG.

To further confirm that changes in matrix and IMS volumes influence the $K_{1/2}$ (ADP), we carried out experiments on skinned fibers in the presence or absence of 8 nM nigericin, with the results shown in Fig. 9. At this low concentration, nigericin, which is a K^+/H^+ antiporter, will cause a mild matrix contraction due to net loss of K^+ until a new, lower steady-state volume is achieved, with consequent expansion of the IMS. Nigericin had little effect on state 2 respiration or on V_{max} (Fig. 9*A*); however, nigericin caused a large (70%) decrease in $K_{1/2}$ (ADP) (Fig. 9*B*), confirming that matrix and IMS volumes also control $K_{1/2}$ (ADP) in skinned fibers. Note also in this experiment that removal of BSA from the medium caused a decrease in $K_{1/2}$ (ADP) from ~400 to 200 μM, showing that the macromolecule effect is also observed in situ.

In summary, these findings on the effect of restoring matrix volume in isolated mitochondria on $K_{1/2}$ (ADP) show *1*) that opening the mitoK_{ATP} channel affects ADP compartmentation, *2*) that this effect is due to small changes in matrix volume, and *3*) that an intact outer membrane is necessary for the effect.

Effects of volume on ATP hydrolysis in ischemic heart mitochondria. Given that volume regulation strongly affects outer membrane permeability to ADP (Figs. 8 and 9) and that mitoK_{ATP} channels can regulate mitochondrial volume even under conditions of ischemia (Fig. 6*B*), we hypothesized that volume regulation should also affect ATP compartmentation and hydrolysis during ischemia. The data shown in Fig. 10 summarize an extensive series of measurements of ATP hydrolysis by nonrespiring mitochondria. Moderate re-

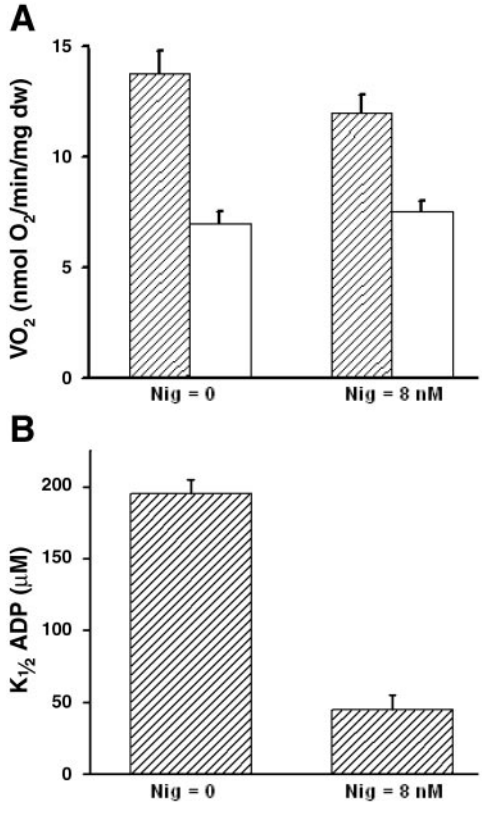

Fig. 9. Matrix contraction decreases $K_{1/2}$ (ADP) in permeabilized fibers. Respiration was evaluated in permeabilized fibers obtained immediately after isolation of the rat heart. *A*: respiration. Shown are the effects of 8 nM nigericin (Nig) on ADP stimulated respiration (V_{max} − V_O) (hatched bars) and on state 2 respiration (V_O) (open bars). V_{max} is respiration in the presence of 2 mM ADP. Nigericin had no significant effect on either value. *B*: $K_{1/2}$ (ADP). $K_{1/2}$ (ADP) was determined in the presence or absence of 8 nM nigericin in *solution B* in the absence of BSA. The results shown are the means ± SE of 4 different preparation.

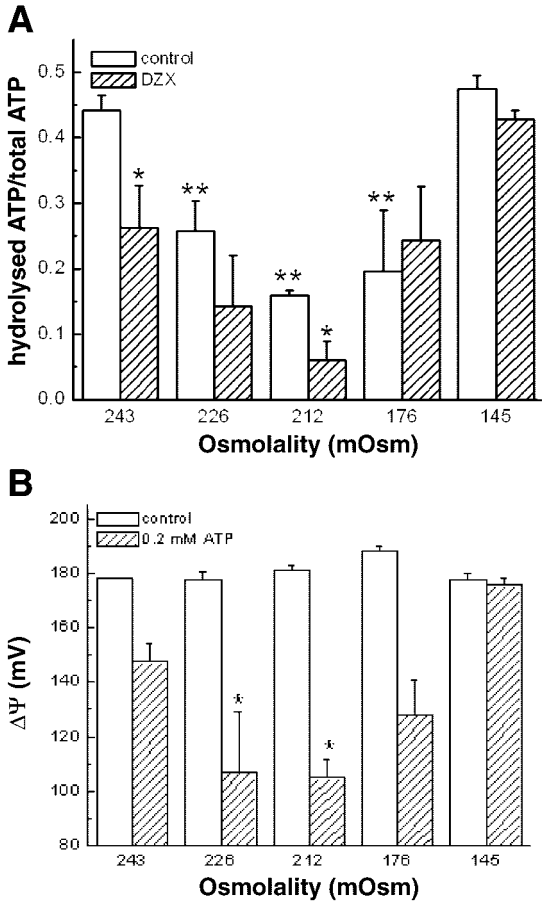

Fig. 10. In vitro regulation of ATP hydrolysis and membrane potential by matrix volume. Rat heart mitochondria (0.5 mg/ml) were incubated in *solution B* supplemented with 200 μM ATP and 2 μM antimycin A at the osmolalities shown. *A*: ATP hydrolysis. ATP hydrolysis after 90 s was determined as described in MATERIALS AND METHODS. Experiments were performed in the absence (open bars) or presence (hatched bars) of 30 μM diazoxide. Mild osmotic swelling progressively reduced ATP hydrolysis, and diazoxide provided additional reduction at each osmolality. At 145 mosM, when the outer membrane was broken, rates of ATP hydrolysis returned to control values. The results shown represent the means ± SE of at least 3 repetitions. Each bar represents data collected from 5 individual experiments ± SE. $^*P < 0.01$ compared with the control at equal osmolality; $^{**}P < 0.01$ compared with control in 243 mosM media. *B*: membrane potential. Mitochondrial membrane potentials (ΔΨ) were determined after 90-s incubation at the osmolalities shown in the absence (open bars) or presence (hatched bars) of 200 μM ATP and 2 μM antimycin A as described in MATERIALS AND METHODS. Each bar represents data collected from 3 individual experiments ± SE. $^*P < 0.01$ compared with open bar at 243 mosM.

ductions in osmolality, with consequent IMS contraction, caused profound reductions in ATP hydrolysis. At 243 mosM, diazoxide reduced ATP hydrolysis by ~45%. At 212 mosM, diazoxide caused a nearly 90% reduction in ATP hydrolysis compared with controls. ATP hydrolysis returned to control values after outer membrane rupture, which resulted in complete loss of the volume sensitivity of ATP hydrolysis (data not shown). As stated in connection with the experiments shown in Fig. 8*A*, we interpret the finding that ATP hydrolysis reached a minimum at 212 mosM as being due to the fact that both hypotonicity and uptake of K^+ salts and water are taking place simultaneously, causing the outer membrane to rupture at higher osmolalities than those observed in the equilibrium experiments shown in Fig. 4. From these studies, we draw conclusions similar to those relating to the volume effect on $K_{1/2}$ (ADP): *1*) opening mitoK$_{ATP}$ channels reduces ATP hydrolysis, *2*) this effect is due to small changes in matrix volume, and *3*) an intact outer membrane is necessary for the effect.

Finally, to determine whether the reduced ATP hydrolysis in mitochondria with restored matrix and IMS volumes is due primarily to reduced inner membrane ion permeability or to a reduction in ΔΨ itself, we measured ΔΨ supported either by respiration (Fig. 10*B*, open bars) or by ATP hydrolysis in the presence of a respiratory chain inhibitor (Fig. 10*B*, hatched bars). We observed that osmotic swelling of mitochondria had no effect on ΔΨ supported by respiration; however, it caused a profound decrease of ΔΨ supported by ATP hydrolysis. On this basis, we conclude that restoration of matrix volume causes a depolarization of ΔΨ, which reduces the inner membrane proton leak that is responsible for ATP hydrolysis. The mechanism of this effect is addressed in the DISCUSSION.

DISCUSSION

This study confirms previous work (16) showing that diazoxide treatment of Langendorff-perfused rat hearts before ischemia improves recovery of cardiac function after reperfusion, delays the onset of contracture, and decreases the amplitude of contracture. The hypothesis that the mitoK$_{ATP}$ channel is the receptor for the cardioprotective actions of K$_{ATP}$ channel openers (17) implies that important aspects of protection occur at the mitochondrial level. Our studies on permeabilized fibers reveal three components of mitochondrial protection by diazoxide: *1*) reduced permeability to exogenous cytC (Fig. 2); *2*) maintenance of low outer membrane permeability to nucleotides, reflected in the high values of $K_{1/2}$ (ADP) in the regulation of respiration (Fig. 3); and *3*) preservation of functional coupling between the ATP/ADP translocator and Mi-CK, reflected in the increased affinity for ADP in the presence of Cr (Fig. 3). The same components of mitochondrial protection were previously described for IPC (31).

To understand these effects, it is necessary to consider the complex sequence of events that follow diazoxide administration. MitoK$_{ATP}$ channel opening in the resting state of the cardiomyocyte, in which oxygen consumption is low and ΔΨ is high, will cause a modest increase in mitochondrial K^+ influx and matrix expansion, as shown in Fig. 6*A*. As we have previously reported (13), the K^+ influx causes a moderate increase in mitochondrial production of ROS, which, in turn triggers the cardioprotective signaling pathway (47). The observation that mitoK$_{ATP}$ channel opening induces ROS production has now been reported by several laboratories (7, 10, 44, 57). We have proposed a

mechanism for this effect (3) and will address this aspect in more detail in future communications.

We hypothesize that the cardioprotective signaling pathway causes two changes in mitochondria, perhaps by phosphorylating mitochondrial proteins: the outer membrane retains its impermeability to cytC and the mitoK_{ATP} channel is opened. It has been proposed that disruption of contact sites between inner and outer membranes promotes cytC release (9), suggesting that mitoK_{ATP} channel opening may mediate this protective effect by preventing contact site disruption. However, it is equally likely that the prevention of cytC release occurs by independent mechanisms. Outer membrane permeability to cytC is known to be controlled by the Bcl-2 family of proteins (65), and there is evidence that translocation of activated Raf-1 to mitochondria protects the cells from apoptosis via regulation of Bcl-2 activity (42, 43, 48) and also that phosphorylation of proapoptotic Bid prevents apoptosis (8). One or both of these mechanisms may be triggered independently by the cardioprotective signaling pathway. We propose that cell signaling causes mitoK_{ATP} channels to be open during ischemia, so that it can act an end effector of cardioprotection (16). Pain et al. (47) have questioned the end effector role of mitoK_{ATP} channels based on the finding that 5-HD administered 5 min before the test ischemia failed to block diazoxide protection. However, Wang et al. (64) found that a higher dose of 5-HD was required at this stage and that 5-HD did block protection when administered after diazoxide washout. Indeed, many studies show that the mitoK_{ATP} channel is required to be open during the ischemic phase (11, 46, 58, 59, 64). The results of a recent study by Tsuchida et al. (59) are particularly convincing in this regard. They show that diazoxide reduced infarct size even when administered after the onset of ischemia, provided that diazoxide was added before the development of necrosis. Several studies indicate that infarct size is reduced even when the mitoK_{ATP} channel is opened at the time of reperfusion (38, 58, 60).

There is evidence that IPC and K^+ channel openers specifically protect mitochondrial function during ischemia. Protected hearts exhibit a reduced rate of ATP loss during ischemia (22, 37, 40). Mitochondrial $\Delta\Psi$ is lower during ischemia, leading to reduced Ca^{2+} accumulation (63, 67), which has been identified as being important for cardioprotection (25, 36). Jennings et al. (26) have shown that adenine nucleotides are rapidly degraded during ischemia and that IPC retards the rate of degradation. This may be particularly important, because the cardiomyocyte cannot survive reperfusion if there is no ADP available to phosphorylate.

To explain these effects, we hypothesize that mitoK_{ATP} channel opening preserves the segregation of adenine nucleotides that normally exists between the mitochondrial and cytosolic compartments. Nucleotide permeability is controlled by VDAC, which is normally in a low conductance state that is poorly permeable to ATP (2, 34, 50), and energy transfers between mitochondria and cytosol are mediated instead by Cr and creatine phosphate (55). Octamers of Mi-CK bridge the IMS between outer membrane VDAC and inner membrane ATP/ADP translocator (62). We hypothesize that binding of octameric Mi-CK to VDAC confers a low conductance to nucleotides and that this binding requires a narrow intermembrane distance. When IMS expands due to matrix contraction, Mi-CK dissociates from VDAC, leading to a high conductance state.

Two measurements that reflect outer membrane permeability to nucleotides are the $K_{1/2}$ (ADP) for respiration (31) and the rate of ATP hydrolysis in nonrespiring mitochondria. The data shown in Figs. 7–10 show for the first time that changes in matrix volume have profound effects on both of these parameters and that an intact outer membrane is required for these effects. Diazoxide caused a massive reduction in the rate of ATP hydrolysis in rat heart mitochondria undergoing simulated ischemia (Fig. 10) and a large increase in $K_{1/2}$ (ADP) (Fig. 8). The effects of matrix volume on $K_{1/2}$ (ADP) were confirmed in skinned, intact cardiac fibers, in which a very low dose of nigericin, to contract the matrix and expand the IMS, caused a 70% drop in the $K_{1/2}$ (ADP) (Fig. 9). Finally, we show that ischemia causes a profound decrease in $K_{1/2}$ (ADP) in fibers after ischemia-reperfusion and that diazoxide prevents this loss of nucleotide compartmentation (Fig. 3*A*).

In order for mitoK_{ATP} channels to play a role during ischemia, it is necessary to postulate that the matrix contracts (and IMS expands) during the initial stages of ischemia and that mitoK_{ATP} channel opening prevents these volume changes. There is no direct experimental evidence for matrix contraction during early ischemia, but it must be noted that a 10% contraction may be too small to detect. Moreover, electron microscopic studies have generally focused on matrix swelling, which we view as a later, pathological event occurring in nonprotected cells. There are theoretical and experimental grounds for postulating ischemia-induced matrix contraction: mitochondria will depolarize due to lack of oxygen, causing a decrease in diffusive K^+ influx. Matrix volume will therefore contract until the K^+/H^+ antiporter is sufficiently inhibited to bring influx and efflux into balance, at which time matrix volume will achieve a new steady state at lower levels. These changes have been demonstrated in isolated heart mitochondria, as shown in Fig. 6*B* and Ref. 29. Therefore, we propose that an open mitoK_{ATP} channel during ischemia, by virtue of its role in regulating matrix and IMS volumes, conserves cellular adenine nucleotides by preserving the normal low conductance state of VDAC.

The mechanism of the volume-dependent decrease in ATP hydrolysis requires further scrutiny. ATP hydrolysis is determined by the rate of ion leaks (primarily of protons) across the inner membrane. Ion leaks, in turn, depend on $\Delta\Psi$ (15), and $\Delta\Psi$ is in equilibrium with the free energy of ATP hydrolysis (ΔG_P). Consequently, the extent of ATP loss at any given time during ischemia will depend on ΔG_P, and the only way to reduce Ca^{2+} uptake and ATP hydrolysis during ischemia is to lower mitochondrial ΔG_P to a greater extent than cytosolic ΔG_P. This is not possible when VDAC are in their high conductance state, because nucleotides are then in

equilibrium across the outer membrane. When mitoK_{ATP} channels are open, however, major perturbations in matrix and IMS volumes are prevented (Fig. 6*B*). The structure-function of the IMS is preserved, and VDAC will be maintained in the low conductance state. Nucleotides will not equilibrate across the outer membrane, and ATP hydrolysis will lead to accumulation of ADP and loss of ATP from the IMS. This will decrease mitochondrial ΔG_P and $\Delta\Psi$, causing reduced proton leak and, consequently, a decreased rate of ATP hydrolysis. This mechanism is confirmed by the finding that $\Delta\Psi$ declined much more rapidly when ATP hydrolysis was inhibited by mild matrix swelling (Fig. 10*B*). (It should be noted that the simultaneous decline of $\Delta\Psi$ and ATP hydrolysis excludes uncoupling as a cause of the decline in $\Delta\Psi$.) According to this hypothesis, the ability of mitoK_{ATP} channel opening to reduce ATP hydrolysis derives from segregation of ATP and ΔG_P between the mitochondrial and external compartments.

These findings support a plausible mechanism by which mitoK_{ATP} channel opening during ischemia *1*) reduces the rate of ATP loss (22, 37, 40), *2*) reduces the rate of adenine nucleotide degradation so that ADP is available for phosphorylation upon reperfusion (26), and *3*) reduces $\Delta\Psi$ and Ca^{2+} accumulation (63, 67). These effects preserve mitochondria so that, upon reperfusion, they can return to their normal function of providing adequate ATP supply to cytosolic ATPases.

We suggest that the rapid recovery of high-energy phosphates after reperfusion of IPC or diazoxide-treated hearts is again due to segregation of mitochondrial ΔG_P. When VDAC are open after ischemia-reperfusion, respiration will be controlled by cytosolic ADP concentration, and restriction of ADP diffusion in the cytosol will result in a limitation of ATP production (54), worsening ischemia-induced damage to the cardiomyocyte. When VDAC are closed in the protected myocyte, reoxygenation will enable mitochondrial ADP to be phosphorylated and the resulting ATP to be converted immediately within the IMS to creatine phosphate, which is then exported to the cytosol (51, 53). In this way, preservation of IMS structure-function during ischemia will lead to a fully functional Mi-CK system upon reperfusion, thereby preparing the heart for resumption of normal function.

In summary, opening of mitoK_{ATP} channels by diazoxide triggers the cardioprotective signaling pathway by inducing ROS production. One molecular consequence of this pathway is to open mitoK_{ATP} channels, which serves to retard the rate of ATP hydrolysis secondary to volume-dependent segregation of ΔG_P by the outer membrane. Upon reperfusion, preservation of ADP and mitochondrial structure permits efficient energy transfer between mitochondria and the cytosol. These effects are mediated by the volume regulatory actions of mitoK_{ATP} channels, which preserve the structure function of the IMS, and, in particular, maintain the normal low permeability of the mitochondrial outer membrane to nucleotides.

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