The direct physiological effects of mitoK$_{\text{ATP}}$ opening on heart mitochondria

Alexandre D. T. Costa, Casey L. Quinlan, Anastasia Andrukhiv, Ian C. West, Martin Jabůrek, and Keith D. Garlid

1Department of Biology, Portland State University, Portland, Oregon; and 2Department of Membrane Transport Biophysics (No. 75), Institute of Physiology, Academy of Sciences of the Czech Republic, Prague, Czech Republic

Submitted 26 July 2005; accepted in final form 30 August 2005


The mitochondrial ATP-sensitive K$^+$ channel (mitoK$_{\text{ATP}}$) has been assigned multiple roles in cell physiology and in cardioprotection. These roles must arise from basic consequences of mitoK$_{\text{ATP}}$ opening that should be observable at the level of the mitochondrion. MitoK$_{\text{ATP}}$ opening has been proposed to have three direct effects on mitochondrial physiology: an increase in steady-state matrix volume, respiratory stimulation (uncoupling), and matrix alkanilization. Here, we examine the evidence for these hypotheses through experiments on isolated rat heart mitochondria. Using perturbation techniques, we show that matrix volume is the consequence of a steady-state balance between K$^+$ influx, caused either by mitoK$_{\text{ATP}}$ opening or valinomycin, and K$^+$ efflux caused by the mitochondrial K$^+$/H$^+$ antiporter. We show that increasing K$^+$ influx with valinomycin uncouples respiration like a classical uncoupler with the important difference that uncoupling via K$^+$ cycling soon causes rupture of the outer mitochondrial membrane and release of cytochrome c. By loading the potassium binding fluorescent indicator into the matrix, we show directly that increasing K$^+$ influx with either valinomycin or diazoxide causes matrix alkalinization. Finally, by comparing the effects of mitoK$_{\text{ATP}}$ openers and blockers with those of valinomycin, we show that four independent assays of mitoK$_{\text{ATP}}$ activity yield quantitatively identical results for mitoK$_{\text{ATP}}$-mediated K$^+$ transport. These results provide decisive support for the hypothesis that mitochondria contain an ATP-sensitive K$^+$ channel and establish the physiological consequences of mitoK$_{\text{ATP}}$ opening for mitochondria.

ATP-sensitive potassium channels; diazoxide; 5-hydroxydecanoate; volume regulation; potassium channel openers; cardiac ischemia; uncoupling; cytochrome c

The mitochondrial ATP-sensitive K$^+$ channel (mitoK$_{\text{ATP}}$) is a receptor for K$_{\text{ATP}}$ channel openers (24) and blockers (30). Their identification and subsequent use in the perfused heart model (23) provided strong evidence for the hypothesis that mitoK$_{\text{ATP}}$ opening protects the perfused heart against ischemia-reperfusion injury. Thus the mitoK$_{\text{ATP}}$-selective openers diazoxide and BMS-191095 were found to be cardioprotective (23, 27), and the mitoK$_{\text{ATP}}$-selective blocker 5-hydroxydecanoate (5-HD) was found to block protection by both ischemic preconditioning (2, 26) and K$_{\text{ATP}}$ channel blockers (23). MitoK$_{\text{ATP}}$ has been proposed to play multiple roles in cardioprotection (21, 40), and the mechanisms underlying these roles must arise from one of the direct effects of mitoK$_{\text{ATP}}$ opening on mitochondrial physiology. We have proposed three effects of increasing K$^+$ influx in normoxic mitochondria: matrix swelling, mild uncoupling, and matrix alkalinization (22). Important aspects of cardioprotection have been attributed to each of these effects.

Matrix swelling is a simple osmotic consequence of net uptake of K$^+$ and phosphate into the matrix. The K$^+$/H$^+$ antiporter is designed to balance K$^+$ uptake and thereby provide volume homeostasis (22). We have proposed that the K$^+$/H$^+$ antiporter can only be activated by increased volume itself, so it follows that increasing K$^+$ influx should cause volume to move to a higher steady-state value with greater underlying K$^+$ cycling, which, in turn, will lead to increased energy dissipation (uncoupling). The concept that uncoupling through K$^+$ cycling is necessarily associated with matrix swelling has not previously been explored.

It has been known for some time that scavengers of reactive oxygen species (ROS) block cardioprotection (3, 10, 42, 44), leading Downey and coworkers (42) to propose that mitoK$_{\text{ATP}}$ opening is a trigger of cardioprotection via mitoK$_{\text{ATP}}$-dependent ROS signaling. Our finding that mitoK$_{\text{ATP}}$ opening leads to a moderate increase in mitochondrial production of ROS in cardiomyocytes (17, 43) has been confirmed in vascular smooth muscle cells (35) and perfused hearts (15, 39). Thus mitoK$_{\text{ATP}}$ opening causes an increase in mitochondrial ROS, which serve as second messengers to activate kinases within the cardioprotective signaling pathway (11). We have hypothesized that the increased ROS production is caused by matrix alkalinization secondary to increased K$^+$ influx (22); however, matrix alkalinization secondary to mitoK$_{\text{ATP}}$ opening has not yet been demonstrated.

We have investigated the physiological consequences of increasing the K$^+$ conductance of the inner membrane in isolated rat heart mitochondria, with the following results. 1) Steady-state perturbation experiments show that changes in K$^+$ conductance cause mitochondrial volume to move between true steady states in which total K$^+$ influx equals total K$^+$ efflux via the K$^+$/H$^+$ antiporter. 2) The increased K$^+$ cycling caused by mitoK$_{\text{ATP}}$ opening leads to very limited uncoupling that is associated with matrix swelling. We have found a very narrow margin of safety between K$^+$ uptake due to mitoK$_{\text{ATP}}$ opening and K$^+$ uptake that causes cytochrome c loss due to matrix swelling and outer membrane rupture. 3) K$^+$ influx caused by mitoK$_{\text{ATP}}$ opening is demonstrated directly, as measured with the matrix-loaded potassium binding fluorescent indicator (PBFI). 4) MitoK$_{\text{ATP}}$ opening leads to matrix alkalinization, as measured with the matrix-loaded fluores-
cience probe BCECF. 5) The effects of ATP, diazoxide, and 5-HD on K⁺ flux in heart mitochondria are shown to be qualitatively and quantitatively the same when measured by four independent techniques: light scattering, respiration, K⁺ flux, and H⁺ flux. These results lay the groundwork for studies of how the direct physiological consequences of mitoK_ATP opening are translated into cardioprotection.

METHODS

Mitochondrial isolation. Two male Sprague-Dawley rats (220–240 g) were anesthetized with CO₂ and immediately decapitated. The hearts were removed and washed in ice-cold buffer A (250 mM sucrose, 10 mM HEPES, pH 7.2, and 5 mM K-EGTA). The tissue was finely minced in the presence of 1 mg/ml protease (type XXIV Sigma), and the suspension was diluted threefold with buffer A supplemented with 0.5% fatty acid-free BSA. We observed that mitoK_ATP activity depends critically on the time between decapitation and completion of homogenization. This period was kept as brief as possible and was completed within 2 min. The suspension was homogenized with a motorized teflon pestle and centrifuged for 3 min at 1,500 g. The supernatant was centrifuged for 5 min at 9,000 g, and the resulting pellets were resuspended in buffer A lacking BSA and centrifuged for 3 min at 2,300 g. This supernatant was centrifuged for 5 min at 9,000 g. For respiration measurements and at least one of each light-scattering experiment, mitochondria were further purified in a self-generating 28% Percoll gradient. The compact, hemoglobin-free mitochondrial pellet was resuspended at 35–40 mg protein/ml and kept on ice. Mitochondrial protein concentration was estimated using the Biuret reaction (25). This procedure is in accordance with the American Physiological Society “Guiding Principles in the Care and Use of Animals” and was approved by Institutional Animal Care and Use Committee at Portland State University.

Measurements of mitochondrial matrix volume and oxygen consumption. Changes in mitochondrial matrix volume, which accompany net salt transport into mitochondria, were followed using a quantitative light-scattering technique. This technique is based on the observation that the inverse absorbance of the mitochondrial suspension (1/A), when corrected for the extrapolated value at infinite protein concentration (1/A₀), is linearly related to matrix volume within well-defined regions (5, 19). Light-scattering changes of 0.1 mg/ml mitochondrial suspensions were followed at 520 nm and 25°C and are reported as β, which is inverse absorbance normalized for protein concentration, P₂₅:

\[ \beta = P_25 / (1/A) \]

Mitochondria were suspended in a buffered salt medium containing K⁺, tetraethylammonium (TEA⁺), Na⁺, or Li⁺ salts of Cl⁻ (120 mM); HEPES (10 mM); EGTA (0.1 mM); succinate (10 mM); and phosphate (P₂₅) (5 mM), pH 7.2. The osmolality of these media ranged between 275 and 280 mosmol/kgH₂O. For assays of nonelectrolyte transport, mitochondria were suspended in the same medium, except that 264 mM erythritol or malonamide was substituted for the Cl⁻ salt. All media also contained 0.5 mM MgCl₂, 25 μM rotenone (to inhibit reverse electron transfer from complex II to complex I) and 0.67 μM oligomycin (to inhibit ATP synthesis by the F₁F₂-ATP synthase and the consequent decrease in membrane potential).

In our experiments, diazoxide (in DMSO) is always added to the suspension 2 s after mitochondria to ensure even distribution of this hydrophobic compound. It should also be noted that diazoxide is ineffective unless ATP is already present because the opener cannot open an already open channel. Similarly, 5-HD is ineffective unless ATP and an opener, diazoxide, are also present because 5-HD does not itself block the channel, but rather it prevents the opening effect of diazoxide (30). The same observations hold for cromakalim and glibenclamide. Light-scattering traces were initiated by addition of the mitochondrial suspension; the first 5–7 s of each trace, containing the transient from the mitochondrial addition, were omitted for clarity.

Oxygen consumption was measured with a Clark-type electrode (Yellow Springs Instruments) in a temperature-controlled chamber, by using the same buffered KCl medium described above and in the presence of ATP (200 μM). Oxygen concentration dissolved in our media was taken to be 480 ng atom O/ml at 25°C.

Measurement of K⁺ influx into PBFI-loaded mitochondria. The final mitochondrial pellet was resuspended to 20 mg/ml in buffer A supplemented with 10 mM pyruvate and 10 mM malate. This suspension was incubated with 20 μM potassium binding fluorescent indicator acetoxyethyl ester (PBFI AM) under stirring for 10 min at 25°C. PBFI AM was dissolved in DMSO and mixed in a 2:1 ratio (vol/vol) with the nonionic surfactant pluronic F-127 before addition to the mitochondrial suspension. The mitochondrial suspension was then diluted to 5–7 mg/ml with TEA⁺ medium containing (in mM) 175 sucrose, 50 TEA-Cl, 10 HEPES, 5 pyruvate, 5 malate, 5 succinate, 5 P₂₅, 0.1 EGTA, and 0.5 MgCl₂ and incubated for 2 min under stirring at 25°C. This incubation was designed to substitute matrix K⁺ with TEA⁺ to bring matrix [K⁺] into the sensitivity range of PBFI; under our experimental conditions, PBFI exhibited an apparent KD for K⁺ of ~8 mM, determined as in Ref. 31. The brief period of TEA⁺ loading had no effect on respiration or respiratory control. The 2-ml suspension was then diluted with ice-cold buffer A to 35 ml and centrifuged twice at 10,000 g for 3 min. The resulting pellet was resuspended in buffer A at 30 mg/ml and kept on ice. For the assays, aliquots of this suspension (0.25 mg/ml) were transferred to 2 ml of the same potassium medium used for the light-scattering assay, at 25°C. PBFI fluorescence, which increases with increasing K⁺ concentration (31), was measured with an SLM/Aminco 8000C fluorescence spectrophotometer using an excitation ratio technique [excitation wavelength (λex) = 340/380 nm, emission wavelength (λem) = 500 nm] in which the signal at 340 nm corresponds to the maximal sensitivity of the probe to K⁺ and the signal at 380 nm corresponds to the isosbestic point of the probe. Ratiometric measurements reduce variations in the measured fluorescence intensity that may arise from competing factors, indicator concentration, excitation path length, excitation intensity, and detection efficiency (9). A calibration was carried out on each preparation. The fluorescence ratios R were found to be hyperbolically dependent on potassium concentrations and were converted to potassium concentration by using the equation [K⁺] = KD × (R – R₀)/(Rmax – R), where KD is the dissociation constant (~8 mM) experimentally determined, as previously described (31); R₀ is the fluorescence signal after lysis; and Rmax is the maximum signal obtained after probe saturation with KCl.

Measurement of pH changes in BCECF-loaded mitochondria. Matrix pH was measured in isolated rat heart mitochondria as described by Jung et al. (32). Briefly, isolated rat heart mitochondria were incubated with 8 μM BCECF AM in buffer A for 10 min at room temperature with stirring and oxygen access. The suspension was then diluted 10-fold with buffer A and centrifuged at 9,000 g for 5 min to remove excess probe. Mitochondria were then resuspended in buffer A and stored on ice. Assays were carried out with 0.25 mg/ml mitochondrial protein at 30°C. BCECF fluorescence, which increases with increasing pH, was measured with an SLM/Aminco 8000C fluorescence spectrophotometer (λex = 509 nm, λem = 535 nm).

Statistical analysis. Data are presented as means ± SD. Data were analyzed using unpaired Student’s t-test of the means using Microcal Origin software (Northampton, MA). A value of P < 0.05 was considered statistically significant.

Chemicals. PBFI AM and BCECF AM were obtained from Molecular Probes (Eugene, OR). Pluronic F-127 was obtained from Calbiochem (San Diego, CA). All other chemicals used were from Sigma Chemical (St. Louis, MO).
RESULTS

Effects of ATP, diazoxide, and 5-HD on matrix swelling are potassium specific. The diagram in Fig. 1 describes the underlying processes by which respiring mitochondria take up $K^+$/H$^+$, phosphate, and osmotically obligated water when suspended in potassium medium. $K^+$/H$^+$ influx is driven by the proton pumps of the electron transport chain (ETS) and occurs via the parallel pathways of diffusion (leak) and the mitochondrial ATP-sensitive $K^+$ channel (mitoK$_{ATP}$, shown as K$_{ATP}$) is also balanced by $K^+$ efflux on the K'/H$^+$ antiporter (K/H). The phosphate transporter (P$i$) is highly active, such that P$i$ is in near-equilibrium with the pH gradient ($\Delta \text{pH}$). Thus there is no net P$i$ movement associated with steady-state $K^+$ cycling because there is no change in $\Delta \text{pH}$. Increasing $K^+$ influx, either by opening mitoK$_{ATP}$ or by adding valinomycin, results in an imbalance between $K^+$ influx and efflux, causing matrix pH to increase. This leads to net uptake of phosphoric acid via the phosphate transporter. Net salt uptake is associated with osmotically obligated water, resulting in matrix swelling. Matrix alkalization releases the K'/H$^+$ antiporter from allosteric inhibition by protons (6), and its activity increases to match the new level of $K^+$ influx. The system then reaches a new steady state at a higher matrix volume. IMS, intermembrane space.

Matrix swelling is proportional to net salt influx and leads to an increase in the light-scattering parameter $\beta$ (5, 19). Figure 2A contains typical light-scattering traces from heart mitochondria respiring in vivo or in vitro, electrorethoric K$^+$ or tetraethylammonium (TEA)$^+$ influx is always balanced by electrogenic H$^+$ ejection by the electron transport chain (ETS). During steady-state $K^+$ cycling, $K^+$ influx via diffusion (leak) and the mitochondrial ATP-sensitive $K^+$ channel (mitoK$_{ATP}$, shown as K$_{ATP}$) is also balanced by $K^+$ efflux on the K'/H$^+$ antiporter (K/H). The phosphate transporter (P$i$) is highly active, such that P$i$ is in near-equilibrium with the pH gradient ($\Delta \text{pH}$). Thus there is no net P$i$ movement associated with steady-state $K^+$ cycling because there is no change in $\Delta \text{pH}$. Increasing $K^+$ influx, either by opening mitoK$_{ATP}$ or by adding valinomycin, results in an imbalance between $K^+$ influx and efflux, causing matrix pH to increase. This leads to net uptake of phosphoric acid via the phosphate transporter. Net salt uptake is associated with osmotically obligated water, resulting in matrix swelling. Matrix alkalization releases the K'/H$^+$ antiporter from allosteric inhibition by protons (6), and its activity increases to match the new level of $K^+$ influx. The system then reaches a new steady state at a higher matrix volume. IMS, intermembrane space.

Matrix swelling is proportional to net salt influx and leads to an increase in the light-scattering parameter $\beta$ (5, 19). Figure 2A contains typical light-scattering traces from heart mitochondria respiring in vivo or in vitro, electrorethoric K$^+$ or tetraethylammonium (TEA)$^+$ influx is always balanced by electrogenic H$^+$ ejection by the electron transport chain (ETS). During steady-state $K^+$ cycling, $K^+$ influx via diffusion (leak) and the mitochondrial ATP-sensitive $K^+$ channel (mitoK$_{ATP}$, shown as K$_{ATP}$) is also balanced by $K^+$ efflux on the K'/H$^+$ antiporter (K/H). The phosphate transporter (P$i$) is highly active, such that P$i$ is in near-equilibrium with the pH gradient ($\Delta \text{pH}$). Thus there is no net P$i$ movement associated with steady-state $K^+$ cycling because there is no change in $\Delta \text{pH}$. Increasing $K^+$ influx, either by opening mitoK$_{ATP}$ or by adding valinomycin, results in an imbalance between $K^+$ influx and efflux, causing matrix pH to increase. This leads to net uptake of phosphoric acid via the phosphate transporter. Net salt uptake is associated with osmotically obligated water, resulting in matrix swelling. Matrix alkalization releases the K'/H$^+$ antiporter from allosteric inhibition by protons (6), and its activity increases to match the new level of $K^+$ influx. The system then reaches a new steady state at a higher matrix volume. IMS, intermembrane space.

Fig. 1. The $K^+$ cycle in heart mitochondria. In mitochondria respiring in vivo or in vitro, electrorethoric K$^+$ or tetraethylammonium (TEA)$^+$ influx is always balanced by electrogenic H$^+$ ejection by the electron transport chain (ETS). During steady-state $K^+$ cycling, $K^+$ influx via diffusion (leak) and the mitochondrial ATP-sensitive $K^+$ channel (mitoK$_{ATP}$, shown as K$_{ATP}$) is also balanced by $K^+$ efflux on the K'/H$^+$ antiporter (K/H). The phosphate transporter (P$i$) is highly active, such that P$i$ is in near-equilibrium with the pH gradient ($\Delta \text{pH}$). Thus there is no net P$i$ movement associated with steady-state $K^+$ cycling because there is no change in $\Delta \text{pH}$. Increasing $K^+$ influx, either by opening mitoK$_{ATP}$ or by adding valinomycin, results in an imbalance between $K^+$ influx and efflux, causing matrix pH to increase. This leads to net uptake of phosphoric acid via the phosphate transporter. Net salt uptake is associated with osmotically obligated water, resulting in matrix swelling. Matrix alkalization releases the K'/H$^+$ antiporter from allosteric inhibition by protons (6), and its activity increases to match the new level of $K^+$ influx. The system then reaches a new steady state at a higher matrix volume. IMS, intermembrane space.

Matrix swelling is proportional to net salt influx and leads to an increase in the light-scattering parameter $\beta$ (5, 19). Figure 2A contains typical light-scattering traces from heart mitochondria respiring in vivo or in vitro, electrorethoric K$^+$ or tetraethylammonium (TEA)$^+$ influx is always balanced by electrogenic H$^+$ ejection by the electron transport chain (ETS). During steady-state $K^+$ cycling, $K^+$ influx via diffusion (leak) and the mitochondrial ATP-sensitive $K^+$ channel (mitoK$_{ATP}$, shown as K$_{ATP}$) is also balanced by $K^+$ efflux on the K'/H$^+$ antiporter (K/H). The phosphate transporter (P$i$) is highly active, such that P$i$ is in near-equilibrium with the pH gradient ($\Delta \text{pH}$). Thus there is no net P$i$ movement associated with steady-state $K^+$ cycling because there is no change in $\Delta \text{pH}$. Increasing $K^+$ influx, either by opening mitoK$_{ATP}$ or by adding valinomycin, results in an imbalance between $K^+$ influx and efflux, causing matrix pH to increase. This leads to net uptake of phosphoric acid via the phosphate transporter. Net salt uptake is associated with osmotically obligated water, resulting in matrix swelling. Matrix alkalization releases the K'/H$^+$ antiporter from allosteric inhibition by protons (6), and its activity increases to match the new level of $K^+$ influx. The system then reaches a new steady state at a higher matrix volume. IMS, intermembrane space.

Fig. 2. $K^+$-selective effects of ATP, diazoxide (DZX), and 5-hydroxydecanoate (5-HD) on matrix swelling. Light-scattering traces ($\beta$) of rat heart mitochondria respiring on succinate. A: mitochondria were respiring in K$^+$ medium. Trace in the absence of ATP (None) and the trace with ATP + DZX are nearly superimposable, as are traces for ATP and ATP + ATP + DZX + 5-HD. B: mitochondria were respiring in TEA$^+$ medium. All 4 traces are superimposable. C: mitochondria were respiring in K$^+$ medium, and ATP was present in all traces except tetraphenylphosphonium cation (TPP$^+$). Traces for valinomycin (Val), Val + TPP$^+$, and Val + 5-HD are nearly superimposable, and the traces. None (ATP only), TPP$^+$, and DZX + 5-HD are superimposable. Mitochondria were suspended at 0.1 mg/ml and assayed as described in METHODS. ATP (200 $\mu$M), TPP$^+$ (0.5 $\mu$M), and 5-HD (300 $\mu$M) were present at the beginning of each corresponding trace. DZX (30 $\mu$M) and Val (0.9 pmol/mg of protein) were added 2 s after the addition of mitochondria. These traces are representative of at least 8 independent experiments.
μM) + 5-HD, or cromakalim + glibenclamide (5 independent experiments for each combination, data not shown).

Figure 2B contains typical light-scattering traces from heart mitochondria respiring in potassium-free TEA+ medium. Electrophoretic influx of TEA+ occurs solely by diffusion. Because there is no efflux pathway for this cation, mitochondria take up TEA+, phosphate, and water until K+ efflux via the K+/H+ antiporter equals TEA+ influx, at which point a steady-state volume is achieved (at 60–90 s, Fig. 2B). This steady state is short lived because mitochondria soon lose all their K+ via K+/H+ exchange, a phenomenon first described in 1979 (18). After matrix potassium is exhausted (at ~120 s, Fig. 2B), uptake of TEA+ can no longer be balanced by K+ extrusion, and matrix swelling proceeds at a rate proportional to the uncompensated TEA+ influx. Note that light scattering in TEA+ medium is unaffected by ATP, diazoxide, or 5-HD (Fig. 2B) and is also unaffected by cromakalim or glibenclamide (not shown). We have also found in experiments not shown that light scattering in Li+ or Na+ media is similarly unaffected by these agents.

Figure 2C contains light-scattering traces from mitochondria suspended in the same K+ medium as was used in Fig. 2A. These data show that inhibition of K+ influx by ATP or tetraphenylphosphonium cation (TPP+) is reversed by the presence of 0.9 pmol valinomycin/mg of protein and that the matrix swelling induced by this concentration of valinomycin is qualitatively and quantitatively similar to that induced by diazoxide in Fig. 2A. Moreover, 5-HD did not inhibit swelling caused by valinomycin, confirming the mitoKATP-specific effects of 5-HD. Glibenclamide was also not able to inhibit swelling caused by valinomycin (not shown).

To further investigate whether ATP has artifactual effects on the light-scattering signal, we examined the effects of ATP on nonelectrolyte transport in respiring mitochondria, with typical results contained in Fig. 3. Heart mitochondria rapidly take up erythritol and malonamide with osmotically obligated water, and malonamide is transported ~5 times faster than erythritol, as previously shown in liver mitochondria (19). Note that the presence of 200 μM ATP had no effect on the rate of swelling, confirming that ATP does not introduce an artifact into the light-scattering assay when added before the mitochondria. We also observed no effect of diazoxide or 5-HD on matrix swelling in erythritol and malonamide (data not shown).

Perturbation of steady-state volume by modifying K+ flux. We stated above that matrix volume approaches a steady state that reflects underlying K+ cycling and that the K+/H+ antiporter is volume dependent. We tested these assertions through steady-state perturbation experiments. First, the putative steady state was perturbed with quinine, which inhibits the K+/H+ antiporter (20, 38). (Quinine may have other effects on mitochondria; however, it behaves in the predicted manner as a K+/H+ antiporter inhibitor under all experimental conditions tested.) It can be seen in Fig. 4A that addition of quinine is followed by a rapid swelling due to uncompensated K+ uptake, confirming that the observed constant volume is the result of steady-state potassium cycling. Subtle changes in steady-state volume can also be elicited by increasing K+ influx or efflux with low concentrations of valinomycin or nigericin, respectively (13). Typical results from such experiments (Fig. 4B) show, as expected, that valinomycin induces matrix expansion while nigericin induces matrix contraction. When quinine was added at the steady state, the resulting net flux must correspond to net K+ influx, which in turn must equal that part of the K+/H+ exchange removed by quinine inhibition. The results in Fig. 4, A and B, therefore demonstrate that K+/H+ antiporter activity increases as matrix volume is increased.

The consequences of uncoupling via increasing activity of the K+ cycle. The K+ cycle (Fig. 1) catalyzes net proton influx, as do the classical protonophoretic uncouplers, but the two processes are not identical in all their effects. Figure 5 contains data from mitochondria in which respiration was titrated with the potassium ionophore valinomycin (Fig. 5A) or the protonophoretic uncoupler m-chlorophenylhydrazone (CCCP; Fig. 5B) in the presence and absence of 10 μM cytochrome c. A typical feature of uncoupling, seen in Fig. 5, A and B, is that respiration increases up to the Vmax of electron transport, after which respiration is constant (at high concentrations, CCCP inhibits electron transport, as seen in the slight decline in rates at 500 nM CCCP). Note that valinomycin is expressed in units per milligram of protein, whereas CCCP is expressed in molar units. The practical reason for this is that the valinomycin partition coefficient between membrane and aqueous medium is very high, so that most of the ionophore is in the mitochondria. CCCP is much less hydrophobic, and most of this ionophore is in the aqueous medium.

Several features of the data in Fig. 5A are noteworthy. 1) The respiratory rate induced by our standard concentration of diazoxide (dashed line in Fig. 5A) is about the same as that induced by 1 pmol valinomycin/mg of protein, a result similar to that obtained with light scattering (Fig. 2) (34). Similar rates of oxygen consumption were observed when 50 μM cromakalim was used instead of diazoxide (n = 3, data not shown). 2) The respiratory stimulation induced by diazoxide is a very small fraction (~5%) of respiratory capacity, which is similar in magnitude to diffusive leak of K+. 3) In the absence

Fig. 3. ATP has no effect on nonelectrolyte transport in heart mitochondria. Light-scattering traces (β) of rat heart mitochondria respiring on succinate in malonamide or erythritol medium in the presence or absence of 200 μM ATP. Mitochondria were suspended at 0.1 mg/ml and assayed as described in METHODS. In these experiments, mitochondria swell due to uptake of permeant nonelectrolyte and osmotically obligated water. The paired traces (+ ATP) for each nonelectrolyte are nearly superimposable. These traces are representative of 4 independent experiments.
of added exogenous cytochrome c, no further acceleration of respiration occurs at valinomycin concentrations >1.5 pmol/mg. Our interpretation is that the swelling consequent on increased K⁺ cycling permeabilizes or ruptures the outer mitochondrial membrane with the release of endogenous cytochrome c. The inner membrane clearly remains intact. 4) The fact that exogenous cytochrome c restores the acceleration of respiration confirms that the outer membrane (OM) has become permeable to cytochrome c.

In contrast to the results with valinomycin, CCCP does not exhibit dependence on added cytochrome c (Fig. 5B). CCCP does not rupture the OM at any concentration because uncoupling by means of increased proton cycling has no direct effect on matrix volume, and the OM maintains its integrity.

Net K⁺ uptake causes matrix alkalization. Net K⁺ influx is balanced exactly by net electrogenic proton ejection by the electron transport chain, leading to matrix alkalization. This will be partially compensated by electroneutral Pi uptake, which delivers protons to the matrix. However, the cytosolic concentration of potassium is much greater than those of Pi and other substrate anions that undergo electroneutral exchange across the inner mitochondrial membrane. Consequently, these anion movements cannot fully compensate the alkalinizing

Fig. 4. Steady-state perturbations of mitochondrial matrix volume by Val, nigericin (Nig), and quinine. Light-scattering traces (β) of rat heart mitochondria respiring on succinate in K⁺ medium. A: the 2 superimposed top traces were obtained with no additions and with DZX + ATP. The 2 superimposed bottom traces were obtained with ATP and with DZX + ATP + 5-HD. To inhibit the K⁺/H⁺ antipporter, quinine (500 µM) was added where indicated. B: after a steady-state volume was achieved in the absence of ATP, 1 pmol/mg protein of Nig or Val was added (ionophores). After the new steady state was achieved, quinine was added. Two superimposable traces for each experiment are presented. Mitochondria were suspended at 0.1 mg/ml and assayed as described in METHODS. Other conditions and concentrations of tested compounds were exactly as described for Fig. 2. These traces are representative of at least 4 independent experiments.

Fig. 5. Uncoupling of respiration by K⁺ cycling causes rupture of the outer mitochondrial membrane. Oxygen consumption rates (V̇_resp) of rat heart mitochondria respiring on succinate in K⁺ medium. A: Val titrations in the presence (●) or absence (○) of cytochrome c. Dashed line represents the rate of diazoxide (Dz)-induced oxygen consumption [in the presence (●) or absence (○) of cytochrome c], being equal to the rate induced by −1 pmol Val/mg of protein. B: m-chlorophenylhydrazone (CCCP) titration in the presence (●) or absence (○) of cytochrome c. Exogenous cytochrome c had little effect on oxygen consumption rates induced by CCCP; thus the data points are nearly superimposable. Mitochondria were suspended at 0.25 mg/ml and assayed as described in METHODS. Data are shown as average ± SD of 5 independent experiments.
effect of net K⁺ influx. We have hypothesized that this normal imbalance must inevitably lead to matrix alkalinization as a consequence of net K⁺ uptake via mitoKATP (22).

To test this hypothesis, we measured changes in both matrix [K⁺] and matrix pH by loading the matrix with the K⁺-selective fluorescent probe PBFI (31) and the pH-selective fluorescent probe BCECF (8). PBFI is insensitive to changes in matrix K⁺ at normal levels of matrix [K⁺] (~150 mM) because the probe (Kᵦ ~ 8 mM) is saturated at these levels, so we first depleted K⁺ by a brief incubation in TEA⁺ salts with substrate (18), resulting in a matrix [K⁺] of 10–20 mM. This substitution had no effect on respiration rate or respiratory control (data not shown). One of the earliest reported features of the K⁺/H⁺ antiporter is that it responds to changes in pH and volume, but not K⁺ (16, 18). Thus, when part of matrix K⁺ is replaced by TEA⁺, the K⁺/H⁺ antiporter responds exactly as if the matrix contained all K⁺. This predicts that the TEA⁺ + K⁺ mitochondria will reach a steady state and that the steady-state K⁺ will move higher in response to additional K⁺ uptake. This is exactly what is observed, as shown in the K⁺ influx data in Fig. 6A. The results of experiments measuring matrix pH are reported in Fig. 6B. The results obtained with diazoxide and 5-HD (Fig. 6) were duplicated by 50 μM cromakalim and 10 μM glibenclamide, respectively (n = 3, data not shown). These results show directly that K⁺ channel openers increase net K⁺ influx and that net uptake of K⁺ by respiring mitochondria causes matrix alkalinization (22).

Four independent assays of mitoKATP activity in isolated mitochondria. Our studies show that the effects of ATP, diazoxide, cromakalim, glibenclamide, 5-HD, and valinomycin on light scattering (Fig. 2, A and C) are qualitatively identical to their effects on K⁺ influx (Fig. 6A) and H⁺ efflux (Fig. 6B). These results also demonstrate that the effect of valinomycin on these parameters affords a means of quantitating mitoKATP-dependent K⁺ flux.

Figure 7 summarizes the results of experiments comparing diazoxide with valinomycin in the light-scattering assay (Fig. 7A) and the respiration assay (Fig. 7B). In both assays, mitoKATP activity corresponded, by interpolation, to 1.0 ± 0.2 pmol valinomycin/mg of protein (5 independent experiments). Figure 8 summarizes the results from the PBFI (Fig. 8A) and BCECF (Fig. 8B) experiments, together with a comparison with the effects of 1 pmol valinomycin/mg of protein. In both assays, mitoKATP activity corresponded again to 1.0 ± 0.2 pmol valinomycin/mg of protein (3 independent experiments). Thus the data contained in Figs. 7 and 8 show that four independent assays yield quantitatively identical results, establishing not only the capacity of the endogenous ATP-inhibitable and diazoxide-openable K⁺ channel but also the validity of each of these assays in monitoring that capacity. These experiments were also duplicated by substituting cromakalim and glibenclamide for diazoxide and 5-HD (as described for Fig. 2A, data not shown).

**DISCUSSION**

MitoKATP has been assigned multiple roles in cell physiology in general and in cardioprotection in particular (21). These include providing efficient energy transfer between mitochondria and cellular ATPases (14), preservation of ADP from deamination during ischemia (14), prevention of Ca²⁺ over-
Although a significant uncoupling during ischemia cannot be excluded, we have shown that the mitoKATP-dependent depolarization observed in simulated ischemia is associated with reduced ATP hydrolysis. This depolarization cannot be due to uncoupling, which would increase ATP hydrolysis (14).

**MitoKATP increases matrix volume during normoxia.** As described in Fig. 1, mitoKATP opening adds a parallel inner membrane K⁺ conductance leading to increased K⁺ influx. K⁺ is accompanied by Pi and water, leading to matrix swelling, as shown by the light-scattering traces in Fig. 2A and in Ref 34. The increase in matrix volume is inhibited by ATP; ATP inhibition is reversed by KATP channel openers such as diazoxide; and channel opening is prevented by KATP channel blockers, such as 5-HD. Attribution of these responses to mitoKATP activity is supported by the finding that they do not

---

**Fig. 8. Quantitative comparison of PBFI and BCECF assays of mitoKATP activity.**

**A:** relative rates of PBFI-detected K⁺ influx of rat heart mitochondria respiring on succinate in K⁺ medium. Mitochondria were loaded with either PBFI or BCECF and assayed at 0.25 mg/ml in K⁺ medium as described in METHODS. For both experiments, ATP (200 μM), DZX (30 μM), Val (0.5, 1, or 1.5 pmol/mg of protein), and 5-HD (300 μM) were present as indicated. Error bars represent SDs from the average of at least 3 independent experiments. *P < 0.05 vs. control, **P < 0.05 vs. ATP, ***P < 0.05 vs. DZX.

**B:** changes in matrix pH (%). Mitochondria were loaded with either PBFI or BCECF and assayed at 0.25 mg/ml in K⁺ medium as described in METHODS. The rates in the presence of ATP were set as 100%. For both sets of experiments, ATP (200 μM), DZX (30 μM), Val (1 pmol/mg of protein), and 5-HD (300 μM) were present as indicated. Error bars represent SDs from the average of at least 3 independent experiments. *P < 0.05 vs. ATP, **P < 0.05 vs. DZX.
occur in K+-free medium (Fig. 2B). Nor do they occur in K+ medium when K+ influx is increased with valinomycin (Fig. 2C). As shown in Fig. 2C, mitochondria treated with valinomycin exhibit the same response as mitochondria treated with diazoxide, except that the effects of valinomycin do not require ATP and are not inhibited by 5-HD.

On the basis of the known behavior of the K+/H+ antiporter, we have asserted that the increase in matrix K+ due to mitoKATP opening simply shifts matrix volume to a higher steady-state value (22). The K+/H+ antiporter is not very susceptible to regulation by changes in its substrate concentrations, because [K+] and [H+] do not change very much, even with rather large amounts of net K+ uptake (16). Instead, the antiporter is regulated allosterically by matrix [Mg2+] (16) and [H+] (6), which sense changes in matrix volume (22). This means that the K+/H+ antiporter will mitigate, but not prevent, volume changes due to increased K+ uptake. Figure 4 contains strong support for this hypothesis. Matrix volume was perturbed by opening and closing mitoKATP (Fig. 4A) or by adding ionophores (Fig. 4B). The effects of quinine, which inhibits the K+/H+ antiporter (38), confirm that the preceding volume was a true steady state and also demonstrate that K+/H+ antiporter activity increases with increasing matrix volume.

MitoKATP opening causes mild uncoupling. The steady-state K+ cycle described in Fig. 1 is a futile cycle and dissipates energy. We have found that, provided K+ influx and efflux are balanced with the aid of ionophores, the result of the futile K+ cycle is exactly the same as that of a futile protonophoretic cycle caused by adding an uncoupler such as CCCP (data not shown). However, when K+ influx and efflux must rely on endogenous pathways, the results are not identical, as shown in Fig. 5. As valinomycin-mediated uncoupling increases, the volume must also increase, as described in the preceding section. This will eventually cause the outer membrane to rupture with loss of cytochrome c and inhibition of respiration (Fig. 5A). This is not observed during CCCP-mediated uncoupling (Fig. 5B), which is not associated with matrix swelling. Valinomycin-induced uncoupling causes loss of outer membrane integrity at ~1.5 pmol valinomycin/mg of protein, and mitoKATP opening causes uncoupling to an equivalent valinomycin dose of ~1 pmol/mg of protein (dashed line in Fig. 5A). Thus mitochondria have a very narrow margin of safety with respect to K+ cycling, and a low mitoKATP activity is essential for preserving the integrity of the outer membrane. Indeed, the K+ flux catalyzed by mitoKATP is so small that it is estimated to cause a decrease in mitochondrial membrane potential of only 1–2 mV in normoxic hearts (34). For this reason, we believe that a decrease in membrane potential is not one of the significant effects of mitoKATP opening in the normoxic heart. Other laboratories have also shown that mitoKATP opening does not cause detectable uncoupling of respiration in vivo or in isolated myocytes (28, 36, 41).

MitoKATP opening causes matrix alkalinization. The theoretical basis for the hypothesis that net K+ uptake results in matrix alkalinization is best understood in the context of Fig. 1. Because overall transport must be electroneutral, electrophoretic K+ influx will be balanced exactly by the electrogenic H+ efflux driven by electron transport. If this were all that happened, the K+ for H+ exchange would increase matrix pH by an amount determined by the buffering power of the matrix (22). For example, if 20 mM K+ were added to the matrix by this process, matrix pH would increase by ~1 pH unit. In fact, however, this loss of matrix protons is partially compensated by electroneutral uptake of phosphoric acid via the Pi transporter. The compensation is limited, however, by the fact that Pi is in equilibrium with the pH gradient and, because the cytosolic concentration of Pi is much lower than that of K+, Pi uptake is limited by its equilibrium distribution. For these reasons, the Pi equivalents taken up will always be less than those of K+ during net K+ uptake, and it is this imbalance that leads to matrix alkalinization. The data in Fig. 6, A and B, show directly that the increase in matrix K+, mediated either by mitoKATP or by valinomycin, is accompanied by matrix alkalinization. This effect is germane to the role of mitoKATP in cardioprotection because matrix alkalinization appears to be the cause of mitoKATP-dependent increases in production of ROS (A. Andrukhiv and K. D. Garlid, unpublished observations).

MitoKATP: its existence and its assay. The theoretical, experimental, and quantitative bases of the light-scattering assay for ion transport in mitochondria were developed 20 years ago (5, 19), and application of this assay to the study of mitoKATP was described 12 years ago (7). Despite a robust body of evidence in support of this application (4, 7, 24, 30, 34), its validity was recently questioned by Halestrap and coworkers (12). They carried out light-scattering experiments with heart mitochondria in which they observed ATP inhibition of matrix swelling in K+ medium but failed to observe any effects of diazoxide or 5-HD. The latter finding caused these authors to raise the question of whether mitochondria possess a KATP channel. To explain the effect of ATP, they attributed the apparent inhibition by ATP to a light-scattering artifact rather than to inhibition of K+ flux.

The results in this study address these concerns as follows. 1) ATP, diazoxide, cromakalim, glibenclamide, and 5-HD affect matrix swelling only in K+ medium and not in TEA+, Li+, Na+, or nonelectrolyte media (Fig. 2A and B; Fig. 3; and data not shown). 2) ATP, diazoxide, and 5-HD have no effect in K+ medium when K+ conductance is increased by valinomycin. 3) Direct measurement of K+ influx (Fig. 6A) and the accompanying matrix alkalinization (Fig. 6B) confirm the effects of ATP, diazoxide, cromakalim, glibenclamide, and 5-HD on K+ influx in mitochondria. 4) With the use of valinomycin-mediated K+ influx for calibration, mitoKATP-mediated K+ influx induced by diazoxide or cromakalim was found to be quantitatively identical in four independent assays: light scattering, respiration, K+ influx, and H+ efflux (Figs. 7 and 8). In our judgment, these findings provide decisive support for the existence of mitoKATP and for the validity of the light-scattering technique for its assay. This is very important because light scattering is the only practical means of studying K+ transport in isolated mitochondria, and such studies are essential for future progress toward understanding the role of mitoKATP in cardioprotection.

In summary, it seems likely that all of the effects of mitoKATP opening arise from increasing the K+ conductance of the inner mitochondrial membrane, although unrecognized consequences of protein-protein interactions cannot be excluded. We believe that the only known direct effect of mitoKATP opening during ischemia is to maintain matrix and IMS...
volumes (14). We have now established three direct effects of mitoK<sub>ATP</sub> opening that occur during normoxia: matrix swelling, mild uncoupling, and matrix alkalization. The unknown mechanisms by which mitoK<sub>ATP</sub> opening confers cardioprotection must ultimately derive from one of these effects, and it is hoped that these findings will help in the search for these mechanisms.

ACKNOWLEDGMENTS

We express gratitude for the technical and administrative assistance of Craig Semrad and Brian Corry.

GRANTS

This work was supported by National Heart, Lung, and Blood Institute Grants HL-35673 and HL-67842.

REFERENCES


