ATP-sensitive K\(^+\) channels in renal mitochondria

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Renal tissue is also protected by ischemic preconditioning (the protective effect of short, nonlethal ischemic episodes on a longer, potentially damaging ischemic event), because this process was inhibited by mitoK\(_{ATP}\) antagonists such as 5-hydroxydecanoate and glibenclamide (3, 20, 24). The cardioprotective effect of mitoK\(_{ATP}\) is probably related to the ability of this channel to prevent ATP loss during ischemia and increase the efficiency of postischemic oxidative phosphorylation (6, 11).

Renal tissue is also protected by ischemic preconditioning (30, 37), suggesting that mitoK\(_{ATP}\) may be present in this tissue. However, to our knowledge, no previous attempt to characterize mitoK\(_{ATP}\) and establish its regulatory properties in renal mitochondria has been made. In this manuscript, we describe a K\(^+\) transport pathway inhibited by adenine nucleotides in kidney mitochondria and measure ATP-sensitive K\(^+\) transport rates and the effects of this transport on mitochondrial function.

MATERIALS AND METHODS

Mitochondrial isolation. Kidneys were rapidly removed from male Wistar rats weighing between 150 and 250 g.

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finely minced, and homogenized in ice-cold buffer containing 210 mM mannitol, 70 mM sucrose, 5 mM Na+-HEPES, 2 mM Na+-EGTA, and 0.1% BSA, pH 7.2. The suspension was then centrifuged at 800 g for 4 min, the resulting supernatant was centrifuged at 12,000 g for 7 min, and the pellet was resuspended in the same buffer. Both centrifugations were repeated, and the final pellet was resuspended in 300–500 µl of buffer. Mitochondrial protein concentration was determined using the Biuret reaction.

**Mitochondrial swelling.** Changes in light scattering, reflecting changes in mitochondrial volume (5, 24, 36), were followed using a temperature-controlled Hitachi F4500 spectrophotometer, operating with continuous stirring at excitation and emission wavelengths of 520 nm, with 2.5-nm slits. Mitochondrial membrane potentials were estimated by following safranin O (5 µM) fluorescence (1) at 495-nm excitation and 586-nm emission on a Hitachi F4500 spectrophotometer. A calibration curve was constructed using the K⁺ ionophore valinomycin (0.1 µg/ml) and known concentrations of K⁺, assuming matrix K⁺ concentration to be ~150 mM (1).

### Measurement of mitochondrial respiration.

Respiration was measured using a computer-interfaced Clark-type oxygen electrode from Hansatech equipped with magnetic stirring. Oxygen solubility at 37°C was taken to be 203 nmol/ml. When fixed potentials were needed (Fig. 3), 0.1 µg/ml valinomycin and known K⁺ concentrations were used to manipulate ΔΨ, calculated using the Nernst equation, assuming matrix K⁺ concentration to be ~150 mM (27).

**ATP measurements.** ATP concentrations were determined by light emission at 560 nm on a Hitachi F4500 spectrophotometer using a commercial luciferin-luciferase kit (Promega FF2021). Light emission during the first 120 s following the addition of luciferin-luciferase was integrated, and data were calibrated using known concentrations of ATP.

**Reagents.** Safranin O, EGTA, succinate, BSA, FCCP, rotenone, valinomycin, cyclosporin A, oligomycin, glibenclamide, diazoxide, 5-hydroxydecanoic acid, ADP, ATP, GTP, and antimony A were purchased from Sigma.

**Data analysis.** Replicate experiments were performed in mitochondria isolated from separate animals, each representing an n of 1. Data are expressed as means ± SE. The inhibition of mitochondrial swelling promoted by ADP and ATP was normalized to control and analyzed by comparison to one using t-tests for a single sample. The remaining swelling data were analyzed with one-way ANOVA multiple t-tests for planned comparisons between mean values. Planned comparisons were DZX + ATP vs. ATP; glibenclamide + DZX + ATP vs. DZX + ATP; 5-hydroxydecanoate + DZX + ATP vs. DZX + ATP; and GTP + ATP vs. ATP. ΔΨ and oxygen consumption variation data (Fig. 2) were compared with zero, and ATP measurements were normalized to control and compared with one using t-tests for a single sample.

### RESULTS

**Regulation of renal mitochondrial K⁺ transport.** Mitochondrial isolation promotes K⁺ loss and contraction of the matrix, a process reversed when the organelles are incubated in a medium containing physiological concentrations of K⁺ (24, 28). We measured increases in mitochondrial volume promoted by K⁺ uptake and the entrance of osmotically obligated water by following light scattering in isolated kidney mitochondrial suspensions incubated in hypotonic media rich in K⁺. K⁺ uptake increased mitochondrial volume, decreasing light scattering in a time-dependent manner. Swelling due to K⁺ uptake was complete after 20 to 60 s and was followed by mitochondrial contraction, probably due to the activity of the mitochondrial K⁺/H⁺ antiporter (results not shown). ATP significantly decreased the swelling rate compared with control (P < 0.001, see typical traces in Fig. 1A and averages in Fig. 1B). The K₁/₂ for ATP inhibition of K⁺ uptake was ~20 µM (data not shown), and 200 µM ATP was sufficient to attain the maximal inhibitory effect. These results indicate that an ATP-sensitive pathway for K⁺ uptake is present in kidney mitochondria. This pathway is selective for K⁺, because swelling in Li⁺ or Na⁺ salts, which are not transported by mitoKATP (28), was less extensive and unaltered by ATP (empty and hatched columns). K⁺ uptake in kidney mitochondria was also prevented by ADP (P < 0.001), which inhibits mitoKATP in brain, liver, and heart (4).

The inhibitory effect of ATP on mitochondrial swelling in K⁺ salts was reversed by GTP (P < 0.005), a physiological mitoKATP activator (33), and DZX (P < 0.001), a pharmacological mitoKATP agonist that acts...
selectively on mitochondrial channels at the concentration used (19). As previously described (24), GTP and DZX had no effect in the absence of ATP and/or K+ (data not shown). The DZX effect could be prevented by the concomitant presence of glibenclamide (P < 0.005), a mitoKATP antagonist (24), demonstrating that renal ATP-sensitive transport is also regulated by sulphonylureas, and must, therefore, be mediated by a channel similar to previously described mitoKATP (4, 19, 24, 32, 33). Another effective inhibitor of the DZX-induced swelling was 5-hydroxydecanoate (P < 0.02), which is thought to be specific for mitoKATP (24). These inhibitors were also ineffective in the absence of ATP and DZX (data not shown), as previously seen (24).

**Effect of ATP-sensitive K+ transport on respiration and ΔΨ.** With the use of safranin O fluorescence to measure ΔΨ, we found an increase of 11.4 ± 1.5 mV (n = 4; P < 0.005 vs. 0 mV) when 200 μM ATP was added to the reaction medium to inhibit mitoKATP (Fig. 2A). An increment of 16.2 ± 4.9 mV (n = 5; P < 0.03 vs. 0 mV; P > 0.05 vs. the previous group) in ΔΨ after a 200 μM ATP addition was measured in a similar reaction mixture containing fatty acid-free BSA (1 g/l), demonstrating that the ATP effect was not due to an inhibition of fatty acid-stimulated uncoupling (26). Cyclosporin A, an inhibitor of the mitochondrial permeability transition (38), also did not eliminate the ATP effect (data not shown), suggesting that our ΔΨ increases were not due to prevention of permeability transition by ATP. In addition, 200 μM ATP did not significantly increase ΔΨ (0.8 ± 2.0 mV, n = 4; P > 0.05 vs. 0 mV) when added to a reaction medium that did not contain K+. Thus K+ transport through an ATP-sensitive pathway results in ΔΨ decreases of −10 mV. In all experiments, the proton ionophore FCCP was added at the end of the run to dissipate ΔΨ and ensure that safranin O fluorescence returned to similar levels.

Associated with the changes in ΔΨ, we found that mitochondria incubated in K+ salts in the absence of ATP presented higher respiratory rates than those in which K+ transport was inhibited by ATP (Fig. 2B), in a manner unaltered by the presence of BSA (not shown). With the use of succinate as a respiratory substrate, ATP-sensitive increments in respiration observed in K+-rich media were 11.9 ± 1.4 nmol O2·min⁻¹·mg protein⁻¹ (n = 10; P < 0.001 vs. 0), which corresponds to an estimated K+ transport rate of 143 nmol O2·min⁻¹·mg protein⁻¹, considering that K+/H+ exchange is electroneutral and 6 protons are pumped per oxygen atom consumed (4).

A verification of the compatibility of our data for ΔΨ and respiration rate changes promoted by mitoKATP was conducted by measuring oxygen consumption rates in the presence of fixed ΔΨs, as described in MATERIALS AND METHODS and shown in Fig. 3. By fitting the ΔΨ data obtained with respiratory rates lower than the maximal rates, we found that respiration and ΔΨ are related by a slope of 1.067 ± 0.012 nmol O2·mV⁻¹·min⁻¹·mg protein⁻¹, a result fully compatible with the finding that mitoKATP alters respiration and ΔΨ by 1.04 ± 0.18 nmol O2·mV⁻¹·min⁻¹·mg protein⁻¹. (See MATERIALS AND METHODS.)

**Effect of K+ transport on the reverse activity of the F0F1 ATP synthase.** Opening ATP-sensitive K+ channels in heart mitochondria changes ADP and ATP transport across mitochondrial membranes, increases the efficiency of oxidative phosphorylation, and preserves tissue ATP levels during ischemia (6, 11). To verify whether the same effect was present in kidney, we incubated renal mitochondria in the presence of antimycin A (a complex III inhibitor), to simulate an ischemic condition, and measured ATP loss promoted by hydrolysis through the reverse activity of the F0F1 ATP synthase. Initially, ATP hydrolysis by the F0F1 ATP synthase was measured by following the ΔΨ generated by proton pumping by this protein. We observed that the ΔΨ sustained by ATP hydrolysis was lower and more rapidly lost when K+ transport was stimulated by the presence of DZX (a representative trace of

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**Fig. 2.** ATP-sensitive K+ transport promotes small changes in inner membrane potential (ΔΨ) and respiration. Isolated renal mitochondria (~0.5 mg/ml) were added to a reaction medium at 37°C containing 150 mM KCl, 10 mM inorganic phosphate, 0.1 mM EGTA, 1 mM Mg2+, 5 mM HEPES, 2 mM succinate, pH 7.4 (KOH), 1 μg/ml oligomycin, and 1 μM rotenone in the presence (ATP) or absence (control) of 200 μM ATP. ΔΨ (A) and oxygen consumption (B) were measured as described in MATERIALS AND METHODS. FCCP (1 μM) was added where indicated.
DISCUSSION

We characterized a regulated K⁺ import pathway in renal mitochondria that promotes mitochondrial swelling. This swelling is inhibited by ATP and ADP, in a manner reversed by the K⁺TPP agonists DZX and GTP. The DZX and GTP effect is prevented by 5-hydroxydecanoate and glibenclamide (Fig. 1). Although the specificity of these drugs toward mitoK⁺TPP and validity of light-scattering measurements of mitochondrial volume have recently been challenged (9, 22, 28), we found the mitoK⁺TPP agonists and antagonists used here to be effective only when mitochondria were suspended in K⁺-containing buffers, a strong indication that they change mitochondrial light-scattering characteristics by altering K⁺ transport. Furthermore, we found our light-scattering data to be reproducible and statistically significant, whereas measurements of mitochondrial volume used previously (9) probably present errors larger than the volume changes promoted by these channels. Indeed, our findings regarding ATP-sensitive K⁺ uptake in renal mitochondria are comparable to those described in mitochondria isolated from liver, heart, and brain (4, 19, 24, 32, 33), in which mitoK⁺TPP has been isolated and reconstituted and strongly support the idea that kidney mitochondria present a mitoK⁺TPP similar to that described in other tissues.

ATP-sensitive K⁺ transport in renal mitochondria results in mild respiratory rate enhancement and a partial decrease in ∆Ψ (Fig. 2), as would be expected for the uptake of a cation into the negatively charged mitochondrial matrix. ∆Ψ Changes promoted by ATP in renal mitochondria (10–20 mV) were somewhat greater than those described in heart (1–2 mV; 28) and in brain (3–6 mV; 4). On the other hand, kidney mitoK⁺TPP transport rates (~140 nmol·min⁻¹·mg protein⁻¹), estimated by measuring respiratory rate differences, were larger than those found in heart (~30 nmol·min⁻¹·mg protein⁻¹; 28) and slightly lower than brain K⁺ transport rates (~170 nmol·min⁻¹·mg protein⁻¹; 4), suggesting that renal tissue expresses intermediate mitoK⁺TPP protein levels. In kidney and all other tissues studied to date (4, 24), the respiration and ∆Ψ effects of mitoK⁺TPP activation were small and certainly insufficient to hamper oxidative phosphorylation or Ca²⁺ uptake, indicating that large-scale ∆Ψ and respiratory regulation must not be the central role of mitoK⁺TPP. On the other hand, a major role of mitoK⁺TPP may be to regulate mitochondrial volume, which can change up to 20% in response to ATP-regulated K⁺ uptake rates as limited as those found in heart (7, 24, 28).

In addition to altering volume, respiration, and ∆Ψ in respiring mitochondria, DZX-stimulated K⁺ transport in renal mitochondria was associated with lower ATP hydrolysis through the reverse activity of the F₀F₁ ATP synthase under nonrespiring conditions (Fig. 4). We previously obtained a similar result in heart mitochondria (6), and there is evidence that this effect is linked to volume changes (11). Indeed, we found that perfused hearts subjected to ischemia presented higher ATP levels when treated with DZX, atracyloside (an
inhibitor of nucleotide transport into the mitochondrial matrix), or oligomycin (which inhibits the mitochondrial FoF1 ATP synthase). This is strong evidence that limitation of ATP hydrolysis by the FoF1 ATP synthase can be relevant for the maintenance of high-energy phosphate levels during ischemia and that mitoKATP may contribute to ischemic protection by preventing ATP hydrolysis secondarily to changes in mitochondrial volume (6, 11).

The reproduction of this finding in kidney mitochondria suggests that this channel may also have a protective effect in renal ischemia. Unfortunately, although we attempted to obtain a protective effect against ischemic damage using DZX in renal tissue and cell lines, we have not yet been able to confirm this hypothesis due to the toxic effect of this drug on these models (Cancherini, Trabuco, Kowaltowski, and Rebouças, unpublished observations). Pinacidil, another mitoKATP agonist frequently used to study the protective effects of this channel (2, 8, 25, 34), previously failed to protect the kidney against ischemia (30).

Although a possible protective role for renal mitoKATP under pathological conditions remains to be verified, our data clearly indicate that this channel may have important functions under physiological conditions. We found that renal mitoKATP can increase mitochondrial matrix volume by stimulating K+ uptake rates (Fig. 1). This provides a regulated mechanism through which renal mitochondria can swell and counteract the contraction promoted by the K+/H+ exchanger (16), which appears to be highly active in this tissue (results not shown). MitoKATP activation thus allows for the maintenance of mitochondrial volume when K+ leak through the inner membrane is decreased due to oxidative phosphorylation (28). In fact, the maintenance of adequate matrix volume and a narrow intermembrane space is important to ensure the preferential transport of creatine phosphate in relation to ATP across mitochondrial membranes in heart (11). Matrix swelling can also significantly activate the electron transport chain (21).

The concomitant activity of both mitoKATP and the K+/H+ exchanger also results in a small decrease in mitochondrial ΔΨ and increased respiration, which may be another important function for this channel. Although insufficient to hamper oxidative phosphorylation or Ca2+ uptake (28), “mild uncoupling” such as that promoted by mitoKATP may be important to generate heat, regulate metabolism, and even prevent reactive oxygen species generation by mitochondria (12, 35). Indeed, uncoupling proteins, whose sole known function is to promote mild uncoupling, exist ubiquitously (13, 26), attesting to the importance of limited physiological ΔΨ regulation.

In conclusion, we characterized a controlled K+ import pathway in renal mitochondria similar to mitoKATP previously described in other tissues. These putative renal mitoKATP channels regulate mitochondrial volume, respiration, ΔΨ, and FoF1 ATP synthase activity. The possible importance of the regulation of these mitochondrial functions in cellular physiology and pathology remains to be determined.

DISCLOSURES

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