Lack of manifestations of diazoxide/5-hydroxydecanoate-sensitive K_{ATP} channel in rat brain nonsynaptosomal mitochondria

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Pharmacological modulation of the mitochondrial ATP-sensitive K^+ channel (mitoK_{ATP}) sensitive to diazoxide and 5-hydroxydecanoate (5-HD) represents an attractive strategy to protect cells against ischaemia/reperfusion- and stroke-related injury. To re-evaluate a functional role for the mitoK_{ATP} in brain, we used Percoll-gradient-purified brain nonsynaptosomal mitochondria in a light absorbance assay, in radioisotope measurements of matrix volume, and in measurements of respiration, membrane potential ($\Delta\Psi$) and depolarization-induced K^+ efflux. The changes in mitochondrial morphology were evaluated by transmission electron microscopy (TEM). Polyclonal antibodies raised against certain fragments of known sulphonylurea receptor subunits, SUR1 and SUR2, and against different epitopes of K^+ inward rectifier subunits Kir 6.1 and Kir 6.2 of the ATP-sensitive K^+ channel of the plasma membrane (cellK_{ATP}), were employed to detect similar subunits in brain mitochondria. A variety of plausible blockers (ATP, 5-hydroxydecanoate, glibenclamide, tetraphenylphosphonium cation) and openers (diazoxide, pinacidil, chromakalim, minoxidil, testosterone) of the putative mitoK_{ATP} were applied to show the role of the channel in regulating matrix volume, respiration, and $\Delta\Psi$ and K^+ fluxes across the inner mitochondrial membrane. None of the pharmacological agents applied to brain mitochondria in the various assays pinpointed processes that could be unequivocally associated with mitoK_{ATP} activity. In addition, immunoblotting analysis did not provide explicit evidence for the presence of the mitoK_{ATP}, similar to the cellK_{ATP}, in brain mitochondria. On the other hand, the depolarization-evoked release of K^+ suppressed by ATP could be re-activated by carboxyatractyloside, an inhibitor of the adenine nucleotide translocase (ANT). Moreover, bongkrekic acid, another inhibitor of the ANT, inhibited K^+ efflux similarly to ATP. These observations implicate the ANT in ATP-sensitive K^+ transport in brain mitochondria.

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A K^+ channel of mitochondrial origin, with properties resembling the properties of the ATP-sensitive K^+ channel of the plasma membrane (cellK_{ATP}), was first demonstrated in 1991 in patch-clamp studies using fused liver mitoplasts (Inoue *et al.* 1991). It has been shown that this mitochondrial K^+ channel (mitoK_{ATP}) is sensitive to openers and blockers of the cellK_{ATP}. Later, the mitoK_{ATP} was isolated, purified and thoroughly studied in the reconstituted system (Paucek *et al.* 1992; Garlid *et al.* 1996; Jaburek *et al.* 1998; Bajgar *et al.* 2001; Mironova *et al.* 2004). From studies with isolated mitochondria, it was suggested that the only function of the mitoK_{ATP} is to regulate mitochondrial volume (Kowaltowski *et al.* 2001). Changes of mitochondrial membrane potential ($\Delta\Psi$) and respiration caused by ATP or by pharmacological modulators of the mitoK_{ATP} were found to be minimal in heart mitochondria (Kowaltowski *et al.* 2001). However, in brain mitochondria, substantial changes of mitochondrial respiration associated with opening and closing of the mitoK_{ATP} were reported (Bajgar *et al.* 2001). This could be due to a sevenfold higher amount of mitoK_{ATP} in brain mitochondria than in liver or heart mitochondria (Bajgar *et al.* 2001).

From the studies with reconstituted mitoK_{ATP}, it was proposed that this channel consists of two types of subunit: a 55 kDa inwardly rectifying channel subunit 'mitoKir', analogous to a Kir 6.X subunit of the cellK_{ATP}, and a 63 kDa sulphonylurea receptor subunit 'mitoSUR', corresponding to SUR1 or SUR2 of the cellK_{ATP} (Grover & Garlid, 2000; Bajgar *et al.* 2001). Several laboratories have performed immunoblotting analysis by applying polyclonal antibodies raised against peptides of known subunits of

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cellK_{ATP}, and detected Kir 6.1, Kir 6.2 and SUR subunits in isolated mitochondria (Suzuki *et al.* 1997, 2002; Lacza *et al.* 2003*a*,*b*; Tai *et al.* 2003). However, these antibodies did not react with proteins in the reconstitutively active purified fraction of the mitoK_{ATP} (Grover & Garlid, 2000). Thus, the molecular identity of the mitochondrial subunits of the putative mitoK_{ATP} remains unknown.

An implication of the mitoK_{ATP} in cardio- and neuroprotection against ischaemia/reperfusion- and stroke-related injuries dramatically raised the interest in its regulation and physiological role (Domoki *et al.* 1999; Pacher *et al.* 2002; Shimizu *et al.* 2002; Liu *et al.* 2003; O'Rourke, 2004; Sato *et al.* 2004). The mitoK_{ATP} was also suggested to be involved in the preconditioning of cardiomyocytes and neurones (O'Rourke, 2000; Nagy *et al.* 2004), but recently the role and existence of the mitoK_{ATP} have been questioned (Ovide-Bordeaux *et al.* 2000; Hanley *et al.* 2002; Das *et al.* 2003; Kis *et al.* 2003; Kopustinskiene *et al.* 2003; Holmuhamedov *et al.* 2004). Several reports cast doubt on the hypothesis that the mitochondrial effects of pharmacological openers or blockers of the mitoK_{ATP} are necessarily related to mitoK_{ATP} activity. For example, diazoxide, an opener of the mitoK_{ATP} (Garlid *et al.* 1996), can depolarize mitochondria due to inhibition of succinate dehydrogenase (Grimmsmann & Rustenbeck, 1998; Lim *et al.* 2002; Hanley *et al.* 2002). Diazoxide and pinacidil uncouple oxidative phosphorylation in heart mitochondria (Kowaltowski *et al.* 2001; Kopustinskiene *et al.* 2002; Holmuhamedov *et al.* 2004). Glibenclamide, a blocker of the putative mitoK_{ATP}, was also found to uncouple rat liver mitochondria (Szewczyk *et al.* 1997). 5-Hydroxydecanoate (5-HD), a derivative of free fatty acid, and one of the most often used blockers of the putative mitoK_{ATP}, is readily converted into 5-HD-CoA, which can be further metabolized in the β-oxidation pathway (Hanley *et al.* 2002; Lim *et al.* 2002).

In the present study, we sought to visualize manifestations of the mitoK_{ATP} using various experimental approaches to determine to what extent functioning of the mitoK_{ATP} contributes to regulation of matrix volume, respiration, $\Delta\Psi$, and uncoupler-induced K^+ efflux from isolated brain nonsynaptosomal mitochondria. Surprisingly, none of the pharmacological agents applied to brain mitochondria in the various assays pinpointed processes that could be unequivocally associated with the mitoK_{ATP}. In addition, immunoblotting analysis did not provide explicit evidence for the presence of the mitoK_{ATP}, similar to the cellK_{ATP}, in purified brain mitochondria.

Methods

Materials

Diazoxide, glibenclamide, 5-HD, testosterone and ^3H_2O were purchased from Sigma (St Louis, MO, USA). [^{14}C]Sucrose was purchased from Moravek Biochemicals (Brea, CA, USA). Pinacidil, chromakalim and minoxidil were from Tocris (Ellisville, MO, USA). Tetraphenylphosphonium chloride was obtained from Fluka (Buchs, Switzerland). Carboxyatractyloside (CAT) and bongkrekic acid (BKA) were the generous gift of Professor Martin Klingenberg.

Isolation of brain mitochondria

Male Sprague-Dawley rats, 225–250 g (Harlan, Indianapolis, IN, USA), were killed by decapitation, according to an Institutional Animal Care and Use Committee approved protocol. Nonsynaptosomal mitochondria derived from the brains were isolated in mannitol–sucrose (MS) medium and purified on a discontinuous Percoll gradient (Sims, 1990; Brustovetsky *et al.* 2002*a*). Mitochondrial protein was determined by the Bradford method (Bradford, 1976) using BSA as a standard.

Measurements of mitochondrial light absorbance, $\Delta\Psi$ and respiration

Changes in light absorbance of the mitochondrial suspension were monitored at 525 nm in a 0.3 ml chamber at 37°C, with continuous stirring. $\Delta\Psi$ was monitored by following the distribution of tetraphenylphosphonium cation (TPP^+) between the external medium (initially 1.8 μM TPP^+-Cl) and the mitochondrial matrix with a TPP^+-sensitive electrode (Kamo *et al.* 1979) in the KCl-, LiCl- or MS-based incubation medium containing 125 mM KCl, 125 mM LiCl or 215 mM mannitol, plus 70 mM sucrose, respectively. All media contained 2.5 mM $MgCl_2$, 10 mM Hepes, pH 7.4 (adjusted with KOH in KCl-based medium, or with Tris in LiCl- and MS-based media), 0.1% BSA (free from fatty acids) and 1 μM oligomycin, unless stated otherwise. In the experiments with adenine nucleotides, oligomycin was used to block oxidative phosphorylation. A similar approach has been employed by others (Kowaltowski *et al.* 2001). In addition, the KCl-based medium contained 3 mM KH_2PO_4, while the LiCl- and MS-based media contained 3 mM NaH_2PO_4. The incubation media were supplemented with 3 mM pyruvate plus 1 mM malate, or with 3 mM succinate plus 3 mM glutamate. All substrate solutions were K^+ free, and pH was adjusted with Tris. A decrease in the external TPP^+ concentration corresponded to mitochondrial polarization, while an increase in the TPP^+ concentration in the medium corresponded to depolarization. Using a Clark-type electrode in a closed 0.3 ml chamber, oxygen consumption was monitored separately or simultaneously with $\Delta\Psi$, under identical conditions. The $\Delta\Psi$ and respiratory measurements were performed at 37°C in a

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continuously stirred 0.3 ml chamber. All data traces shown are representative of at least three separate experiments.

Matrix volume measurements

Measurements of mitochondrial matrix volume were performed using the radioisotope method (Lim *et al.* 2002; Brand, 2002). In the control experiments designed to determine matrix volumes at time zero, mitochondria (0.2 mg protein), ^3H_2O (1 μCi) and [^{14}C]sucrose (0.1 μCi) were added to 0.3 ml of ice-cold KCl-based incubation medium, and matrix volumes were measured immediately. In the experiments with different pretreatments, mitochondria (0.2 mg protein) were incubated with various substances for 5 min at 37°C in 0.3 ml KCl-based incubation medium supplemented with ^3H_2O (1 μCi) and [^{14}C]sucrose (0.1 μCi). Then, mitochondria were pelleted at 15 800 *g* for 2 min, and 200 μl of supernatant was transferred into a fresh tube and mixed with 200 μl of distilled water. The pellets were solubilized in 50 μl of 20% Triton X-100, and 350 μl of distilled water was added to equalize pellet and supernatant sample volumes. Both pellet and supernatant samples were transferred into scintillation vials with 3.5 ml of scintillation liquid (EcoLite; MP Biochemicals, Irvine, CA, USA). ^{3}H and ^{14}C were determined using a Tri-Carb 2100TR liquid scintillation analyser (Packard Instrument Co., Meriden, CT, USA). Matrix volume was calculated as previously described (Brand, 2002) and expressed in microlitres per milligram of mitochondrial protein.

Fluorescence of flavoproteins

Fluorescence of total mitochondrial flavoproteins was monitored in a Perkin-Elmer LS 55 luminescence spectrometer equipped with a bio-kinetics accessory using excitation/emission wavelengths 450/515 nm (Richards-Kortum & Sevick-Muraca, 1996). The experiments were performed in a 400 μl cuvette at room temperature (22°C) under continuous stirring.

K^+ efflux

K^+ efflux from depolarized brain mitochondria was monitored by following the concentration of K^+ in the K^+-free MS-based medium supplemented with either 3 mM succinate plus 3 mM glutamate, or 3 mM pyruvate plus 1 mM malate, using a miniature K^+-selective electrode in a continuously stirred 0.3 ml chamber at 37°C. The miniature K^+-selective electrode was constructed in our laboratory using a K^+-selective membrane purchased from Fluka (Buchs, Switzerland). K^+ efflux was initiated by 1 μM carbonyl cyanide *p*-(trifluoromethoxy) phenylhydrazone (FCCP).

Transmission electron microscopy (TEM)

Purified brain mitochondria were incubated with or without 50 μM diazoxide or 0.4 nM valinomycin in 125 mM KCl-based medium supplemented with 3 mM succinate plus 3 mM glutamate at 37°C, prior to fixation in 2.5% glutaraldehyde in the same incubation medium at room temperature for 15 min. BSA was omitted from the medium. Mitochondria were then centrifuged at 15 800 *g* for 5 min, and the supernatant was discarded. The pellet was layered with fresh 2.5% glutaraldehyde solution and left overnight at 4°C. The samples were postfixed in 1% osmium tetroxide for 1 h and dehydrated in a graded series of ethanol before embedding in Polybed 812. Sections were cut on a Reichert Ultracut S microtome, and stained with a methanolic solution of uranyl acetate plus lead citrate. Electron micrographs were obtained using a JEOL-1200 EX electron microscope. To quantitatively evaluate the morphological changes in each experimental protocol, four different TEM micrographs, representing randomly chosen areas in the grid, were selected and analysed in a blinded fashion using the previously described approach (Scorrano *et al.* 2002). Total mitochondrial population was categorized into three groups according to their morphology: (i) condensed, (ii) intermediate and (iii) swollen. Mitochondria with characteristics bridging morphological groups were assigned to the lower category. Mitochondria were counted and morphological distribution was statistically analysed using one-way ANOVA followed by Bonferroni's post test (GraphPad Prism, 4.0).

Immunoblotting

Brain homogenates and isolated mitochondria pretreated with Protease Inhibitor Cocktail (Sigma) were solubilized by incubation in NuPAGE LDS sample buffer (Invitrogen, Carlsbad, CA, USA) supplemented with a reducing agent at 70°C for 15 min. Bis-Tris Mops gels (4–12%; Invitrogen) were used for electrophoresis (20 μg protein per lane). After electrophoresis, proteins were transferred to Hybond-ECL nitrocellulose membrane (Amersham Biosciences). Blots were incubated with one of the following: primary goat anti-SUR1, anti-SUR2, anti-Kir 6.1 (R14), anti-Kir 6.2 (G16) or anti-Kir 6.2 (N18), or with primary rabbit anti-Kir 6.2 (H55) or anti-Kir 6.1 (H80) antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA,) at 1:500 dilution for 1 h at room temperature in 8% donkey serum, phosphate-buffered saline, pH 7.2, and 0.15% Triton X-100. An incubation of blots with primary antibodies at 4°C overnight produced similar results (not shown). Blots were developed using donkey anti-goat or goat anti-rabbit IgG (1:20000) coupled with horseradish peroxidase (Jackson ImmunoResearch Laboratories, West Grove, PA, USA) and Supersignal

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West Pico chemiluminescent reagents (Pierce, Rockford, IL, USA). When available, blocking peptides were used to distinguish specific and nonspecific bands, as recommended by the manufacturer. Primary antibodies were incubated with the appropriate blocking peptides (1:5 ratio) for 2 h at room temperature. These mixtures were then incubated with corresponding blots for 1 h at room temperature. Molecular mass markers SeeBlue Plus 2 Standards (5 μl) and MagicMark Western Protein standards (10 μl) (Invitrogen) were used to determine molecular masses of the bands.

Statistics

Statistical analyses of experimental data consisted of one-way ANOVA followed by Bonferroni's post test (GraphPad Prism, 4.0). Data represent means ± s.e.m. of at least three separate experiments.

Results

Immunobloting analysis

In our experiments, polyclonal anti-SUR1 antibody recognized several immunoreactive bands on the blotting membranes with transferred homogenate and mitochondrial samples (Fig. 1*A*). Three bands with molecular masses of about 13, 30 and 97 kDa appeared to be enriched in the mitochondrial fraction. A faint 45 kDa band appeared in both homogenate and mitochondrial samples. Only the 45 and 97 kDa bands were eliminated by the blocking peptide. Polyclonal anti-SUR2 antibody detected a significant enrichment of immunoreactive material, with an approximate molecular mass of 97 kDa (Fig. 1*B*). This band was eliminated by the blocking peptide. The amount of 97 kDa immunoreactive material detected with anti-SUR2 antibody greatly exceeded the amount detected with anti-SUR1 antibody (Fig. 1*A* and *B*). In both cases, with anti-SUR1 and anti-SUR2 antibodies, we detected mitochondrially enriched bands of about 13 and 30 kDa, which were insensitive to the blocking peptide. Thus, 13 and 30 kDa bands represented nonspecific binding of antibodies with irrelevant polypeptides. Of note, similar nonspecific bands were found with all antibodies (anti-SUR and anti-Kir 6.X) used in this work and will not be discussed further.

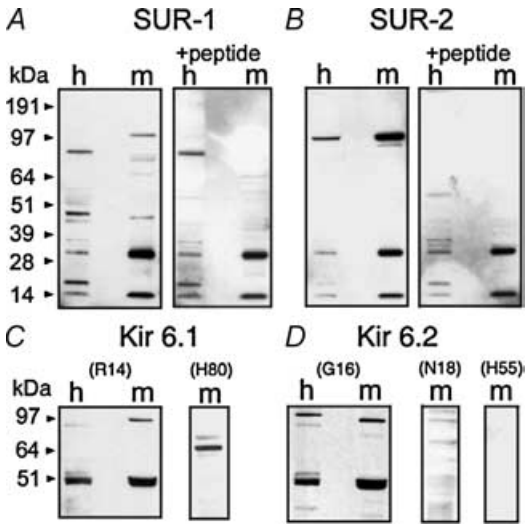

Figure 1. Immunoblotting analysis of brain homogenates (h) and purified mitochondrial fractions (m) with polyclonal antibodies against fragments of SUR1 and SUR2, and different epitopes of Kir 6.1 and Kir 6.2, revealed multiple immunoreactive bands
Blocking peptides (+peptide) were used to discriminate nonspecific bands. Blocking-peptide-insensitive 13 and 30 kDa bands, seen in *A* and *B* with anti-SUR antibodies, were also observed in all immunoblots with anti-Kir 6.X antibodies (not shown).

Polyclonal anti-Kir 6.1 antibody (R14) raised against a peptide corresponding to a near C-terminal region of Kir 6.1 detected two enriched blocking-peptide-sensitive bands in mitochondrial samples with molecular masses of about 51 and 97 kDa (Fig. 1*C*). However, another anti-Kir 6.1 antibody (H80), raised against a peptide corresponding to the C-terminal of Kir 6.1, did not recognize bands with similar molecular masses (Fig. 1*C*). Instead, this antibody detected a highly enriched 65 kDa band, and a faint band at approximately 70 kDa. Anti-Kir 6.2 antibody (G16) raised against a peptide corresponding to an internal region of Kir 6.2 recognized two enriched bands in the mitochondrial fraction with molecular masses of about 51 and 97 kDa (Fig. 1*D*). However, anti-Kir 6.2 antibodies raised against peptides corresponding to the C-terminal fragment (N18) or to N-terminal fragments of Kir 6.2 (H55) did not recognize these bands (Fig. 1*D*). All these antibodies produced distinct bands with polypeptides recommended by the manufacturer as positive controls (not shown).

Light absorbance measurements and radioisotope assay

It was suggested that K^+ influx through the mitoK_{ATP} could be monitored by following light absorbance of mitochondrial suspension (Beavis *et al.* 1985, 1993; Garlid & Beavis, 1985). Since the mitoK_{ATP} in isolated mitochondria incubated without exogenous ATP are supposed to be in an open state, addition of mitochondria into the medium with high K^+ and oxidative substrates should cause massive K^+ influx, accompanied by a concurrent water influx leading to mitochondrial swelling (Beavis *et al.* 1993; Kowaltowski *et al.* 2001). In our experiments, we observed a decrease in light absorbance of mitochondrial suspension after adding brain mitochondria into the KCl-based incubation

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medium (Fig. 2*A*). However, in our experiments, we were unable to suppress the decrease with 0.5 mM ATP, either with 0.5 mM 5-HD, or with 4 μM TPP^+, which is a recently discovered blocker of the reconstituted putative mitoK_{ATP} (Mironova *et al.* 2004). The initial fall in light absorbance was also observed in K^+-free MS-based medium (not shown). Despite the decrease in light absorbance, the radioisotope assay did not reveal statistically significant changes in the matrix volume (Fig. 2*B*). Thus, the initial decrease in light absorbance of the suspension of brain mitochondria appeared to be independent of matrix volume changes, insensitive to modulators of the mitoK_{ATP}, and therefore could not be attributed to the mitoK_{ATP} activity.

In the subsequent experiments, we addressed to what extent pharmacological openers of the mitoK_{ATP} could induce swelling of brain mitochondria. Following a presumed blockade of the putative mitoK_{ATP} with 0.5 mM ATP, the openers of the mitoK_{ATP} were added to mitochondria to activate the channel and to induce K^+ influx into mitochondria leading to mitochondrial swelling. Valinomycin, a K^+-selective ionophore, was used to demonstrate mitochondrial swelling due to K^+ influx. Valinomycin (0.4 nM) produced both a distinct decrease in light absorbance and an increase in matrix volume measured with isotopes (Fig. 3). However, none of the tested openers (diazoxide, chromakalim, pinacidil, minoxidil and testosterone, each at 50 μM) produced a decrease in light absorbance indicative of mitochondrial swelling or increased matrix volume determined with isotopes. All other tested concentrations of the openers (10, 100 and 250 μM) were also without effect (not shown). In addition, neither ATP (0.5 mM) nor 5-HD (0.5 mM), added to mitochondria incubated in the absence of ATP, induced substantial changes in light absorbance or in matrix volume, as determined with isotopes (Fig. 3). The results obtained in the present study with brain mitochondria mirror the liver and heart mitochondria data obtained by Das *et al.* (2003). Thus, the light absorbance measurements and radioisotope assay did not produce evidence for the involvement of the putative mitoK_{ATP} in the regulation of matrix volume of brain nonsynaptosomal mitochondria.

TEM

TEM was used to substantiate light absorbance and matrix volume measurements. Most mitochondria in the control sample appeared to be in a condensed state with a dark electron-dense matrix (Fig. 4*A*). Diazoxide (50 μM) did not cause matrix expansion (Fig. 4*B*), while valinomycin (0.4 nM) resulted in swelling of the mitochondrial matrix (Fig. 4*C*). Morphometric analysis did not reveal statistically significant changes in morphological distribution between control and diazoxide-treated mitochondria (Fig. 4*D*). On the other hand, valinomycin significantly decreased the number of condensed mitochondria and increased the number of swollen mitochondria (Fig. 4*D*). Thus, TEM confirmed the lack of expansion of mitochondrial matrices after diazoxide treatment, and visualized valinomycin-induced swelling.

Measurements of respiration and $\Delta\Psi$

The lack of swelling of brain mitochondria incubated in KCl-based medium could be due to concurrent activation of the K^+/H^+ exchanger leading to extrusion of excessive

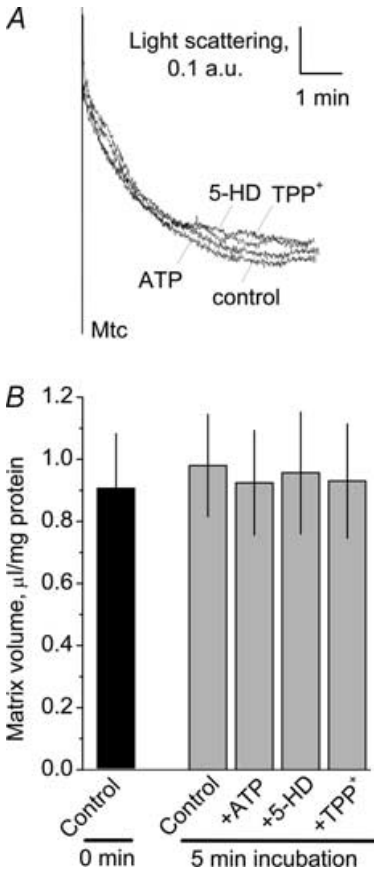

Figure 2. Blockers of the putative mitochondrial ATP-sensitive K^+ channel (mitoK_{ATP}) did not affect an initial decrease in light absorbance of suspension of isolated brain mitochondria (*A*) or mitochondrial matrix volume measured with radioisotopes (*B*)
Mitochondria were incubated in the KCl-based incubation medium supplemented with 3 mM succinate and 3 mM glutamate. *A*, representative traces obtained with 0.5 mM ATP, 0.5 mM 5-hydroxydecanoate (5-HD) and 4 μM tetraphenylphosphonium cation (TPP^+), and without (control). The blockers were added to the medium prior to adding mitochondria (Mtc). *B*, summary of the radioisotope measurements of the mitochondrial matrix volume. Matrix volume at time zero was measured on ice as outlined in Methods. In other experiments, mitochondria were incubated for 5 min with or without the blockers of the putative mitoK_{ATP} at 37°C prior to determination of the matrix volume. Data are means ± S.E.M., $n = 5$.

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K^+ from the mitochondrial matrix (Bernardi & Azzone, 1983; Brierley *et al.* 1984). Simultaneous K^+ influx through the putative mitoK_{ATP}, and K^+ efflux through the K^+–H^+ exchanger, constitute a continuous K^+ cycle

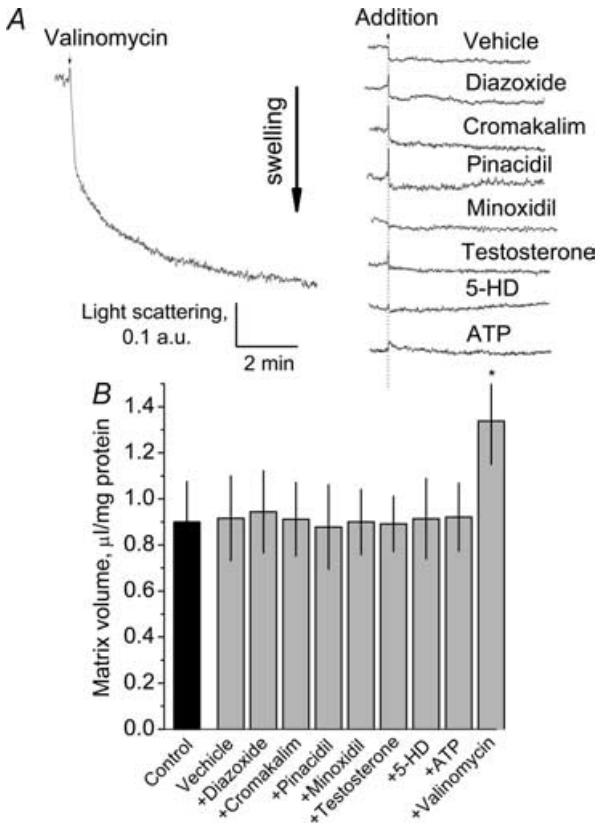

Figure 3. Openers and blockers of the putative mitoK_{ATP} did not affect light absorbance of the suspension of isolated brain mitochondria (*A*) or mitochondrial matrix volume measured with radioisotopes (*B*)

Mitochondria were incubated in the KCl-based incubation medium supplemented with 3 mM succinate and 3 mM glutamate. *A*, representative traces obtained with and without openers and blockers of the mitoK_{ATP}, and with valinomycin (0.4 nM). Openers (50 μM each) and blockers (0.5 mM 5-HD, 0.25 mM ATP) of putative mitoK_{ATP} were added as indicated to mitochondria 4–5 min after addition of mitochondria into the chamber, when the initial decrease in light absorbance was completed. The openers were added in the presence of 0.5 mM ATP. Higher and lower doses of the openers (10 and 100 μM) did not produce an effect beyond the deflection caused by a vehicle (1.5 μl DMSO, 0.5% final concentration) alone. The presence of the higher concentrations of DMSO (1–1.5% final concentration) in the medium produced a larger effect of the vehicle, but did not improve the effects of diazoxide or other DMSO-dissolved openers. Exclusion of BSA from the medium did not result in changes in the effect of the openers or blockers (not shown). Diazoxide, cromakalim and pinacidil were solubilized in DMSO. Valinomycin, minoxidil and testosterone were solubilized in ethanol. Ethanol as a vehicle (final concentration 0.5%) did not affect light absorbance measurements (not shown). *B*, summary of the radioisotope measurements of the mitochondrial matrix volume with and without the openers of the mitoK_{ATP}, and with valinomycin. Matrix volume in the control was determined in the ice-cold medium. All other matrix volume measurements were performed at 37°C. Vehicle was 0.5% DMSO. $^*P < 0.05$ *versus* vehicle. Data are means ± S.E.M., $n = 3$.

that may partially depolarize mitochondria and activate mitochondrial respiration (Kowaltowski *et al.* 2001; Bajgar *et al.* 2001). It has been proposed that the mitoK_{ATP} is selective for K^+ and does not conduct Li^+ (Kowaltowski *et al.* 2001). In K^+-free MS-based medium, the mitoK_{ATP} should also be inoperative because of the absence of K^+. Thus, if K^+ cycling involving mitoK_{ATP} is supposed to be active in brain mitochondria, omitting K^+ from the incubation medium should stop the K^+ cycling, polarize mitochondria, and decrease respiratory rate. The respiratory rates of brain mitochondria incubated in KCl-, LiCl- and MS-based media, with or without ADP or 2,4-dinitrophenol (2,4-DNP), were similar (Fig. 5). These experiments were performed in the absence of oligomycin. Accumulation of TPP^+ indicative of $\Delta\Psi$ was also comparable in KCl- and LiCl-based media (Fig. 5*A* and *B*). This suggested a lack of K^+-dependent processes that could affect respiration and $\Delta\Psi$ in these experiments. In addition, the lack of any difference in light scattering measurements conducted in K^+-containing and K^+-free medium argues against the K^+–H^+ antiporter compensating for any effects of mitoK_{ATP}.

With succinate plus glutamate, consecutive additions of 50 μM diazoxide depolarized mitochondria in both KCl- and LiCl-based media (Fig. 6*A* and *B*). In these experiments, succinate was the main substrate, while glutamate was used to prevent oxaloacetate-induced inhibition of SDH (Wojtczak *et al.* 1969) by eliminating oxaloacetate in the transaminase reaction (Lehninger *et al.* 1993). Opening of the putative mitoK_{ATP} was not involved in these depolarizations since they were observed in both KCl- and LiCl-based media. The diazoxide-induced depolarizations were not accompanied by concomitant activation in respiration (Fig. 6*A* and *B*). Diazoxide inhibits succinate dehydrogenase (SDH) (Grimmsmann & Rustenbeck, 1998; Lim *et al.* 2002; Hanley *et al.* 2002) and uncouples oxidative phosphorylation acting as a weak protonophore (Kowaltowski *et al.* 2001; Kopustinskiene *et al.* 2002; Holmuhamedov *et al.* 2004). Therefore, depolarization without activation of respiration could be explained by the inhibition of SDH combined with uncoupling. The inhibition of SDH could also account for the suppression of the uncoupled respiration by diazoxide. While basal respiration with succinate plus glutamate remained virtually unaffected, respiration stimulated by 60 μM 2,4-DNP was inhibited by diazoxide: 320 ± 6 nmol O_2 min^{-1} (mg protein)$^{-1}$ without diazoxide *versus* 152 ± 7 nmol O_2 min^{-1} (mg protein)$^{-1}$ with 50 μM diazoxide (mean ± S.E.M., $P < 0.001$, $n = 5$). An opener, pinacidil (150 μM), and a blocker, glibenclamide (150 μM), depolarized brain mitochondria, oxidizing 3 mM pyruvate plus 1 mM malate in both KCl- and LiCl-based media (Fig. 6*C*–*F*). In this case, however, the depolarizations were accompanied by activation of respiration. Similar results were obtained

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when mitochondria were fuelled by succinate plus glutamate (not shown). Interestingly, in our experiments, glibenclamide inhibited the uncoupling effect of 2,4-DNP. The mechanism of this inhibition is unclear.

Earlier, an inhibition of respiration by ATP followed by reactivation with diazoxide and consecutive suppression with 5-HD or glyburide was demonstrated, and considered to be evidence for the presence of the mitoK_{ATP} in heart and brain mitochondria (Kowaltowski *et al.* 2001; Bajgar *et al.* 2001). In our hands, the respiration of brain mitochondria in KCl- or LiCl-based media supplemented with pyruvate and malate was not affected by 0.5 mM ATP, while 50 μM diazoxide slightly activated respiration (Fig. 7*A* and *B*). These experiments were performed without addition of TPP^+ into the media to avoid possible interaction of TPP^+ with the putative mitoK_{ATP} (Mironova *et al.* 2004). 5-HD (0.5 mM) was added after diazoxide without effect. Figure 7*C* shows the dose–response curves for the effect of diazoxide on respiration in KCl- and in LiCl-based media. The effect of diazoxide on respiration of brain mitochondria was K^+ independent, and therefore could not be attributed to the putative mitoK_{ATP}.

Flavoprotein redox status

In our experiments, both diazoxide and 5-HD failed to change redox status of mitochondrial flavoproteins (FP) (Fig. 7*D*). The slight increase in FP oxidation induced by diazoxide might reflect mild uncoupling. The tiny increase in FP oxidation was also observed in LiCl-based medium (not shown). On the other hand, FCCP produced distinct oxidation of FP, while KCN resulted in reduction of FP (Fig. 7*D*). Thus, FP redox status appeared to be independent from the putative changes in the mitoK_{ATP} activity.

Recently, it has been reported that testosterone applied to cardiac myocytes or to cardiac mitoplasts produces 5-HD-sensitive opening of mitoK_{ATP} in a KCl-dependent manner leading to oxidation of FP (Er *et al.* 2004). In our experiments with isolated brain mitochondria, we found no influence of testosterone (10–50 μM) on the light

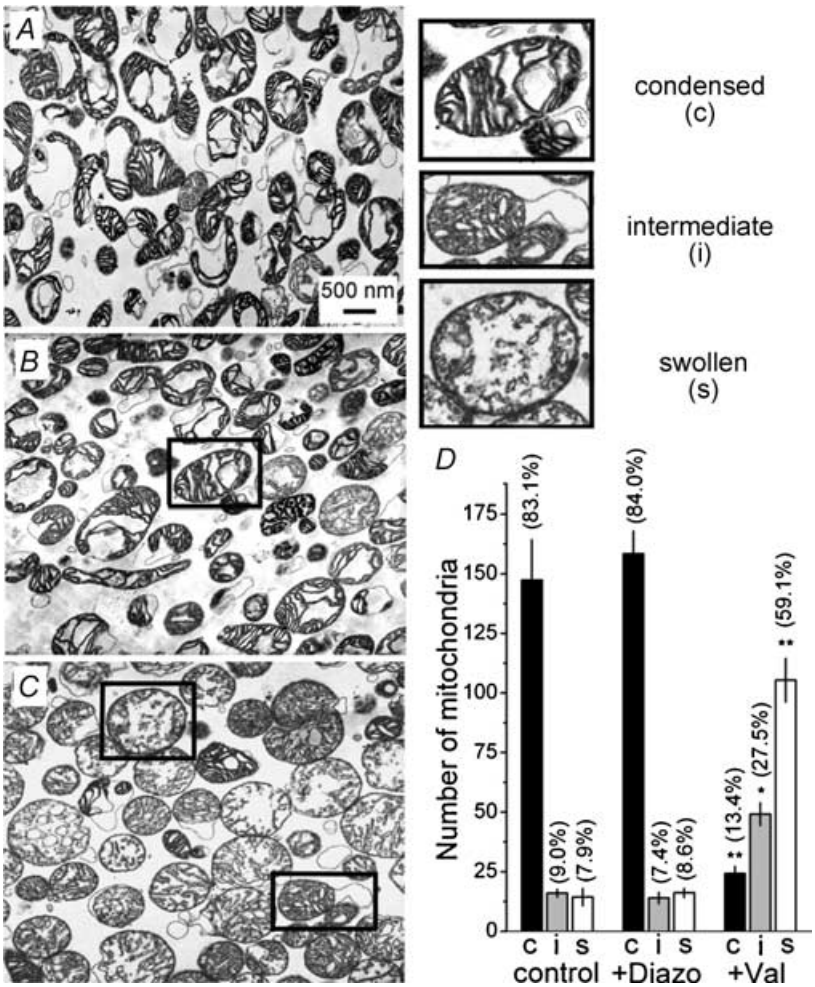

Figure 4. Diazoxide failed to induce swelling of brain mitochondria as judged by transmission electron microscopy (TEM) (*A* and *B*). A low dose of valinomycin produced a distinct expansion of the mitochondrial matrix (*C*)

A, mitochondria were incubated in the KCl-based incubation medium supplemented with 3 mM succinate and 3 mM glutamate before fixation. *B* and *C*, mitochondria were incubated for 5 min in the same medium additionally containing 50 μM diazoxide (*B*) or 0.4 nM valinomycin (*C*). Higher or lower doses of diazoxide (10 and 100 μM) produced similar results (not shown). In all these experiments, BSA was omitted from the medium to avoid possible interference with diazoxide or valinomycin. Frames, representative examples of condensed (c), intermediate (i) and swollen (s) mitochondria. *D*, morphometric analysis of mitochondria incubated with and without 50 μM diazoxide (+Diazo) or 0.4 nM valinomycin (+Val). The percentages of the mitochondrial populations in the respective groups are given in parentheses. $^*P < 0.05$, $^{**}P < 0.01$ *versus* corresponding morphological group in the control without pretreatments. Data are means ± S.E.M., $n = 4$.

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absorbance of mitochondrial suspension, $\Delta\Psi$ and redox status of FP (not shown).

K^+ efflux measurements

Mitochondria incubated in K^+-free medium respond to depolarization by releasing intramitochondrial K^+ (Jung & Brierley, 1981; Baranova *et al.* 2000). Sensitivity of K^+ release to ATP and to pharmacological modulators of mitoK_{ATP} activity could directly implicate this channel in K^+ transport in brain mitochondria. In our experiments, brain mitochondria incubated in K^+-free MS-based medium retained K^+ during the course of the experiment. FCCP-induced depolarization caused a massive release of mitochondrial K^+ into the medium (Fig. 8). The release of K^+ was partially inhibited by 0.15 mM ATP added to mitochondria prior to FCCP. ADP had a similar effect. A higher concentration of ATP or ADP (0.5 mM) did not provide a stronger inhibition (not shown). GTP and GDP (0.15 mM) were without effect (Fig. 8*B* and *C*). Diazoxide (50 μM) added after ATP did not reverse ATP-induced suppression of K^+ efflux. Neither a lower nor a higher dose of diazoxide (10 or 100 μM) affected K^+ release (not shown). Thus, while the release of K^+ from depolarized brain mitochondria could be partially inhibited by ATP, diazoxide proved to be ineffective. However, the release of K^+ from ATP-treated mitochondria could be re-activated by CAT (10 μM), an inhibitor of adenine nucleotide translocase (ANT), applied either prior to (not shown) or 2 min after addition of ATP to mitochondria (Fig. 8*A*). In addition, BKA, another inhibitor of the ANT, partially blocked K^+ release (Fig. 8*B* and *C*). These observations implicate the ANT in the ATP-sensitive FCCP-induced K^+ efflux.

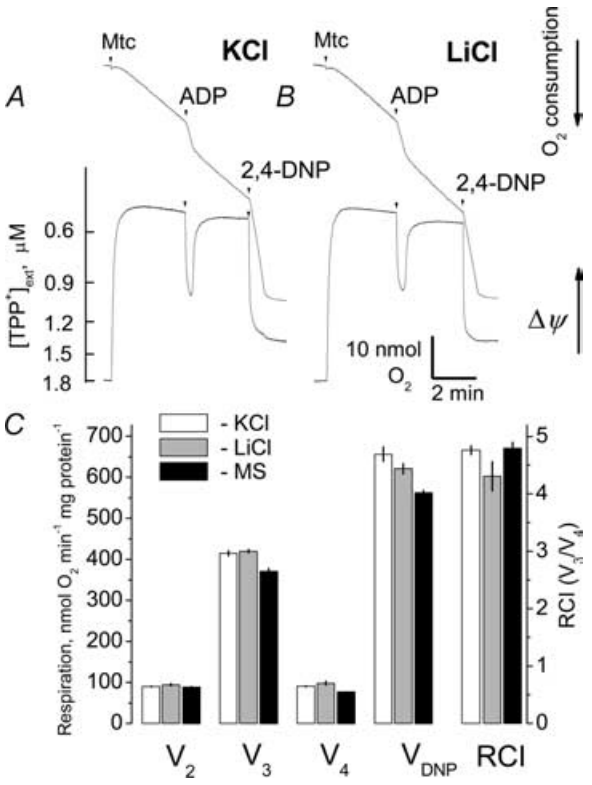

Figure 5. Respiration and membrane potential of isolated brain mitochondria in media of various compositions
A and *B*, representative traces of respiration (upper traces) and change in membrane potential ($\Delta\Psi$; lower traces) obtained with and without 200 μM ADP or 60 μM 2,4-dinitrophenol (2,4-DNP). Mitochondria were incubated in KCl- or LiCl-based media supplemented with 3 mM succinate plus 3 mM glutamate. These experiments were performed in the absence of oligomycin. *C*, summary plot of the respiratory rates, including measurements in mannitol–sucrose (MS)-based medium. V_2 is the rate of respiration prior to addition of ADP, V_3 is the respiratory rate in the presence of ADP, V_4 is the respiratory rate after all ADP was depleted, V_{DNP} is the respiratory rate in the presence of 2,4-DNP. RCI, respiratory control index, the ratio of the respiratory rate with 200 μM ADP to the respiratory rate after all added ADP has been phosphorylated, without addition of oligomicin. Data are means ± S.E.M., $n = 3$.

Discussion

The results presented in this paper cast doubt on a functional role of the mitoK_{ATP} in CNS mitochondria, although we can not completely rule out the existence of mitoK_{ATP} in brain. We presume that if mitoK_{ATP} exists, it has a different molecular identity than cellK_{ATP}. The lack of knowledge at the molecular and genetic levels represents a substantial impediment in the study of the channel. While the molecular structure of mitoK_{ATP} remains unknown, the evidence for its presence and role in mitochondria is almost utterly pharmacological, based on the apparent selectivity of the putative openers and blockers of the mitoK_{ATP}. However, in our study, the pharmacological modulators of the putative mitoK_{ATP} failed to affect functions of brain mitochondria in a manner that could be clearly associated with the activity of the putative mitoK_{ATP}. In addition, immunoblotting analysis did not provide explicit evidence for the presence of Kir 6.1, Kir 6.2, SUR1 and SUR2 in brain mitochondria. On the other hand, CAT-induced reactivation of ATP-suppressed K^+ efflux and BKA inhibition of K^+ release suggest involvement of the ANT in the K^+ transport in brain mitochondria.

Previously, in mitochondria of PC 12 cells, anti-SUR1 antibody recognized a 155 kDa protein, while anti-SUR2 antibody did not detect a band (Tai *et al.* 2003). In contrast, in mitochondria from mouse, rat and piglet brain, the anti-SUR2 antibodies recognized mitochondrially enriched polypeptides with molecular masses around 30 and 130 kDa (Lacza *et al.* 2003*a*). The anti-SUR2 antibodies also recognized a 25 kDa polypeptide in mouse brain and liver mitochondria, and in rat heart mitochondria (Lacza *et al.* 2003*b*).

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With anti-SUR1 and anti-SUR2 antibodies, we found a blocking-peptide-sensitive 97 kDa band enriched in mitochondrial samples. This band did not correspond to the expected molecular masses of SUR1, SUR2 or mitoSUR and, therefore, it could not be attributed to any of these proteins. In contrast to other groups (Shimizu *et al.* 2002; Lacza *et al.* 2003*a*), our experiments with anti-SUR2 antibody, did not reveal the 130 kDa band. In addition, the 30 kDa band detected previously with anti-SUR2 antibody in brain mitochondrial samples (Lacza *et al.* 2003*a*) appeared to be insensitive to the blocking peptide and thus appeared to be nonspecific. Therefore, it could not be attributed to mitoSUR.

Earlier, the 50 kDa polypeptides tentatively corresponding to Kir 6.1 or Kir 6.2 were found in heart mitochondria (Lacza *et al.* 2003*b*). A 50 kDa 'mitochondrial Kir 6.1' but not 'Kir 6.2' was detected in PC 12 cells (Tai *et al.* 2003). Also, a 50 kDa polypeptide putatively representing Kir 6.1 was found in brain, liver, and skeletal muscle mitochondria (Suzuki *et al.* 1997; Shimizu *et al.* 2002; Lacza *et al.* 2003*a*). In contrast, Thomzig *et al.* (2001) did not detect Kir 6.1 in brain mitochondria. In our experiments with purified brain nonsynaptosomal mitochondria, we detected 50 kDa bands enriched in mitochondrial samples with both anti-Kir 6.1 and anti-Kir 6.2 antibodies. These might represent spliced variants of Kir 6.1 or Kir 6.2 subunits of the cellK_{ATP}. However, in contrast to heart mitochondria (Lacza *et al.* 2003*b*), in brain mitochondria we did not detect 50 kDa bands with anti Kir 6.X antibodies raised

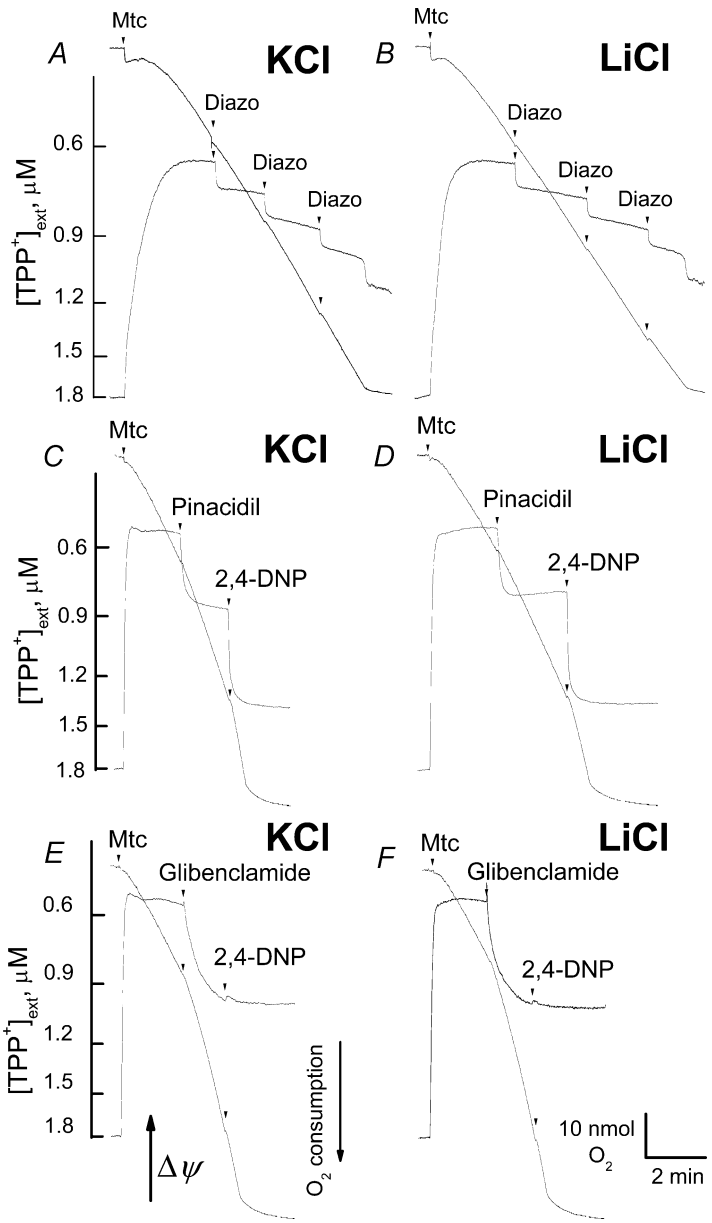

Figure 6. Diazoxide, pinacidil and glibenclamide induce K^+-independent depolarization of brain mitochondria
Pinacidil and glibenclamide activated respiration in a K^+-independent manner concurrently with depolarization. Mitochondria were incubated either in KCl-based (*A*, *C* and *E*) or LiCl-based (*B*, *D* and *F*) medium supplemented either with 3 mM succinate and 3 mM glutamate (*A* and *B*) or with 3 mM pyruvate plus 1 mM malate (*C–F*). BSA was omitted in all experiments. $\Delta\Psi$ and respiration were monitored simultaneously. *A* and *B*, three consecutive additions of diazoxide, 50 μM each, were made as indicated. Each addition of diazoxide introduced 0.5% DMSO into the medium as a vehicle. 0.5–1.5% DMSO alone affected neither mitochondrial respiration nor $\Delta\Psi$ (not shown). *C* and *D*, 150 μM pinacidil was added as indicated. *E* and *F*, 150 μM glibenclamide was added as indicated. 60 μM 2,4-DNP was added at the end of the experiments to induce maximal depolarization and activation of respiration (*C–F*).

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against other epitopes of Kir 6.2 or Kir 6.1. Thus, the 50 kDa bands might not represent either Kir 6.1 or Kir 6.2, but might instead belong to another protein with cross-reactivity to certain anti-Kir 6.X antibodies. In line with this, Grover & Garlid (2000) reported that antibodies against Kir 6.1 reacted with an unknown mitochondrial protein, but did not react with any protein in the reconstitutively active purified fraction of the mitoK_{ATP}.

Based on studies with isolated mitochondria, it has been suggested that the main function of a putative mitoK_{ATP} is to regulate mitochondrial matrix volume (Kowaltowski *et al.* 2001). Opening of the putative mitoK_{ATP} with diazoxide caused an increase in matrix volume evaluated with light absorbance assay (Kowaltowski *et al.* 2001;

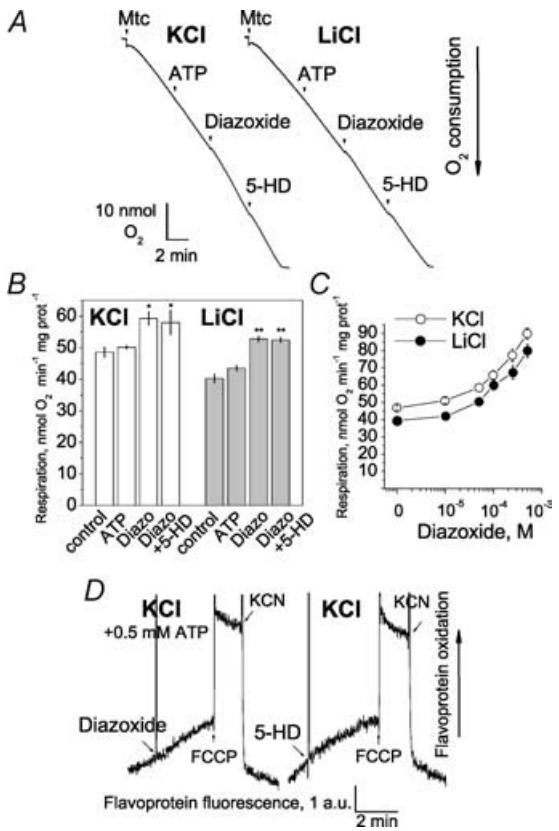

Figure 7. Effects of ATP, diazoxide and 5-HD on the respiration and flavoprotein redox status of isolated brain mitochondria in KCl- and LiCl-based medium
Mitochondria were incubated either in KCl-based medium or in LiCl-based medium (*A* and *B*). In both cases, the medium was supplemented with 3 mM pyruvate plus 1 mM malate. BSA was excluded from the media. 0.5 mM ATP, 50 μM diazoxide and 0.5 mM 5-HD were added to mitochondria as indicated. An addition of diazoxide introduced 0.5% DMSO into the medium as a vehicle. 0.5–1.5% DMSO alone did not affect mitochondrial respiration. The higher final concentrations of the vehicle (1–1.5% DMSO) did not improve the effect of diazoxide (not shown). *B*, summary plot of respiratory rates in KCl- and LiCl-based media. $^*P < 0.05$, $^{**}P < 0.01$ *versus* respiratory rate without additions (V_2). Data are means ± S.E.M., $n = 4$. *C*, diazoxide dose–response curves for the respiration in KCl- and LiCl-media. Substrates: 3 mM pyruvate plus 1 mM malate. Data are means ± S.E.M., $n = 4$. *D*, fluorescence of mitochondrial flavoproteins was monitored in KCl-based medium. Similar results were obtained in LiCl- and MS-based media (not shown). 50 μM diazoxide, 0.5 mM 5-HD, 1 μM FCCP and 2 mM KCN were added as indicated.

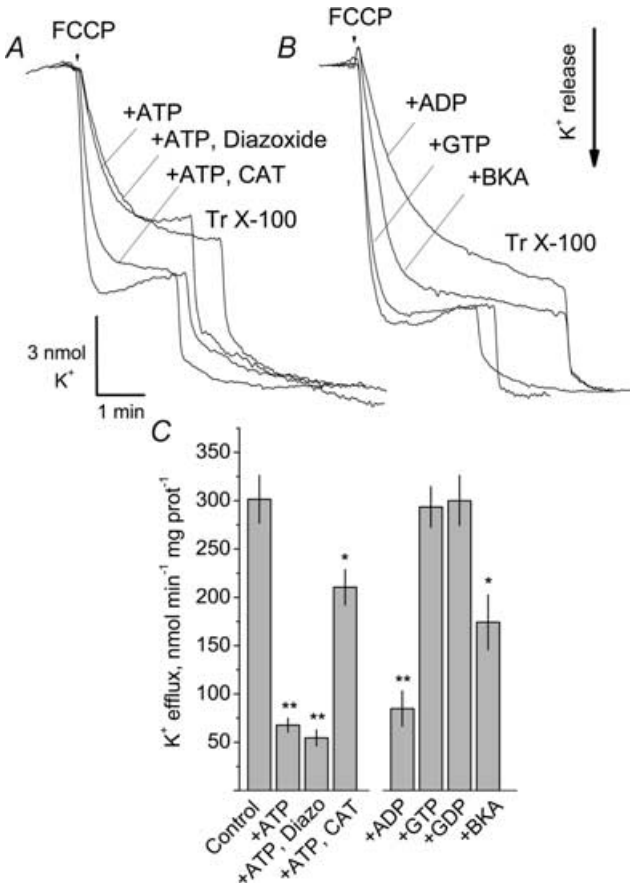

Figure 8. Uncoupler-induced release of K^+ from isolated brain mitochondria incubated in K^+-free MS-based medium
ATP, ADP and bongkrekic acid (BKA) partially inhibited K^+ efflux. Diazoxide did not reactivate the ATP-suppressed release of K^+, while carboxyatractyloside (CAT) reactivated the ATP-suppressed K^+ efflux. GTP and GDP did not influence K^+ efflux. Mitochondria were incubated in the MS-based medium supplemented with 3 mM succinate and 3 mM glutamate. 0.15 mM ATP and 50 μM diazoxide (in the presence of 0.5% DMSO as a vehicle) were added into the chamber prior to mitochondria. Higher and lower doses of diazoxide (10 and 100 μM) produced similar results. The higher final concentrations of the vehicle (1–1.5% DMSO) did not improve the effect of diazoxide (not shown). *A*, 10 μM CAT was added to mitochondria 2 min after addition of ATP. *B*, 0.15 mM ADP, 0.15 mM GTP, 0.15 mM GDP or 20 μM BKA was added prior to mitochondria in the absence of ATP. *A* and *B*, the medium was supplemented with 1 μM oligomycin. In both cases, FCCP (1 μM) was added to depolarize mitochondria and to trigger K^+ efflux. Similar results were obtained with 60 μM 2,4-DNP (not shown). At the end of the experiment, 0.1% Triton X-100 (Tr X-100) was added to release a residual K^+.
C, summary of the results of the experiments. $^*P < 0.05$, $^{**}P < 0.01$ *versus* control. Data are means ± S.E.M., $n = 4$.

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Bajgar *et al.* 2001). However, in the present study, using three different assays (light absorbance measurements, radioisotope measurements and TEM), we did not find evidence for the changes of matrix volume following treatment of brain mitochondria with blockers or openers of the putative mitoK_{ATP}. The recent radioisotope measurements of matrix volume in liver and heart mitochondria also challenged the role of mitoK_{ATP} in the regulation of matrix volume (Das *et al.* 2003). The possible cause of the differences between our results and the previously reported data (Bajgar *et al.* 2001) is not clear. The use of different types of mitochondrial preparations could be one of the possible explanations. In our work, we used Percoll-gradient-purified non-synaptosomal mitochondria, while others used brain mitochondria isolated with the use of digitonin without a further density gradient purification step (Bajgar *et al.* 2001). Although the conflicting data might be caused by irreversible inactivation of the putative mitoK_{ATP} during purification over Percoll gradient, isolated Percoll-gradient-purified brain mitochondria were used in other studies of mitoK_{ATP} without loss of activity (Shimizu *et al.* 2002; Lacza *et al.* 2003*a*). It is also possible that concurrent activation of the K^+–H^+ exchanger compensated K^+ influx through a mitoK_{ATP}, preventing detectable matrix volume changes in our experiments.

It has been reported that diazoxide causes slight activation of mitochondrial respiration, presumably due to acceleration of futile K^+ cycling across the inner mitochondrial membrane (IMM) (Kowaltowski *et al.* 2001; Bajgar *et al.* 2001; Garlid & Paucek, 2003). In heart mitochondria, opening the mitoK_{ATP} resulted in a negligible depolarization (1–2 mV) that was practically impossible to detect (Kowaltowski *et al.* 2001). In isolated brain mitochondria, the amount of the mitoK_{ATP} was estimated to be sevenfold higher than in liver or heart mitochondria (Bajgar *et al.* 2001). Correspondingly, in brain mitochondria, the blockade of a putative mitoK_{ATP} with ATP was accompanied by a stronger inhibition of respiration followed by greater re-activation of respiration induced by diazoxide (Bajgar *et al.* 2001). In contrast to the previous studies with isolated brain mitochondria (Bajgar *et al.* 2001), we did not observe an inhibition of respiration with exogenous ATP. Diazoxide produced subtle K^+-independent activation of respiration without reversal by 5-HD. With succinate, diazoxide noticeably depolarized brain mitochondria. However, this effect of diazoxide was observed in KCl-based as well as in KCl-free media. Therefore, the diazoxide-induced depolarization could not be attributed to opening the putative mitoK_{ATP}. The effects of diazoxide in our experiments could be better explained by protonophoric properties of diazoxide (Kopustinskiene *et al.* 2002; Holmuhamedov *et al.* 2004) and by inhibition of SDH (Schafer *et al.* 1971), rather than by opening the putative mitoK_{ATP}. An increased electron flow in the respiratory chain due to activation of respiration could cause oxidation of mitochondrial FP, including $FADH_2$ and FMN (Nicholls & Ferguson, 2002). In neurones, diazoxide increased FP fluorescence that was interpreted as an indication of opening the mitoK_{ATP}, leading to activation of electron flow in the respiratory chain due to membrane depolarization (Liu *et al.* 1998). 5-HD, added with diazoxide, blocked a putative mitoK_{ATP} and attenuated electron flow preventing oxidation of FP (Liu *et al.* 1998). In contrast, in our experiments with isolated brain mitochondria, neither diazoxide nor 5-HD affected redox status of FP. Our observations are consistent with results from two other groups that reported a failure of diazoxide to induce detectable changes in FP fluorescence in cardiomyocytes (Lawrence *et al.* 2001; Hanley *et al.* 2002). Recently, testosterone has been shown to be an effective opener of the mitoK_{ATP} in cardiomyocytes and cardiac mitoplasts (Er *et al.* 2004). However, in our experiments with brain mitochondria, testosterone did not influence mitochondrial volume, $\Delta\Psi$ or redox status of FP. Thus, the effect of testosterone could be tissue specific. An electrophysiological study of mitochondrial multiprotein complex including SDH, α-subunit of ATP synthase, phosphate carrier, mitochondrial ATP-binding cassette protein 1 and ANT, reconstituted into proteoliposomes or a planar lipid bilayer, revealed a channel sensitive to blockers and openers of a putative mitoK_{ATP} (Ardehali *et al.* 2004). It is unknown whether all five proteins of the multiprotein complex that resembles the putative mitoK_{ATP} are required for channel activity (Ardehali *et al.* 2004). It is also unclear which of them is a pore-forming component of the putative channel. The presence of the ANT in the multiprotein complex invigorates the hypothesis that the ANT might be responsible for the mitochondrial K^+ uniport sensitive to adenine nucleotides (Panov *et al.* 1980; Halestrap, 1989). It has been previously shown that 2,4-DNP-induced release of K^+ from rat liver mitochondria is inhibited by ATP and ADP (Baranova *et al.* 2000). Atractyloside, a specific inhibitor of the ANT, completely eliminated the inhibitory effect of adenine nucleotides. In accord with this observation, in our experiments, CAT, another specific inhibitor of the ANT, reversed ATP–induced inhibition of FCCP-evoked K^+ release from brain mitochondria. Initially, the adenine nucleotide binding site of the mitoK_{ATP} was proposed to face the mitochondrial matrix (Inoue *et al.* 1991). Later, the adenine nucleotide binding site of the putative mitoK_{ATP} was found to face the cytosol (Yarov-Yarovoy *et al.* 1997). If this is the case, the atractyloside- and CAT-sensitive effects of adenine nucleotides could be attributed to the ANT rather than to the putative mitoK_{ATP}. In line with this, BKA, an inhibitor of the ANT, suppressed K^+ efflux induced by depolarization mimicking the effect of adenine nucleotides. Since the ANT-linked channel is regulated by adenine nucleotides (Halestrap, 1989; Brustovetsky *et al.*

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2002*b*), it could be nominally identified as a mitoK_{ATP}. The other constituents of the multiprotein complex described by Ardehali *et al.* (2004) might cooperate with the ANT, providing regulatory mechanisms of a putative mitoK_{ATP}. However, additional studies are required to substantiate this hypothesis.

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