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Identification and Properties of a Novel Intracellular (Mitochondrial) ATP-sensitive Potassium Channel in Brain*

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Protection of heart against ischemia-reperfusion injury by ischemic preconditioning and K_{ATP} channel openers is known to involve the mitochondrial ATP-sensitive K^+ channel (mitoK_{ATP}). Brain is also protected by ischemic preconditioning and K_{ATP} channel openers, and it has been suggested that mitoK_{ATP} may also play a key role in brain protection. However, it is not known whether mitoK_{ATP} exists in brain mitochondria, and, if so, whether its properties are similar to or different from those of heart mitoK_{ATP}. We report partial purification and reconstitution of a new mitoK_{ATP} from rat brain mitochondria. We measured K^+ flux in proteoliposomes and found that brain mitoK_{ATP} is regulated by the same ligands as those that regulate mitoK_{ATP} from heart and liver. We also examined the effects of opening and closing mitoK_{ATP} on brain mitochondrial respiration, and we estimated the amount of mitoK_{ATP} by means of green fluorescence probe BODIPY-FL-glyburide labeling of the sulfonylurea receptor of mitoK_{ATP} from brain and liver. Three independent methods indicate that brain mitochondria contain six to seven times more mitoK_{ATP} per milligram of mitochondrial protein than liver or heart.

The inner membranes of liver and heart mitochondria contain an ATP-sensitive K^+ channel (mitoK_{ATP}),[1] whose regulation has been studied in both intact mitochondria and liposomes containing reconstituted, purified mitoK_{ATP} (1–6). MitoK_{ATP} is inhibited by ATP, ADP, long-chain CoA esters, glyburide, and 5-hydroxydecanoate (5-HD). The ATP-inhibited channel is opened by GTP, GDP, cromakalim, diazoxide, and other K_{ATP} channel openers. $K_{½}$ values for regulation of K^+ flux by these ligands are virtually identical in heart and liver mitoK_{ATP}. The same set of ligands regulates K_{ATP} channels found in plasma membranes (cellK_{ATP}); however, in some cases the effects are different. For example, cellK_{ATP} is opened by ADP and long-chain CoA esters (7), whereas mitoK_{ATP} is blocked by these ligands (1, 4). There are also important pharmacological differences: cellK_{ATP} from cardiac sarcolemma is essentially insensitive to diazoxide and 5-HD, whereas mitoK_{ATP} is sensitive to both agents (3).

It has been known for some time that K_{ATP} channel openers protect the heart against ischemia-reperfusion injury and that K_{ATP} channel blockers prevent this protection (8–10). In a study on cardiac ischemia-reperfusion injury, we exploited the pharmacological differences between cellK_{ATP} and mitoK_{ATP} in heart to show that mitoK_{ATP} mediates the cardioprotective effects of K_{ATP} channel openers (11).

Ischemia-reperfusion injury in brain is an important medical problem. Several studies have shown that K_{ATP} channel openers such as cromakalim and diazoxide are protective in brain models of ischemia-reperfusion (12, 13), and Domoki *et al.* (14) have suggested that the mechanism of tissue protection in brain is similar to that in heart and may be mediated by the opening of mitoK_{ATP}. It is important, therefore, to establish whether or not brain mitochondria contain a K_{ATP} channel and to determine its properties and regulation.

In this work, we report that rat brain contains an active mitoK_{ATP} whose regulation is qualitatively identical to regulation of mitoK_{ATP} from heart and liver. We also observed that brain mitochondria appeared to be significantly enriched in mitoK_{ATP}. This was verified with a novel technique for labeling the mitochondrial sulfonylurea receptor (mitoSUR). The labeling studies indicate that brain mitochondria contain approximately seven times more mitoK_{ATP} per milligram of mitochondrial protein than heart and liver mitochondria.

EXPERIMENTAL PROCEDURES

Mitochondrial Isolation—Mitochondria were isolated by differential centrifugation from rat brain cortex (15) and liver (16). The brain mitochondrial preparation utilizes digitonin to disrupt synaptosomal vesicles and is considered to provide a population that is representative of both glial and neuronal tissue (15). Mitochondrial protein was estimated using the Biuret reaction (17).

Measurement of Mitochondrial Respiration—Respiration was measured at 25 °C with a Clark-type oxygen electrode in K^+- and TEA^+-based media containing 0.5 mg of mitochondrial protein/ml, 2.77 mM $CaCl_2$, 1.38 mM $MgCl_2$, 0.5 mM dithiothreitol, 20 mM imidazole, 2 mM malate, 5 mM pyruvate, 3 mM phosphate, and 10 mM EGTA, pH 7.1 (adjusted by KOH or TEAOH).

Measurement of Mitochondrial Volume—Changes in mitochondrial matrix volume, due to net K^+ salt transport into mitochondria, were monitored by quantitative light scattering, as described previously (3, 5,

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[1] The abbreviations used are: mitoK_{ATP}, mitochondrial ATP-sensitive K^+ channel; cellK_{ATP}, plasma membrane ATP-sensitive K^+ channel; KIR, inwardly rectifying K^+ channel; 5-HD, 5-hydroxydecanoate; CCCP, carbonyl cyanide *m*-chlorophenyl; SUR, sulfonylurea receptor; IMS, mitochondrial intermembrane space; PBFI, potassium-binding benzofuran isophthalate; ROS, reactive oxygen species; TEA, tetraethylammonium; VDAC, voltage-dependent anion channel; $\Delta\Psi$, electrical membrane potential; TES, 2-{[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]amino}ethanesulfonic acid; PAGE, polyacrylamide gel electrophoresis; BODIPY-FL-glyburide, green fluorescence probe BODIPY-FL-glyburide.

16). Mitochondria (0.1 mg/ml) were incubated in K^+ salts of 135 mM chloride, 5 mM TES, 5 mM glutamate, 1 mM malate, 2.5 mM inorganic phosphate, 0.5 mM EGTA, and 0.5 mM $MgCl_2$, pH 7.4. A comparison of the linear osmotic responses of matrix water content, W_m, and the light scattering parameter was used to convert the values to matrix water content, as described previously (16).

Solubilization and Fractionation of the Mitochondrial K^+ Channel—30 mg of rat brain mitochondria was centrifuged at 15,000 × *g* for 10 min, and the pellet was solubilized in 10 ml of 3% Triton X-100, 0.1% β-mercaptoethanol, 0.2 mM EGTA, and 50 mM Tris-HCl, pH 7.2. After incubation on ice for 90 min, the mixture was centrifuged at 180,000 × *g* for 40 min. The supernatant was loaded onto a 10-ml DEAE-cellulose column pre-equilibrated with column buffer, which contained 0.5% Triton X-100, 0.1% β-mercaptoethanol, 1 mM EDTA, and 50 mM Tris-HCl, pH 7.2. The column was washed sequentially with column buffer containing 0, 100, 180, 250, and 500 mM KCl, two column bed volumes each, at 0.2 ml/min. Column eluate was continuously monitored for UV absorption and conductivity and collected in 1-ml fractions. Appropriate selected fractions were dialyzed overnight against *column buffer*, photolabeled by BODIPY-FL-glyburide, analyzed by SDS-PAGE, and reconstituted into liposomes for transport activity studies. Electrophoresis was carried out using 10% polyacrylamide gels (18), with gel patterns visualized by Coomassie Brilliant Blue R-250.

To further purify mitoK_{ATP}, the DEAE-cellulose mitoK_{ATP} fractions were combined (5 ml) and dialyzed overnight at 4 °C with 1 ml of ATP-agarose against column buffer containing 1 mM $MgCl_2$. The dialysate was poured into a small column (1 ml) and washed sequentially with the dialyzing buffer alone, buffer with 200 mM NaCl, dialyzing buffer alone, and buffer with 20 mM Tris-buffered ATP (three bed volumes each). After dialysis, the fractions eluted with ATP were reconstituted into liposomes and analyzed by SDS-PAGE.

Reconstitution of MitoK_{ATP}—Reconstitution of mitoK_{ATP} proteins into PBFI-loaded liposomes was performed as described previously (1, 19). *Internal medium* contained 100 mM TEA-SO_4, 1 mM EDTA, 25 mM TEA-HEPES, pH 6.8, and 300 μM PBFI. Kinetic studies were performed in *external medium* containing 150 mM KCl, 1 mM EDTA, 1 mM MgCl, and 25 mM TEA-HEPES, pH 7.2, at a proteoliposome concentration of 0.4 mg of lipid/ml. K^+ flux through mitoK_{ATP} was initiated by 0.5 μM CCCP, which provides charge compensation for the electrophoretic K^+ flux. Fluorescence changes of the K^+-sensitive probe PBFI were monitored using an SLM/Aminco 8000C fluorescence spectrophotometer ($\lambda_{ex}/\lambda_{em}$ = 345/485 nm), with fluorescence signals calibrated to K^+ flux as previously described (20). Results were plotted as the normalized values $\Delta J/\Delta J_{max}$, where ΔJ_{max} is the difference between fluxes in the absence and presence of 200 μM (saturating) ATP, and ΔJ is the difference between fluxes in the presence or absence of the mitoK_{ATP} modulator. $K_{½}$ values and Hill coefficients (n_H) were determined from three independent experiments by non-linear regression fits to sigmoidal curves using ORIGIN 6.0 software.

BODIPY-FL-Glyburide Labeling of MitoK_{ATP}—DEAE-cellulose fractions containing mitoK_{ATP} in Triton X-100 micelles were incubated for 60 min at 25 °C with 50 nM BODIPY-FL-glyburide, in the presence or absence of 1 μM unlabeled glyburide (control). Reaction mixtures were UV-irradiated (5000 J/m^2, λ 254 nm) for 6 min at 4 °C (21, 22) and then precipitated to remove unbound probe (23). Precipitated, delipidated proteins were dissolved with 5% SDS in 50 mM Tris-HCl, pH 6.8, then diluted 20 times with 50 mM Tris-HCl, pH 6.8, and analyzed directly for fluorescence ($\lambda_{ex}/\lambda_{em}$ = 493/515 nm).

Chemicals—PBFI and BODIPY-FL-glyburide were purchased from Molecular Probes; electrophoresis chemicals were obtained from Bio-Rad; column resins and other chemicals were from Sigma Chemical Co.

RESULTS

Isolation and Reconstitution of Brain MitoK_{ATP}—We reconstituted brain mitoK_{ATP} using protocols identical to those used for mitoK_{ATP} from heart and liver (1). Fig. 1*A* shows the reconstitutively active mitoK_{ATP} fraction that was eluted from a DEAE-cellulose column. This fraction contains several protein bands, including 55- and 63-kDa proteins, similar to those observed in active fractions obtained from heart or liver mitochondria. Further purification of this fraction on an ATP affinity column yielded a reconstitutively active fraction containing only 55- and 63-kDa proteins (Fig. 1*B*). Upon reconstitution, the proteoliposomes exhibited K^+ flux characteristic of mitoK_{ATP} (Fig. 2). CCCP was required for K^+ flux (*trace a versus trace d*), confirming that the flux was electrophoretic.

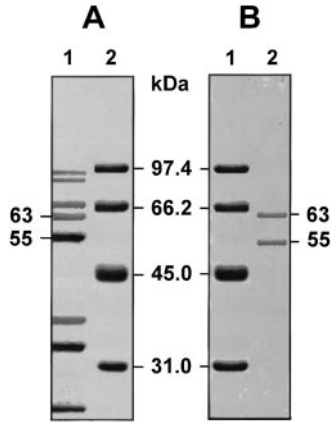

FIG. 1. **Purification of brain mitoK_{ATP}.** MitoK_{ATP} was first purified on a DEAE-cellulose column and eluted with 250 mM KCl (*panel A*, *lane 1*). This fraction was further purified on an ATP-affinity column (*panel B*, *lane 2*). The reconstitutively active fraction separates on 10% SDS-PAGE as two protein bands of 63 and 55 kDa.

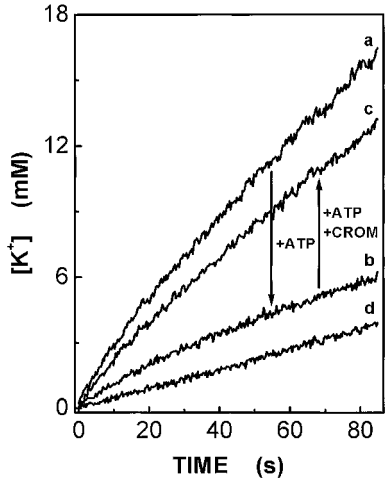

FIG. 2. **K^+ flux in liposomes reconstituted with brain mitoK_{ATP}.** The figure contains representative traces of intraliposomal K^+, determined from PBFI fluorescence *versus* time. Electrophoretic K^+ uptake into liposomes was initiated by the addition of 0.5 μM CCCP to provide charge compensation via H^+ flux. K^+ flux through mitoK_{ATP} (*trace a*) was inhibited by 200 μM ATP (*trace b*), and ATP inhibition was reversed by 50 μM cromakalim (*trace c*). *Trace d* represents a control experiment in which CCCP was omitted.

K^+ flux was inhibited by 200 μM ATP (*trace b*), and this inhibition was reversed by 50 μM cromakalim, a K_{ATP} opener (*trace c*). As previously observed with mitoK_{ATP} from liver, ATP did not inhibit in the absence of Mg^{2+} ion (1).

Regulation of Reconstituted Brain MitoK_{ATP}—Fig. 3 contains the results of experiments designed to determine the dependence of K^+ flux on ATP (●) and GTP (○) concentrations. The $K_{½}$ for ATP inhibition was 43 ± 3 μM (see Table I). The $K_{½}$ for GTP opening in the presence of 200 μM ATP was 3.2 μM. MitoK_{ATP} was also released from ATP inhibition by the K_{ATP} channel openers diazoxide ($K_{½}$ = 0.78 μM) and cromakalim ($K_{½}$ = 11 μM) (Fig. 4). The pharmacologically open channel (in the presence of 200 μM ATP and 2 μM diazoxide) was inhibited by 5-HD ($K_{½}$ = 71 μM) or glyburide ($K_{½}$ = 56 nM) (Fig. 5). As previously observed, 5-HD did not inhibit unless Mg^{2+}, ATP, and diazoxide were all present (3). These and other data are summarized in Table I. It can be seen that all mitoK_{ATP} modulators were effective at concentrations similar to those found for heart and liver mitoK_{ATP}.

Effects of MitoK_{ATP} Opening and Closing on Brain Mitochondrial Matrix Volume—Matrix swelling secondary to K^+ influx

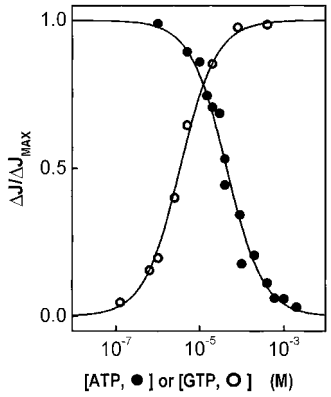

FIG. 3. **ATP and GTP regulate reconstituted mitoK$_{ATP}$.** The normalized mitoK$_{ATP}$ flux ratio $\Delta J/\Delta J_{max}$, defined under "Experimental Procedures," is plotted *versus* concentrations of ATP (●) or GTP (○). ATP inhibited the channel with $K_{½}$ = 43 μM and n_H = 1. GTP reversed inhibition by 200 μM ATP with $K_{½}$ = 3.2 μM and n_H = 1. These results are representative of three separate experiments.

TABLE I
Comparison of mitoK$_{ATP}$ kinetic parameters among liver, heart and brain

Data are compared from rat brain mitoK$_{ATP}$ (n = 3) with data obtained from heart and liver mitoK$_{ATP}$. The former data were obtained from experiments such as those contained in Figs. 3–6. The latter data were previously published (references are in parentheses).

Modulator	Effect	$K_{1/2}$		
		Brain	Liver	Heart
ATP	Inhibition	43.0 ± 3.0 μM	43.0 μM (1)	20.0 μM
GTP	Opening	3.2 ± 0.2 μM	6.9 μM (4)	4.0 μM
Diazoxide	Opening	780.0 ± 20.0 nM	370.0 nM (2)	490.0 nM (11)
Cromakalim	Opening	11.0 ± 2.0 μM	1.0 μM (2)	1.1 μM (11)
Glyburide	Inhibition	56.0 ± 5.0 nM	62.0 nM (1)	56.0 nM (11)
5-HD	Inhibition	71.0 ± 2.0 μM	85.0 μM (3)	83.0 μM (11)

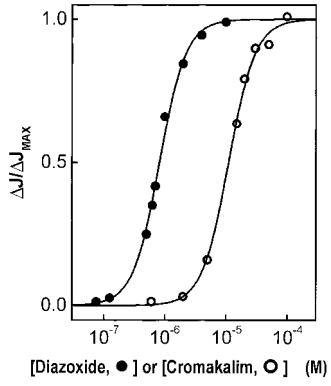

FIG. 4. **Opening of brain mitoK$_{ATP}$ by diazoxide and cromakalim.** The normalized mitoK$_{ATP}$ flux ratio $\Delta J/\Delta J_{max}$, defined under "Experimental Procedures," is plotted *versus* concentrations of the K$_{ATP}$ channel openers diazoxide (●, $K_{½}$ = 0.78 μM, n_H = 2) and cromakalim (○, $K_{½}$ = 11 μM, n_H = 2) in the presence of 200 μM ATP. These results are representative of three separate experiments.

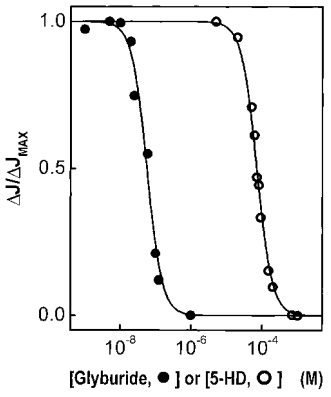

FIG. 5. **Inhibition of brain mitoK$_{ATP}$ by glyburide and 5-hydroxydecanoate.** The normalized mitoK$_{ATP}$ flux ratio $\Delta J/\Delta J_{max}$, defined under "Experimental Procedures," is plotted *versus* concentrations of the K$_{ATP}$ channel blockers glyburide (●, $K_{½}$ = 56 nM, n_H = 2) and 5-hydroxydecanoate (○, $K_{½}$ = 71 μM, n_H = 2) in the presence of 200 μM ATP and 2 μM diazoxide. These results are representative of three separate experiments.

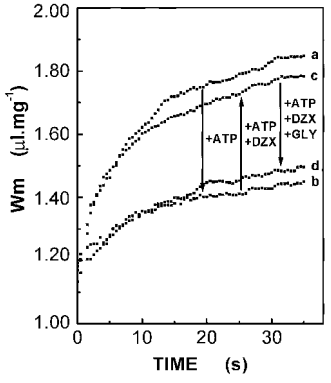

FIG. 6. **Brain mitoK$_{ATP}$ regulates matrix volume.** Time traces of matrix water content in brain mitochondria respiring in K^+ medium, as described under "Experimental Procedures." K^+ uptake restores the K^+ lost during isolation and eventually achieves a steady-state balance between K^+ influx and K^+ efflux via the K^+/H^+ antiporter (*trace a*). Steady-state volume is decreased by 400 μM ATP (*trace b*), which inhibits mitoK$_{ATP}$. Volume is increased by 10 μM diazoxide in the presence of 400 μM ATP (*trace c*), and decreased by 2 μM glyburide in the presence of 400 μM ATP and 10 μM diazoxide (*trace d*). Results are representative of five separate experiments.

in respiring brain mitochondria was followed by light scattering (16), with the results shown in Fig. 6. There is an initial respiration-driven uptake of K^+ salts and water, which acts to restore the matrix K^+ that was lost during mitochondrial isolation (24). A steady-state volume is reached, which reflects a zero net flux balance between K^+ influx and K^+ efflux via the mitochondrial K^+/H^+ antiporter (25). Matrix swelling was decreased in rate and extent by 400 μM ATP (*trace b*), and the control fluxes were restored by addition of 10 μM diazoxide (*trace c*). Matrix swelling was inhibited by further addition of 2 μM glyburide (*trace d*). No effects of ATP, diazoxide, or glyburide were observed when Li^+ or TEA^+ were substituted for medium K^+ (data not shown). Thus, these changes are specific for K^+ and attributable to opening and closing of mitoK$_{ATP}$.

Effects of MitoK$_{ATP}$ Opening and Closing on Brain Mitochondrial Respiration—The effects of mitoK$_{ATP}$ on mitochondrial state 2 respiration in brain mitochondria are shown in Fig. 7. Respiration was compared in K^+ (*closed bars*) and TEA^+ (*open bars*) media. We previously showed that opening of heart mitoK$_{ATP}$ is associated with small changes in respiration that translate to a K^+ influx of only 24–30 nmol/mg·min (26). Brain mitochondria exhibited a significantly larger change in respiration, amounting to 16–17 ng of atom O/mg·min (Fig. 7). Assuming an H^+/O stoichiometry of 10 (27), this corresponds to 160–170 nmol of K^+/min·mg, about seven times larger than that observed in rat heart mitochondria. Although large, this rate of K^+ influx does not greatly depolarize the mitochondrial membrane potential. Measurements using Safranin O fluorescence (28) indicated that ΔΨ decreased by only 3–6 mV, which is consistent with the magnitude of the respiratory stimulation.

Relative Abundance of Brain MitoK$_{ATP}$—During reconstitutions of brain mitoK$_{ATP}$, we were struck by the fact that much smaller amounts of starting material were required to achieve transport rates comparable to heart and liver. Indeed, 15 mg of

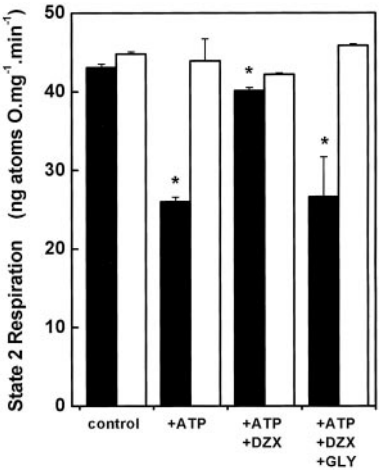

FIG. 7. **Effects of brain mitoK$_{ATP}$ opening/closing on mitochondrial respiration.** Oxygen consumption rates of rat brain mitochondria (0.5 mg/ml) respiring in pyruvate media, described under "Experimental Procedures." Media were made up as K^+ (■) or TEA^+ (□) salts in the presence of no additions (control); 200 μM ATP (+ATP); 200 μM ATP and 10 μM diazoxide (+ATP +DZX +GLY); or 200 μM ATP, 10 μM diazoxide, and 1 μM glyburide (+ATP +DZX). *Bars* represent the mean and S.D. from three independent experiments.

rat brain mitochondria yielded rates similar to rates from 100 mg of either rat liver or heart mitochondria. This observation was consistent with the ratio of K^+ fluxes calculated from respiration rates. We decided to examine the abundance of mitoK$_{ATP}$ using an independent approach. Kramer *et al.* (21) had shown that the β-cell sulfonylurea receptor could be labeled in detergent micelles, and we applied the same approach to photoaffinity labeling of mitoK$_{ATP}$. The results of these studies are contained in Table II. MitoSUR was seven times more abundant (per milligram of mitochondrial protein) in brain than in liver, consistent with the above studies, indicating greater transport activity in brain mitochondria. In further studies being prepared for publication, we labeled the ATP column eluate with BODIPY-FL-glyburide ($\pm$1.0 μM glyburide) and fractionated the proteins by preparative SDS-PAGE. The 63-kDa protein was specifically labeled, whereas the 55-kDa protein was not labeled.[2]

TABLE II

Relative abundance of mitoK$_{ATP}$ in brain and liver mitochondria

MitoK$_{ATP}$ was covalently labeled in detergent micelles by BODIPY-FL-glyburide, as described in text, and the fluorescence was normalized to starting protein (n = 3).

	Liver	Brain	Ratio
Starting protein content	92.4 mg	9.8 mg	9.4:1
BODIPY-FL-glyburide fluorescence (arbitrary units)	2044 ± 343	1457 ± 224	1:1.4
BODIPY-FL-glyburide fluorescence/starting protein content	22 ± 3	149 ± 22	1:7

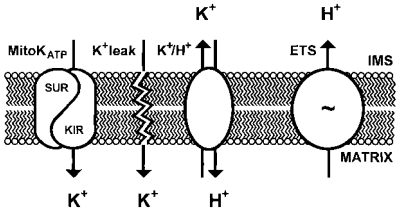

FIG. 8. **Mitochondrial K^+ cycle.** Protonmotive force (Δp) is generated by proton ejection via the electron transport system. $\Delta p = \Delta\Psi + 2.3(RT/F)\Delta$pH, where R is the gas constant, T is temperature, F is the Faraday constant, $\Delta\Psi$ is the membrane potential (about 190 mV), and ΔpH is the pH gradient (about 0.3 unit) across the inner membrane. $\Delta\Psi$ drives K^+ influx by diffusion ("K^+ leak") and via mitoK$_{ATP}$. Phosphate enters by the electroneutral P_i/OH exchanger (not shown), so that net K^+ transport is accompanied by anions and osmotically obligated water. The electroneutral K^+/H^+ antiporter is regulated on the matrix side to respond to volume changes independently of the means used to change volume (24). The K^+/H^+ antiporter discharges excess K^+ (accompanied by P_i and water) and thereby prevents excessive swelling. We favor the idea that mitoK$_{ATP}$ is regulated physiologically to open during states of high ATP production, which will cause $\Delta\Psi$ to fall. Because diffusive K^+ influx is exponential with $\Delta\Psi$ (59), it is exquisitely sensitive to such fluctuations. Thus, a 30-mV decrease in $\Delta\Psi$ will cause a 50% decrease in diffusive K^+ influx, and the matrix will contract until the K^+/H^+ antiporter comes back into balance at a lower steady-state volume. When mitoK$_{ATP}$ is opened in this condition, it adds a parallel conductance pathway to compensate for the lower driving force. Therefore, its role in high work states is to prevent matrix contraction caused by mild depolarization (26). As described under "Discussion," a similar role is proposed for mitoK$_{ATP}$ opening during ischemia, which is also associated with depolarization. If ischemia occurs without warning, however, there are no endogenous mechanisms for opening mitoK$_{ATP}$, and severe ischemia-reperfusion injury will result.

DISCUSSION

We report identification of an ATP-sensitive K^+ channel in rat brain mitochondria with properties similar to heart and liver mitoK$_{ATP}$ (1–3). ATP inhibition is reversed by GTP, diazoxide, or cromakalim, and the open channel is inhibited by glyburide or 5-HD (Figs. 2–5). The sensitivity to sulfonylureas and the presence of two protein bands in the purified mitoK$_{ATP}$ fraction (Fig. 1) imply that mitoK$_{ATP}$ is a heteromultimer consisting of a 55-kDa inwardly rectifying K^+ channel, mitoKIR (29, 30), and a 63-kDa sulfonylurea receptor, mitoSUR (10).

Participation of mitoK$_{ATP}$ in regulation of matrix volume is confirmed by the effects of ATP, diazoxide, and 5-HD shown in Fig. 6. These effects are similar to those observed in heart mitochondria (26) and are thought to reflect the dynamic volume regulation mediated by the mitochondrial K^+ cycle *in vivo*, as described in the legend to Fig. 8. Increased K^+ cycling due to mitoK$_{ATP}$ opening caused a moderate increase in respiration (Fig. 7), which corresponds to a K^+ flux of 160–170 nmol/mg·min. This degree of uncoupling due to K^+ cycling is relatively small, but it is noteworthy that it is ~7 times greater than that observed in heart or liver mitochondria (26), a difference that was confirmed by BODIPY-FL-glyburide labeling of the partially purified proteins (Table II). The basis for the higher quantity in brain is unknown.

The unitary conductance of mitoKIR is 10 pS (31), which corresponds to a turnover of 10^8 mol of K^+ per mol of channel protein per minute. Dividing V_{max} by the turnover number yields an estimate of 1.6 fmol of channel per mg of brain mitochondrial protein. If there are four mitoSUR and mitoKIR subunits per channel, and mitoK$_{ATP}$ is open 50% of the time during V_{max} measurements, we can estimate that brain mitochondria contain about 13 fmol of mitoSUR and mitoKIR per mg of protein.

There is intense interest in understanding the mechanism of protection against ischemia-reperfusion injury. Considerable evidence suggests that heart and brain share common pathways of ischemic protection, and it is generally agreed that K_{ATP} channels play an important role. Thus, both tissues are protected by ischemic preconditioning in which a brief period of ischemia protects against a subsequent longer period of ischemia (32, 33), and this protection is prevented by blockers of K_{ATP} channels (34–36). Moreover, both tissues are protected from ischemia-reperfusion injury if they are pretreated with pharmacological openers of K_{ATP} channels (12, 37). For both tissues, it was initially assumed that protection was afforded exclusively by the K_{ATP} channel of the plasma membrane (8,

[2] P. Paucek, unpublished data.

38). This assumption was shown to be incorrect in heart by the discovery that the receptor for K_{ATP} channel openers and blockers, which affect ischemic protection, is the mitochondrial ATP-sensitive K^+ channel (11). Although some experiments suggest that plasma membrane K_{ATP} channels may also contribute to protection (39–41), the central role of mitoK_{ATP} is now widely accepted (10, 42–48). It is logical to predict that the same conclusion will apply to brain (14); however, definitive evidence for this hypothesis is lacking.

To understand how mitoK_{ATP} opening protects the ischemic cell, it is necessary to consider a complex sequence of events, beginning with how mitoK_{ATP} can be opened *in vivo*. This occurs either by administering a K_{ATP} channel opener or by endogenous signals that are triggered by ischemic preconditioning. We hypothesize that these signals open mitoK_{ATP} by phosphorylation, but there is no direct evidence for this at present. Opening mitoK_{ATP} will increase K^+ influx under all conditions, but the outcome of this influx will depend on the underlying bioenergetic state of the cell. We will consider first the resting, non-ischemic cell.

When diazoxide is added to normoxic heart cells, it induces a moderate rise in mitochondrial ROS production (49, 50), a phenomenon that may arise in the following way: In isolated mitochondria, we observe increased ROS production in response to mild matrix alkalinization.[3] Matrix alkalinization is a normal concomitant of mitoK_{ATP} opening in the cell, because uptake of P_i equivalents will always be less than uptake of K^+, due to the disparity in their cytosolic concentrations. The increased ROS activates kinases and triggers a signaling cascade that involves protein kinase C and other kinases, one of whose targets is mitoK_{ATP} itself. This signaling cascade is vital for preconditioning in heart (49–54), and scavenging ROS during this period prevents diazoxide's cardioprotective effects (49, 54). After diazoxide treatment, mitoK_{ATP} is open, and the cell is now significantly protected from injury caused by a test ischemia.

How does an open mitoK_{ATP} during ischemia reduce ischemia-reperfusion injury? Mitochondrial ATP hydrolysis accounts for a sizable fraction of the loss of high energy phosphates during ischemia, and ischemic protection in the heart is accompanied by lower rates of ATP hydrolysis (55, 56). We propose that mitoK_{ATP} opening is responsible for this partial preservation of cytosolic ATP by a mechanism that links volume regulation, VDAC conductance state, and ATP hydrolysis. When matrix volume contracts, due to membrane depolarization, IMS will expand reciprocally. Swelling will disrupt the structure-function of the IMS, causing dissociation of mitochondrial creatine kinase from VDAC, and increasing outer membrane permeability to nucleotides, which is mediated primarily by VDAC (57, 58). In this unprotected state (ischemia, closed mitoK_{ATP}, open VDAC), nucleotides will equilibrate across the outer membrane, and all of the cell's ATP will be available to support ATP hydrolysis. The rate of mitochondrial ATP hydrolysis is determined by the rate of ion leaks across the inner membrane. The leaks, in turn, depend exponentially on $\Delta\Psi$ (59), which is in equilibrium with the free energy for ATP hydrolysis, ΔG_P. Consequently, the extent of ATP loss at any given time will depend on ΔG_P. These interrelationships mean that the only way to reduce ionic leak during ischemia is to lower mitochondrial $|\Delta G_P|$ to a greater extent than cytosolic $|\Delta G_P|$. This is not possible when VDAC is in its high conductance state. In the protected state (ischemia, open mitoK_{ATP}, closed VDAC), nucleotides will not equilibrate across the outer membrane, and only mitochondrial ATP can support ATP hydrolysis. This will cause decreases in mitochondrial $|\Delta G_P|$, $\Delta\Psi$, and ion leaks, and, consequently, in the rate of ATP hydrolysis.

We have evidence in support of this hypothesis. In perfused rat hearts, we have shown that the outer membrane becomes permeable to nucleotides after ischemia-reperfusion and that the normal permeability barrier is retained in hearts protected either by ischemic preconditioning (48) or diazoxide.[3] In isolated rat heart mitochondria, in which respiration was inhibited to simulate ischemia, we have shown that mitoK_{ATP} opening with diazoxide reduced the rate of ATP hydrolysis to 50% of the control value. This effect was mimicked by moderate osmotic swelling to decrease IMS volume. When the outer membrane was broken by excessive matrix swelling, the effect disappeared, and ATP hydrolysis became independent of matrix volume. These results show that mitoK_{ATP} opening reduced ATP hydrolysis, that the effect was caused by changes in matrix volume, and that the effect required an intact outer membrane (60). Accordingly, we hypothesize that mitoK_{ATP} opening during ischemia plays an energy-sparing role and that this occurs through preservation of the structure-function of the IMS and the low conductance state of VDAC. It should be noted that energy-sparing and preservation of IMS structure may also contribute to the rapid recovery of oxidative phosphorylation that is observed in protected hearts upon reperfusion (48).

Based on the abundant evidence that mitoK_{ATP} plays a key role in ischemic protection in heart (10, 11, 42–48), it is logical to predict that a similar mechanism will operate in brain (14). This hypothesis can be more readily explored now that brain mitoK_{ATP} has been identified and its regulation partially characterized.

[3] R. Bajgar, S. Seetharaman, A. J. Kowaltowski, K. D. Garlid, and P. Paucek, unpublished data.

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