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Single channel studies of the ATP-regulated potassium channel in brain mitochondria

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Abstract Mitochondrial potassium channels in the brain have been suggested to have an important role in neuroprotection. The single channel activity of mitochondrial potassium channels was measured after reconstitution of the purified inner membrane from rat brain mitochondria into a planar lipid bilayer. In addition to a large conductance potassium channel that was described previously, we identified a potassium channel that has a mean conductance of 219 ± 15 pS. The activity of this channel was inhibited by ATP/Mg^{2+} and activated by the potassium channel opener BMS191095. Channel activity was not influenced either by 5-hydroxydecanoic acid, an inhibitor of mitochondrial ATP-regulated potassium channels, or by the plasma membrane ATP-regulated potassium channel blocker HMR1098. Likewise, this mitochondrial potassium channel was unaffected by the large conductance potassium channel inhibitor iberiotoxin or by the voltage-dependent potassium channel inhibitor margatoxin. The amplitude of the conductance was lowered by magnesium ions, but the opening ability was unaffected. Immunological studies identified the Kir6.1 channel subunit in the inner membrane from rat brain mitochondria. Taken together, our results demonstrate for the first time the single channel activity and properties of an ATP-regulated potassium channel from rat brain mitochondria.

Keywords Mitochondria · Potassium channel · ATP · Potassium channel openers · Rat brain · Planar lipid membrane

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Introduction

Four different types of potassium channels have been described in the inner mitochondrial membrane: an ATP-regulated potassium channel (the mitoK_{ATP} channel), a large Ca^{2+}-activated potassium channel (the mitoBK_{Ca} channel), a voltage-gated potassium channel (the mitoKv1.3 channel) and twin-pore TASK-3 potassium channels (Szewczyk and Wojtczak 2002; Szewczyk et al. 1996, 2006, 2009; Zoratti et al. 2008). It has been postulated that mitochondrial potassium channels play a pivotal role in cardio- and neuroprotection (O'Rourke 2004; Ardehali 2005; Costa and Garlid 2009). Although the details of this cytoprotective mechanism have not yet been fully elucidated, it is believed that an increase in the K^+ flux (via mitochondrial potassium channels) into the mitochondrial matrix, followed by changes in mitochondrial volume or the generation of reactive oxygen species (ROS), may play a triggering role (Kowaltowski et al. 2001; Kis et al. 2003; Busija et al. 2004; Facundo et al. 2005; Kulawiak et al. 2008). Increased understanding of the properties of the mitoK_{ATP} channels

from brain mitochondria may therefore lead to a rational strategy for neuroprotection.

The margatoxin-sensitive voltage-gated Kv1.3 channel was identified in T-lymphocyte mitochondria using patch-clamp electrophysiology, electron microscopy and immunological studies (Szabo et al. 2005). It was shown that Kv1.3 is present in both the plasma and mitochondrial membranes, despite the lack of an N-terminal mitochondrial targeting sequence. The mitochondrial Kv1.3 channel may be an important factor in apoptotic signal transduction (Szabo et al. 2008). The mitoBK$_{Ca}$ channel was originally studied using a patch-clamp technique in glioma cells (Siemen et al. 1999). This channel, with a conductance of 295 pS, is stimulated by Ca^{2+} and inhibited by charybdotoxin (ChTx). Later, the presence of the mitoBK$_{Ca}$ channel was observed using patch-clamp recordings from mitoplasts that were isolated from guinea pig ventricular cells (Xu et al. 2002). Recently, immunological and functional studies have suggested the presence of mitoBK$_{Ca}$ channels in brain mitochondria (Douglas et al. 2006; Piwonska et al. 2008; Skalska et al. 2009).

The mitoK$_{ATP}$ channel was first identified using the patch clamp technique in rat liver mitochondria (Inoue et al. 1991). The mitoK$_{ATP}$ channel was also found in heart tissue (Paucek et al. 1992), human T-lymphocytes (Dahlem et al. 2004), skeletal muscle (Debska et al. 2002), renal (Cancherini et al. 2003) and amoeba mitochondria (Kicinska et al. 2007). These channels are activated by potassium channel openers such as diazoxide and BMS191095 and inhibited by antidiabetic sulfonylureas (e.g., glibenclamide) and 5-hydroxydecanoic acid (5 - HD) (Szewczyk 1998; Szewczyk and Marban 1999). Additionally, functional investigations of isolated mitochondria indicate the presence of mitoK$_{ATP}$ channels in brain mitochondria (Debska et al. 2001; Bajgar et al. 2001).

The molecular identity of the mitoK$_{ATP}$ channel is still unclear. Based on similarities between mitoK$_{ATP}$ and plasma membrane ATP-regulated potassium channels, it is believed that mitoK$_{ATP}$ channels are composed of a pore-forming subunit (similar to the inward rectifying Kir subunit) and a mitochondrial sulfonylurea receptor (mitoSUR). Several observations regarding the pharmacological profile and immunoreactivity of specific antibodies suggest that the cardiac and liver mitoK$_{ATP}$ channels belong to the inward-rectifier K^+ channel family Kir6.x (Suzuki et al. 1997). Additionally, Kir6.1, Kir6.2 and sulfonylurea receptor (SUR2A) subunits have been demonstrated to exist in ventricular myocyte mitochondria (Singh et al. 2003). It was proposed that a complex of mitochondrial proteins such as succinate dehydrogenase and ATP/ADP translocase in the inner membrane is capable of transporting K^+ with properties similar to the mitoK$_{ATP}$ channel (Ardehali et al. 2004). Recently, two proteins that were immunoreactive with Kir6.1 antibodies were identified by mass spectrometry to be identical to NADH dehydrogenase flavoprotein 1 and the NADP-bound form of mitochondrial isocitrate dehydrogenase (Foster et al. 2008).

Previously, the properties of brain mitochondrial mitoK$_{ATP}$ channels were determined using isolated mitochondria or proteoliposomes (Bajgar et al. 2001; Debska et al. 2001; Raval et al. 2007; Fornazari et al. 2008). In this study, we used purified mitochondrial inner membrane particles (SMP) reconstituted into a planar lipid bilayer in order to study the biophysical and pharmacological properties of the brain mitoK$_{ATP}$ channel.

Materials and methods

Materials

L-α-phosphatidyl-choline (asolectin), *n*-decane and bovine serum albumin (BSA) were obtained from Sigma-Aldrich, Germany. Antibodies against Kir6.1 and ANT were obtained from Santa Cruz Biotechnology, UK, and the secondary antibodies were obtained from Sigma-Aldrich, Germany. All other chemicals were of the highest purity commercially available.

Isolation of mitochondria

Brains without cerebella from seven adult Wistar rats (70–80 days old) were used for the inner mitochondrial membrane preparations. Rat brain mitochondria were isolated under ice-cold conditions according to a standard protocol (Kudin et al. 2004). In brief, a male Wistar rat was anesthetized and killed by decapitation. The brain was rapidly removed, washed, and placed in ice-cold buffer containing 225 mM mannitol, 75 mM sucrose, 5 mM HEPES, 1 mg/ml BSA and 1 mM EGTA, pH 7.4. The tissue was then minced with scissors and placed in 10 ml of isolation medium supplemented with nagarase (0.5 mg/ml) and homogenized using a motor-driven Teflon-glass Potter homogenizer. After the homogenate was diluted two-fold, it was then centrifuged at 2,000×g for 4 min. The supernatant was decanted and centrifuged at 12,000 × g for 9 min. In order to permeabilize the synaptosomes, the pellet was suspended in isolation buffer supplemented with digitonin (0.2 mg/ml) and homogenized manually. Finally the suspension was centrifuged at 12,000× g for 11 min. The mitochondrial pellet was resuspended in isolation medium to a final protein concentration of 15–20 mg/ml. All procedures were carried out at 4 °C.

Preparation of submitochondrial particles (SMP)

To obtain SMP, the rat brain mitochondria were sonicated 3×15 s and ultracentrifuged as previously

described (Cino and Del Maestro 1989). The submitochondrial particles were resuspended to a final concentration of about 4 mg protein/ml.

Black lipid membrane measurements (BLMs)

All experiments were performed using planar lipid membrane techniques as previously described (Hordejuk et al. 2004; Bednarczyk et al. 2004, 2005, 2008; Kulawiak and Bednarczyk 2005). In brief, BLMs were formed in a 250 μm diameter hole drilled in a Delrin cup (Warner Instrument Corp., Hamden, CT USA), which separated two chambers (*cis/trans*). The chambers contained either a gradient of 50/450 mM KCl (*cis/trans*) or symmetrical 450/450 mM KCl (*cis/trans*) concentrations with 20 mM Tris-HCl, pH 7.2 (Fig. 1d). The outline of the aperture was coated with a lipid solution and dried over nitrogen before the formation of the bilayer in order to improve the stability of the membrane. BLMs were painted with asolectin in *n*-decane at a final concentration of 25 mg lipid/ml. Rat brain SMP (about 4 mg of protein/ml, 1–3 μl) was then added to the *trans* compartment. The formation and thinning of the bilayer was monitored using capacitance measurements and optical observations. Electrical connections were made using Ag/AgCl electrodes and agar salt bridges (3 M KCl) to minimize liquid junction potentials. Voltage was applied to the *cis* compartment of the chamber and the *trans* compartment was grounded. The current was measured throughout using a bilayer membrane amplifier (BLM-120, BioLogic).

Data analysis

The signals were filtered at 500 Hz. The current was digitized at a sampling rate of 100 kHz (A/D converter PowerLab 2/25, ADInstruments), and the data were transferred to a PC for off-line analysis using Chart v5.2 (ADInstruments) and pCLAMP 9.0 (Axon Laboratory). The channel recordings illustrated are representative of the most frequently observed amplitudes of the opening channels under the given conditions. The conductance was calculated from the current-voltage relationship, averaged from three independent experiments. Single channel currents were recorded at different voltages. The reversal potential was derived from fitting the experimental data to a polynomial, second order curve. The probability of channel opening [P(open)] was calculated using an automatic interval setting. The mean open and closed times were determined by logarithmic binning mode using the Marquardt-LSQ fitting method, order one, with no weighting. The number of total experiments is denoted by *n*. The mean open and closed times as well as P(open) were calculated from segments of continuous recordings lasting 60 s and with N ≥ 1,000 events. Data from the experiments are

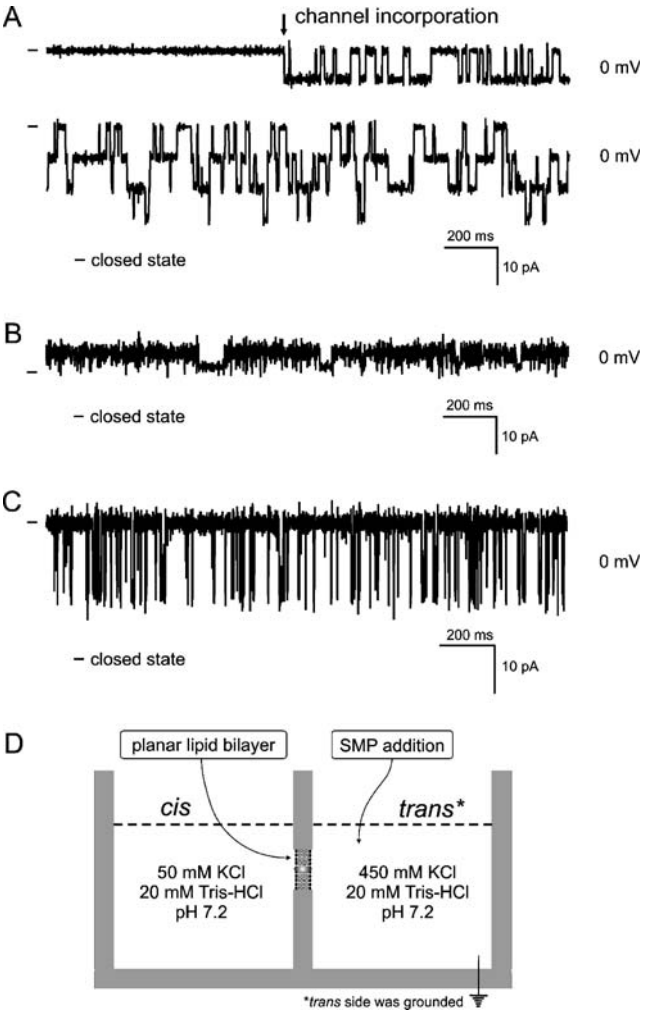

Fig. 1 Single channel recordings of ion channels from rat brain mitochondria in a planar lipid bilayer. **a** Single channel recordings of the rat brain mitoK$_{ATP}$ channel in a 50/450 mM KCl (*cis/trans*) gradient solution, before and after reconstitution of the SMP vesicles at 0 mV. Multichannel recordings in a 50/450 mM KCl (*cis/trans*) gradient solution at 0 mV are shown after the incorporation of three mitoK$_{ATP}$ channels in a planar lipid bilayer. **b** Single channel recordings of the chloride channel of rat brain mitochondria in a 50/450 mM KCl (*cis/trans*) gradient solution at 0 mV. **c** Single channel recordings of the large conductance potassium channel in a 50/450 mM KCl (*cis/trans*) gradient solution at 0 mV and in the presence of 300 μM Ca^{2+} *cis/trans*. "-" indicates the closed state. **d** Configuration of the *cis* and *trans* compartments. The experiments were performed as described under "Materials and Methods"

reported as the mean value ± S.D. (standard deviation). The permeability ratios P_K/P_{Cl} were calculated according to the Goldman-Hodgkin-Katz voltage equation (Hille 2001):

$$E_{rev} = \frac{RT}{zF} \ln \frac{[Cl^-]_{cis} + P_K/P_{Cl}[K^+]_{trans}}{[Cl^-]_{trans} + P_K/P_{Cl}[K^+]_{cis}}, \quad (1)$$

where E_{rev} is the potential at which the current is zero, R is the gas constant, T is the temperature in Kelvin, F is Faraday's constant, P_{ion} is the permeability of the ion, and

[ion] is the respective concentration of the ion in the *cis* and *trans* chambers. Rewriting Eq. 1, the permeability ratios are equal to:

$$P_K/P_{Cl} = \frac{[Cl^-]_{cis} - \xi[Cl^-]_{trans}}{\xi[K^+]_{cis} - [K^+]_{cis}}, \quad (2)$$

where $\xi = \exp\left(\frac{zFE_{rev}}{RT}\right)$.

Western blotting

Proteins were heated in Laemmli loading buffer at 97 °C for 10 min, and 25 μg of total protein from each sample was resolved on 12% SDS-PAGE and electroblotted onto nitrocellulose (Bio-Rad). The membranes were blocked for 1 h at room temperature with 10% fat-free milk in TBS-T buffer (20 mM Tris, 137 mM NaCl, 0.1% Tween 20, pH 7.6) and incubated overnight in the same buffer (plus 3% milk) with a specific primary antibody (Santa Cruz Biotechnology, 1:500). Mitochondrial anti-adenine nucleotide translocase (Anti-ANT, 1:2,000), an affinity-purified goat polyclonal antibody raised against a peptide that maps near the amino terminus of adenine nucleotide translocase 1, was used to estimate the purity of the cellular fraction. After washing, the blots were incubated for 1 h with a horseradish peroxidase-conjugated secondary antibody (Sigma, 1:5000) and detected using ECL (Pierce).

Protein concentration assay

The protein concentrations of the submitochondrial particle (SMP) preparations was determined using the Bio-Rad protein assay kit.

Results

Reconstitution of the inner mitochondrial membrane into a lipid bilayer

SMPs from rat brain were reconstituted into a planar lipid membrane and the characteristic currents for the ion channels were recorded. Fusion of the SMP particles to the bilayer was usually observed within 10–30 min after they were added only to the *trans* compartment. After incorporation of the SMPs, we usually observed three types of ion channels that had different single channel amplitudes and selectivity (n=65). Figure 1a shows recordings of the potassium channel (later characterized as the mitoK$_{ATP}$ channel) as a single or triple active channel in a 50/450 mM KCl (*cis/trans*) gradient solution at 0 mV (n=51). Additionally, we observed a chloride-selective channel (n=10) (Fig. 1b) and a large-conductance potassium channel (n=4, Fig. 1c). Single channel recordings of the large-conductance potassium channel were obtained in the presence of 300 μM Ca^{2+} in both the *cis* and *trans* compartments.

Single channel properties of the brain mitoK$_{ATP}$ channel

In order to describe the electrophysiological properties of the mitoK$_{ATP}$ channel in more detail, we observed the single channel current vs. time traces in a gradient of 50/450 mM KCl (*cis/trans*) and in a symmetrical 450/450 mM KCl (*cis/trans*) solution at different voltages (Figs. 2a and b). Figure 2c shows the current-voltage relationship under gradient (dashed line, ■) and symmetrical (solid line, ●) conditions. According to the Nernst equation the reversal potential was 56 mV in the 50/450 mM KCl (*cis/trans*) gradient solution, thus confirming that the examined channel is cation-selective. The mean reversal potential calculated from curve fitting the experimental data was 55±5 mV (n=5); this means that the channel was not permeable to chloride ions given its permeability ratio P_K/P_{Cl} of 180 (see "Materials and Methods"). The conductance was determined to be 219 ± 15 pS in a symmetric 450/450 mM KCl (*cis/trans*) solution (n=4). We also analyzed the kinetics of the mitoK$_{ATP}$ channel. Figure 3a illustrates the voltage-dependent mean open dwell times and closed dwell times of the channels. The mean open dwell time decreased with increasing voltage, and the mean closed dwell time had a bell-shaped dependence. In Fig. 3b, we show that the probability that the mitoK$_{ATP}$ channel is open [P(open)] in symmetric 450/450 mM KCl (*cis/trans*) solutions was voltage dependent. P(open) decreased from about 0.5 at −70 mV to about 0.2 at 0 mV and remained constant at positive voltages up to 80 mV. Figure 3c shows histograms indicating the mean open dwell-times at 30 mV and −30 mV as 16 ms and 25 ms, respectively; also, mean closed dwell times at 30 mV and −30 mV were found to be 48 ms and 44 ms, respectively. All calculations were performed using basic single channel recordings of the mitoK$_{ATP}$ channel in symmetric 450/450 mM KCl (*cis/trans*) solution.

Pharmacological properties of the brain mitoK$_{ATP}$ channel

Substances known to modulate mitoK$_{ATP}$, mitoBK$_{Ca}$ and mitoKv1.3 channel activities were used to examine the properties of the ion channels. Figure 4a shows single channel recordings using a gradient of 50/450 mM KCl (*cis/trans*) solution at 0 mV under control conditions and after the addition of 1 mM Mg^{2+} plus 2.5 mM ATP to both chambers [*cis* and *trans* (n=8)]. The ATP/Mg^{2+} complex inhibited the channel activity within 1 to 10 min. Addi-

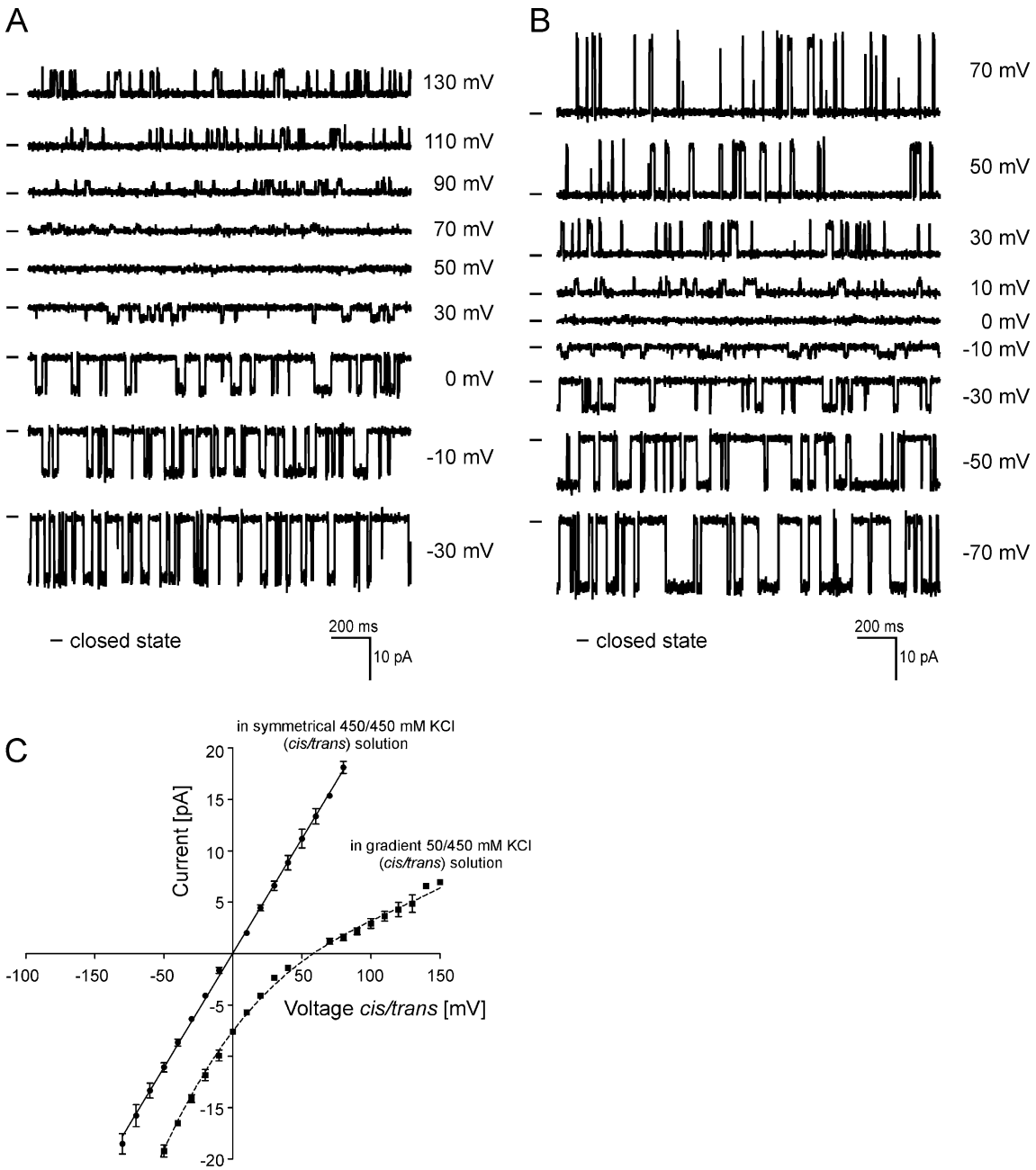

Fig. 2 Single channel recordings of the mitoK$_{ATP}$ channel in a planar lipid bilayer. **a** Single channel recordings in a 50/450 mM KCl (*cis/trans*) gradient solution at different voltages. **b** Single channel recordings in a symmetrical 450/450 mM KCl (*cis/trans*) solution at different voltages. "-" indicates the closed state of the channel. **c** Current-voltage characteristics of single channel events in a 50/450 mM KCl (*cis/trans*) gradient solution (*dashed line*, ■) and in a symmetric 450/450 mM KCl (*cis/trans*) solution (*solid line*, ●). *Error bars* indicate the S.D. from three independent experiments

tionally, we examined the effect of various K$_{ATP}$ channel inhibitors and activators on single channel activity (Fig. 4a). The addition of 150 μM 5-hydroxydecanoic acid (5-HD, n=5), a known inhibitor of cardiac mitoK$_{ATP}$ channels (Zhang et al. 2001; Nakae et al. 2003), and 100 μM HMR1098 (n=3), a specific inhibitor of plasma membrane K$_{ATP}$ channels (Gogelein et al. 2001; Liu et al. 2001), to both the *cis* and *trans* compartments, did not influence brain mitoK$_{ATP}$ channel activity. We also examined the effect of iberiotoxin (IbTx), a potent inhibitor of the BK$_{Ca}$ channel, and margatoxin (MrTx), a mitoKv1.3 channel blocker (Szabo et al. 2005). We observed no effect on channel activity when IbTx (150 nM, n=4) and MrTx (10 nM, n=4) were added to both the *cis* and *trans* sides of the 50/450 mM KCl (*cis/trans*) gradient solution, in separate experiments (Fig. 4b).

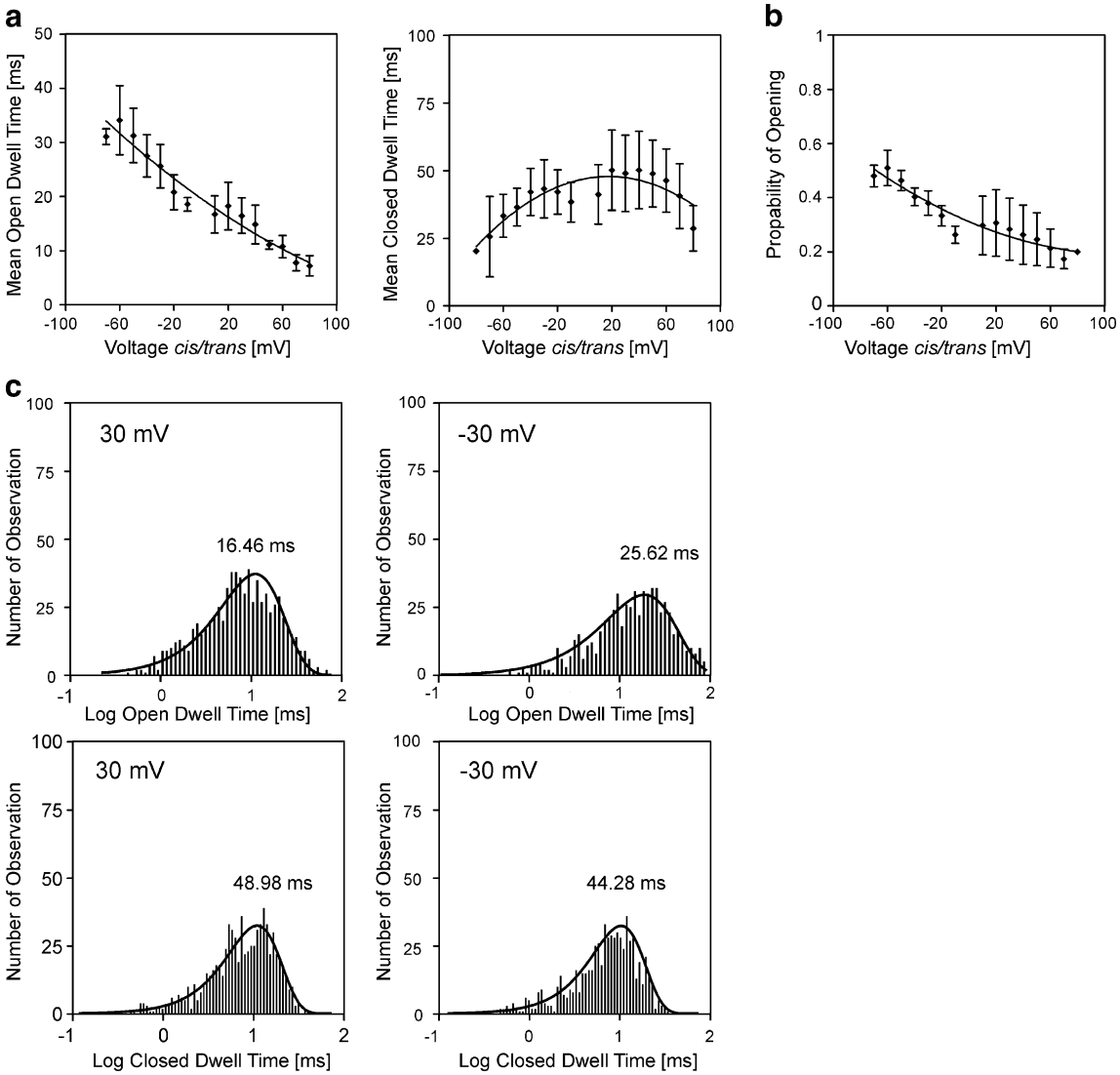

Fig. 3 Kinetic analysis of the mitoK$_{ATP}$ channel activity. **a** Voltage dependence of the mean open dwell time and closed dwell time of the mitoK$_{ATP}$ channel in a symmetric 450/450 mM KCl (*cis/trans*) solution. **b** Probability of opening the mitoK$_{ATP}$ channel in symmetric 450/450 mM KCl (*cis/trans*) solution at different voltages. *Error bars* indicate the S.D. from three independent experiments. **c** The open and closed mean dwell-time distributions in a symmetric 450/450 mM KCl (*cis/trans*) solution at 30 mV and −30 mV. The histograms were fitted using superimposed Gaussian curves

We also tested the influence of BMS191095, a potent activator of mitochondrial potassium channels (Moses et al. 2005; Grover et al. 2001; Grover et al. 2002). Figure 5 illustrates the inhibitory effect of 2.5 mM ATP (in the presence of 1 mM Mg^{2+}), which was reversed by the addition of 30 μM BMS191095 (*cis/trans*) within 10 s after application of the drug (n=6). These effects can also be observed in the amplitude histograms, as shown in Fig. 5b.

In earlier work, we described the regulation of cardiac mitoK$_{ATP}$ channels by magnesium ions (Bednarczyk et al. 2005). We noticed during these experiments that single channel amplitude decreased after the application of Mg^{2+}. Figure 6a presents single channel recordings in a 50/450 mM KCl (*cis/trans*) gradient solution at different voltages under control conditions, and after the addition of 1 mM Mg^{2+} to the *trans* compartment (n=9). Channel activity was not affected by the application of 1 mM Mg^{2+} to the *cis* compartment (n=3, data not shown). Figure 6b shows the current-voltage characteristics of single channel amplitudes in a 50/450 mM KCl gradient solution under control conditions (dashed line, ■), and after the addition of 1 mM Mg^{2+} to the *trans* compartment (solid line, ●). Figure 6b also shows that the K^+ current amplitude was only changed by the addition of Mg^{2+} at negative voltages. In the presence of 1 mM $MgCl_2$ in the *trans* compartment, mitoK$_{ATP}$ channel conductance decreased at negative voltages.

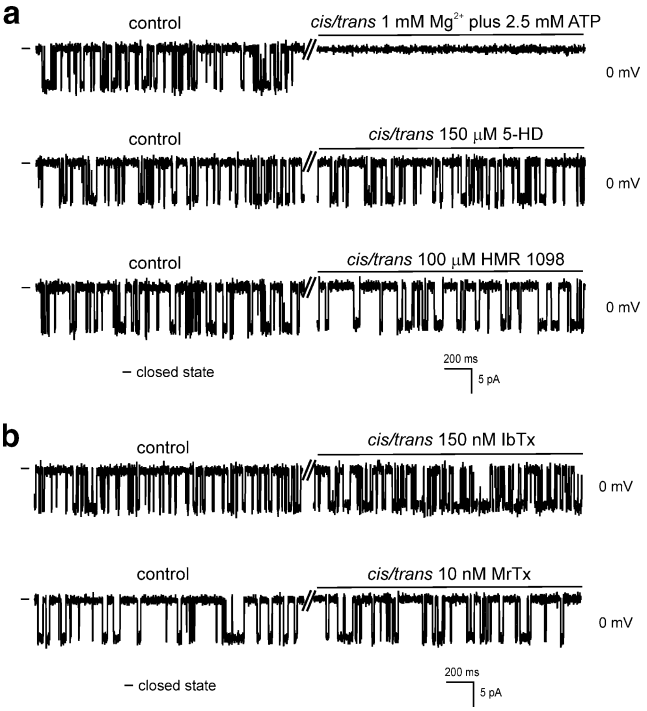

Fig. 4 The effect of an ATP/Mg^{2+} complex, 5-HD, HMR 1098, IbTx and MrTx on the activity of the mitoK$_{ATP}$ channel. **a** Single channel recordings in a 50/450 mM KCl (*cis/trans*) gradient solution at 0 mV under control conditions and after the addition of 1 mM Mg^{2+} plus 2.5 mM ATP, 150 μM 5-HD and 100 μM HMR 1098 to the *cis/trans* compartment. **b** Single channel recordings in a 50/450 mM KCl (*cis/trans*) gradient solution at 0 mV under control conditions and after the addition of 150 nM TbTx and 10 nM MrTx to the *cis/trans* compartment. All drugs were added in separate experiments. "-" indicates the closed state of the channel. The recordings were filtered using a low-pass filter at 500 Hz

The amplitude of the currents and probability of opening under control conditions and after addition of 1 mM Mg^{2+} only to the *trans* compartment at 0 mV is shown in Fig. 6c. Changes in the current amplitude were significant at the $P<0.001$ level, but the probability of opening was not significantly affected.

Immunoreactivity of brain mitochondrial membranes

Rat brain homogenates and purified, isolated mitochondria were used for Western blotting (Fig. 7). Antibodies raised against the K_{ATP} channel subunit Kir6.1 were used for immunoblotting analysis. The purity of rat brain mitochondria was confirmed with the use of an antibody raised against ATP/ADP translocase (ANT), an enzyme marker for the inner mitochondrial membrane. Western blotting showed that ANT was enriched in the mitochondrial fraction. Labeling of the blotting membranes with antibodies raised against Kir6.1 indicated the presence of two 50 kDa bands in the mitochondrial fraction. The lower mitochondrial bands were sensitive to the blocking peptide, indicating that that primary antibody against Kir6.1 was bound specifically near the C-terminal fragment of the applied polypeptide.

Discussion

Previous observations concerning the potential presence and regulation of the mitoK$_{ATP}$ channel were based on the modulation of mitochondrial function or properties (such as matrix volume, mitochondrial potential, flavoprotein oxidation or oxygen consumption) by channel inhibitors or potassium channel openers (Paucek et al. 1992; Liu et al. 1999; Debska et al. 2001, 2002; Skalska et al. 2008). Another experimental approach measured the K^+ flux using potassium-specific fluorescent dyes (Paucek et al. 1992; Garlid et al. 1997; Bajgar et al. 2001) or the radioactive isotope $^{86}Rb^+$, a K^+ analogue (Bednarczyk et al. 2004). The single channel properties of the mitoK$_{ATP}$ channel have been studied successfully by reconstituting the inner membrane of cardiac mitochondria into a planar lipid bilayer (Zhang et al. 2001; Nakae et al. 2003; Bednarczyk et al. 2004, 2008; Skalska et al. 2009). A similar approach was used here to study the properties of the brain mitochondrial mitoK$_{ATP}$ channel.

Until now, the properties of the mitoK$_{ATP}$ channel present in brain mitochondria have been described using isolated mitochondria or proteoliposomes (Bajgar et al. 2001; Debska et al. 2001). In addition, partial purification and reconstitution of the brain mitoK$_{ATP}$ channel has been reported (Bajgar et al. 2001). The K^+ flux in these proteoliposomes has been found to be regulated by the same ligands as the cardiac mitoK$_{ATP}$ channel, and the effects of opening and closing the mitoK$_{ATP}$ channel suggest that there are several fold more brain mitoK$_{ATP}$ channels per milligram of mitochondrial protein as compared to the liver or heart (Bajgar et al. 2001). Hippocampal mitochondria have also been reported to be sensitive to openers and inhibitors (Debska et al. 2001).

In the present study, reconstitution of the inner membrane from brain mitochondria revealed the presence of a chloride-selective channel and two types of potassium-selective channels that have different conductances. Large-conductance potassium channels present in the inner mitochondrial membrane have been recently characterized as calcium-activated potassium channels (Skalska et al. 2009).

The potassium-selective channels have a conductance of ~219 pS in symmetrical 450/450 mM KCl solutions were inhibited by ATP/Mg^{2+} and activated by the potassium channel opener BMS191095. Additionally, the plasma membrane K_{ATP} channel blocker HMR1098 had no effect

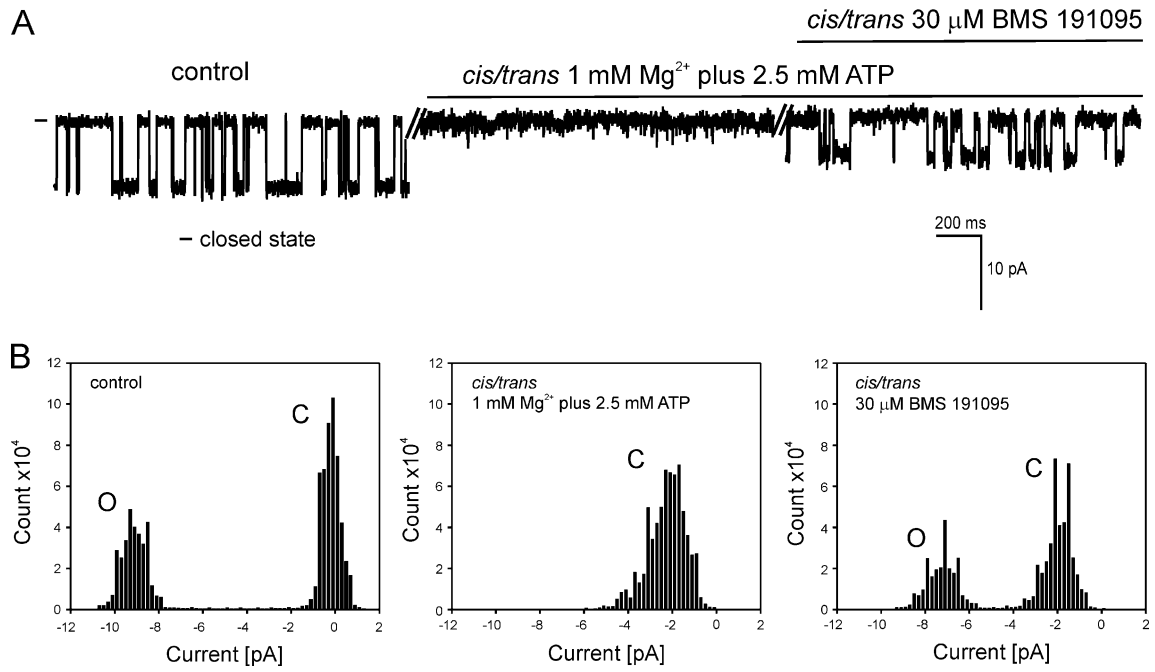

Fig. 5 The ATP/Mg^{2+} complex blocks, and BMS 191095 opens the mitoK$_{ATP}$ channel. **a** Single channel recordings in a 50/450 mM KCl (*cis/trans*) gradient solution at 0 mV under control conditions and after the addition of 1 mM Mg^{2+} plus 2.5 mM ATP and 30 μM BMS 191095 to the *cis/trans* compartment. "-" indicates the closed state of the channel. **b** Amplitude histograms under control conditions and after the addition of 1 mM Mg^{2+} plus 2.5 mM ATP and 30 μM BMS 191095 to the *cis/trans* compartment. "O" indicates the open state and "C" the closed state of the channel

on the recorded channels. These properties confirm that the activity was due to the mitoK$_{ATP}$ channel. The sensitivity of the mitochondrial potassium channels from various tissues to ATP/Mg^{2+} has been shown in isolated mitochondria (Yarov-Yarovoy et al. 1997), proteoliposomes (Paucek et al. 1992) and with the use of single channel recordings (Zhang et al. 2001; Nakae et al. 2003; Bednarczyk et al. 2004; Jiang et al. 2006).

The potassium channel opener BMS191095 is considered to act more specifically than diazoxide, another potassium channel opener, on mitochondria (Grover et al. 2001; Grover et al. 2002; Busija et al. 2005). Later, it was shown that BMS191095 induces preconditioning in cortical neurons (Kis et al. 2004) and decreases the membrane potential in brain mitochondria (Busija et al. 2005).

5-hydroxydecanoic acid (5-HD) is known to be a specific inhibitor of mitoK$_{ATP}$ channels and is distinct from glibenclamide, which is a non-specific channel inhibitor (Schultz et al. 1997). This classification is based mainly on functional measurements of cardiac mitochondria in intact cells (Ichinose et al. 2003; Sato et al. 2004; Matsunga et al. 2005), isolated mitochondria (Korge et al. 2002) and on single channel measurements (Zhang et al. 2001; Nakae et al. 2003; Dahlem et al. 2004; Bednarczyk et al. 2004; Jiang et al. 2006). Surprisingly, when using 5-HD, we did not observe an inhibitory effect on the single channel activity of the brain mitoK$_{ATP}$ channel. Previously, an inhibitory effect of 5-HD was shown on diazoxide-induced activation of the K$_{ATP}$ channel in intact mitochondria (Debska et al. 2001) and proteoliposomes (Bajgar et al. 2001). Inhibition by 5-HD was also reported in neuronal tissue on treatment with diazoxide (Shimizu et al. 2002; Teshima et al. 2003; Liang et al. 2005). In contrast, the lack of diazoxide/5-HD-sensitive K$_{ATP}$ channels was shown in rat brain non-synaptosomal mitochondria (Brustovetsky et al. 2005). It was also shown that 5-HD is converted by metabolically-active mitochondria to 5-HD-CoA and can act as substrate or inhibitor of respiration (Lim et al. 2002). Recently, the lack of 5-HD effect on brain mitochondria swelling was observed (Kupsch et al. 2007).

The single channel conductance in brain mitochondria was 219 pS and was thus much higher than the 10 to 103 pS described previously for liver, cardiac and T-lymphocyte mitochondria (Inoue et al. 1991; Mironova et al. 1999; Nakae et al. 2003; Bednarczyk et al. 2004; Dahlem et al. 2004; Jiang et al. 2006). Since two other potassium channels, including the large conductance potassium channel, have recently been found in the inner mitochondrial membrane (Xu et al. 2002; Szabo et al. 2005), we checked if other potassium channels could contribute to the channel activity described in this paper. No effect was observed when using IbTx or MrTx. The

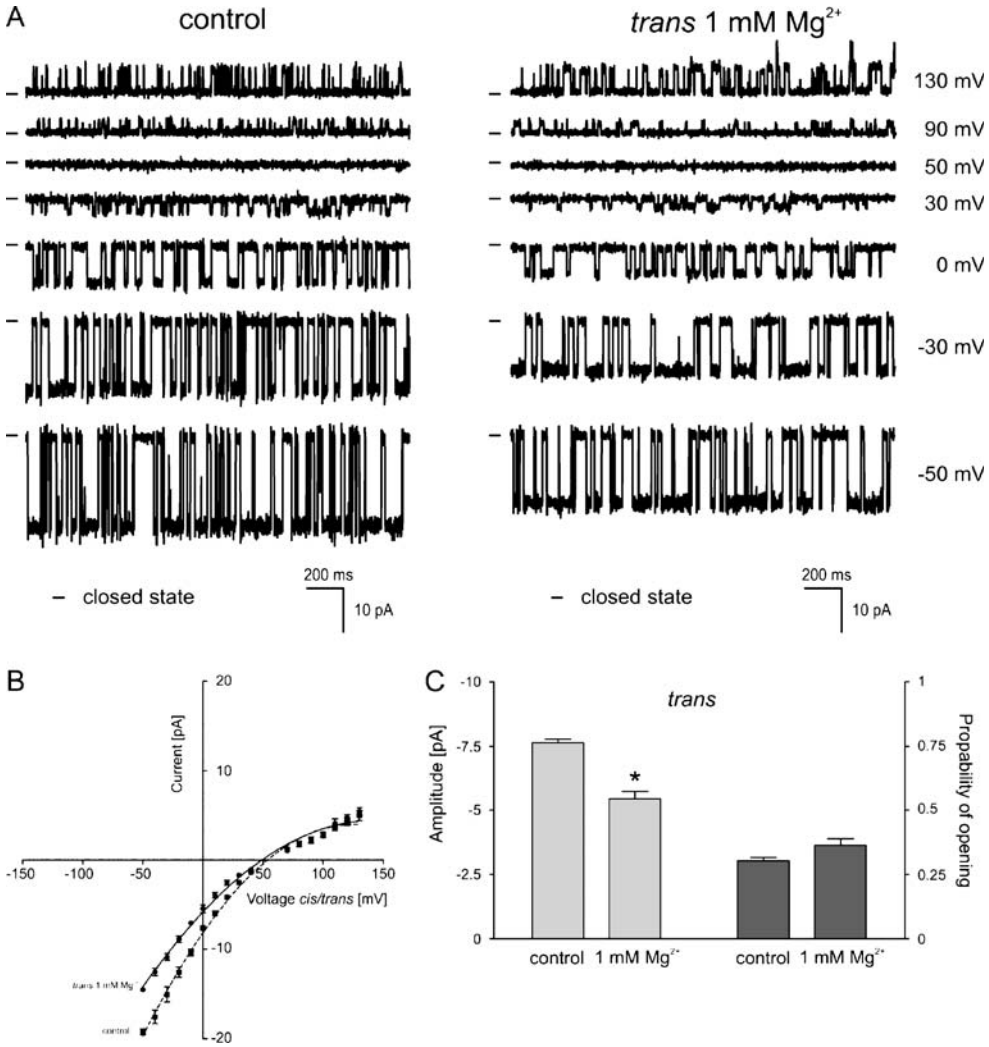

Fig. 6 The effect of Mg^{2+} on the activity of the mitoK$_{ATP}$ channel. **a** Single channel recordings in a 50/450 mM KCl (*cis/trans*) gradient solution at different voltages under control conditions and after the addition of 1 mM Mg^{2+} only to the *trans* compartment. "-" indicates the closed state of the channel. **b** Current-voltage characteristics of single channel events in a 50/450 mM KCl (*cis/trans*) gradient solution under control conditions (*dashed line*, ■) and after the addition of 1 mM Mg^{2+} only to the *trans* compartment (*solid line*, ●). **c** Current amplitude and the probability of opening under control conditions and after the addition of 1 mM Mg^{2+} only to the *trans* compartment, at 0 mV. The results are presented as the mean ± S.D. from three independent experiments. $^{*}P<0.001$ vs. the control

brain mitoK$_{ATP}$ channel was modulated by Mg^{2+} ions in a similar manner to that observed for the cardiac mitoK$_{ATP}$ channel (Bednarczyk et al. 2005). The conductance of both channels is reduced after treatment with Mg^{2+}. The open probability of the brain K$_{ATP}$ channel was not significantly changed in comparison to the cardiac mitoK$_{ATP}$ channel after the addition of magnesium.

The molecular identity of the mitoK$_{ATP}$ channel is still unknown. Immunological studies have identified both pore-forming Kir6.1 and Kir6.2 subunits in brain mitochondria (Zhou et al. 1999; Lacza et al. 2003). Similarly, the presence of the mitochondrial sulfonylurea receptor (mitoSUR) is not clear. The sulfonylurea derivative [^{125}I]-glibenclamide label a 28 kDa protein in heart mitochondria (Szewczyk et al. 1997), and a 64 kDa protein was labeled in brain mitochondria when the fluorescent probe BODIPY-glibenclamide was used (Bajgar et al. 2001).

In rat brain mitochondria, we found that the antibody against Kir6.1 recognized a blocking-peptide-sensitive band with a molecular mass of ~50 kDa. These results partially correspond to the findings of Lacza et al.

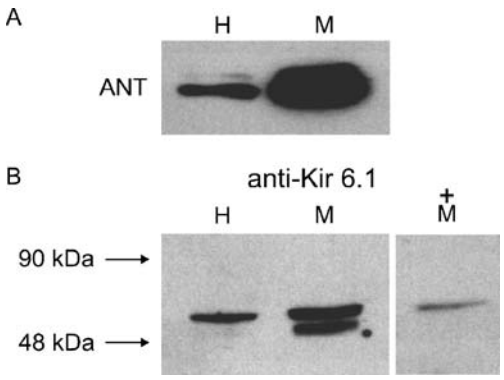

Fig. 7 Western blot analysis of the rat brain homogenate (H) and purified mitochondrial fraction (M). **a** Western blotting shows the mitochondrial enrichment of ANT. **b** Western blotting with an antibody against Kir6.1 demonstrates the presence of two ~ 50 kDa bands in the mitochondrial fraction. A blocking peptide (+) was used to discriminate nonspecific bands

(2003), who observed specific bands at ~50 kDa when labeling rat homogenate and the mitochondrial fraction with anti-Kir6.1 and anti-Kir6.2. Brustovetsky et al. (2005) observed two enriched blocking-peptide-sensitive bands in mitochondrial fractions at approximately 51 kDa after labeling with anti-Kir6.1.

In summary, in the present work we show the first characterization of single channel properties of the brain mitoK_{ATP} channel. Our data suggest that the channel is inhibited by an ATP/Mg^{2+} complex and is activated by BMS191095. Channel activity was not blocked by 5-HD and HMR1098. The channel conductance (but not the channel open probability) was reduced in the presence of magnesium ions.

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