J Bioenerg Biomembr (2008) 40:325–335
DOI 10.1007/s10863-008-9153-y

New properties of mitochondrial ATP-regulated potassium channels

Piotr Bednarczyk · Krzysztof Dołowy · Adam Szewczyk

Received: 3 May 2008 / Accepted: 16 June 2008 / Published online: 31 July 2008

Abstract The ATP-regulated potassium channel is present in the inner membrane of heart mitochondria. In this study, the activity of a single channel was measured after reconstituting the myocardium inner mitochondrial membrane into a planar lipid bilayer. We provide direct evidence of vectorial pH regulation of mitoK$_{ATP}$ channels. When the matrix side was alkalized, this changed the channel conductance, the open probability, and the mean open and closed dwell time distributions. The conductance of the mitoK$_{ATP}$ channel increased from about 110±8 to 145± 5 pS upon changing the pH from 7.2 to 8.2. This effect was reversed by reverting the pH to the neutral value. The mitoK$_{ATP}$ channel activity was not altered by alkalization of the cytosolic side of the planar lipid bilayer. We also observed that acidification from pH 7.2 to 6.2, in either the matrix or cytosolic compartments, decreased the open probability of the channel. This effect was reversed by perfusion with a pH 7.2 medium. Additionally, our results suggest that the mitoK$_{ATP}$ channel is regulated by multiple phosphorylation events. The channel activity was inhibited by an ATP/Mg^{2+} complex, but not by ATP alone, nor by a non-hydrolysable ATP analog, e.g. AMP-PNP/Mg^{2+}. The mitoK$_{ATP}$ channel "run-down" was reversed by incubating with the ATP/Mg^{2+} complex on both sides of the planar lipid bilayer. We conclude that both pH and ATP play an important regulatory role for the cardiac mitoK$_{ATP}$ channel with respect to the phenomenon of ischemia–reperfusion.

Keywords Mitochondria · MitoK$_{ATP}$ channel · Potassium channel openers · Acidosis · Alkalization · Nucleotide

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Introduction

Potassium channels, such as the ATP-regulated potassium channel (mitoK$_{ATP}$ channel) and the high-conductance Ca^{2+}-activated potassium channel (mitoBK$_{Ca}$ channel), are present in the inner membrane of cardiac mitochondria (Paucek et al. 1992; Xu et al. 2002). Potassium transport through the mitochondrial inner membrane was found to trigger cardioprotection (Mattson and Liu 2003; O'Rourke 2004; Busija et al. 2004). Potassium ions control mitochondrial metabolism by regulating the matrix volume (Halestrap 1994), and they indirectly affect the uptake of calcium (Holmuhamedov et al. 1999) and the production of reactive oxygen species (Andrukhiv et al. 2006; Kulawiak et al. 2008). The biophysical and pharmacological properties of mitochondrial potassium channels are similar to those of the potassium channels present in the plasma membrane of various cell types (Piwonska et al. 2008; Skalska et al. 2008). Mitochondrial potassium channels are regulated by the same potassium channel openers and inhibitors as the plasma membrane ATP-regulated (K$_{ATP}$ channel) or high-conductance Ca^{2+}-activated potassium channels (BK$_{Ca}$ channel; Szewczyk et al. 2006; O'Rourke 2007).

The mitoK$_{ATP}$ channel was initially described in liver mitochondria (Inoue et al. 1991). Later, it was also identified in heart (Paucek et al. 1992), brain (Bajgar et al. 2001; Debska et al. 2001), kidneys (Cancherini et al.

2003), skeletal muscle (Debska et al. 2002), human T lymphocyte (Dahlem et al. 2004), and amoeba mitochondria (Kicinska et al. 2007). However, the molecular identity of the mitoK$_{ATP}$ channel is still unknown. Several observations on the pharmacological profile and the immunoreactivity with specific antibodies suggest that the mitoK$_{ATP}$ channel belongs to the inward rectifier K^+ channel family, Kir6.x (Suzuki et al. 1997; Zhou et al. 1999). Using specific antibodies, Kir6.1, Kir6.2, and sulfonylurea receptors (SUR2A) subunits were identified in ventricular myocyte mitochondria (Singh et al. 2003), as well as in brain mitochondria (Lacza et al. 2003). Like the K$_{ATP}$ channel of the plasma membrane, mitoK$_{ATP}$ probably contains a sulfonylurea receptor (mitoSUR). ^{125}I-glibenclamide has been used to label a 28 kDa protein in bovine heart mitochondria (Szewczyk et al. 1997, 1999), whereas the fluorescent probe BODIPY-glibenclamide was used to label a 64 kDa protein in brain mitochondria (Bajgar et al. 2001). Recently, it was hypothesized that a complex of five proteins in the mitochondrial inner membrane, one with characteristics similar to those of the mitoK$_{ATP}$ channel, is capable of transporting K^+ (Ardehali et al. 2004).

Observations on the regulation of the mitoK$_{ATP}$ channel are based on pharmacological modulation of mitochondrial properties, such as matrix volume, mitochondrial potential, or oxygen consumption (Paucek et al. 1992; Szewczyk et al. 1995; Holmuhamedov et al. 1998). Measurements of the K^+ flux were performed using potassium-specific fluorescent dyes (Bajgar et al. 2001; Garlid et al. 1996) or the radioactive isotope $^{86}Rb^+$, a K^+ analogue (Bednarczyk et al. 2004). To study single-channel properties of the mitoK$_{ATP}$ channel, the mitoK$_{ATP}$ channel has been reconstituted into planar lipid bilayers and patch clamp techniques were successfully applied (Bednarczyk et al. 2004; Zhang et al. 2001; Nakae et al. 2003). The patch clamp technique was used to show that the human mitoK$_{ATP}$ channel is modulated by calcium and nitric oxide (Dahlem et al. 2004). By using the planar lipid bilayer technique, it has been shown that oxidative stress activates the mitoK$_{ATP}$ channel, which is inhibited by 5-hydroxydecanoic acid (5-HD) or the sulfhydryl-alkylating compound, *N*-ethylmaleimide (Zhang et al. 2001). Using the same technique, a direct activation of the mitoK$_{ATP}$ channel by the anesthetic isoflurane was observed (Nakae et al. 2003). More recent work has described the regulation of the cardiac mitoK$_{ATP}$ channel by quinine and magnesium ions (Bednarczyk et al. 2004, 2005).

In this study, we characterize the regulation of the mitoK$_{ATP}$ channel by protons and adenine nucleotides. The effects of ATP and pH on the activity of a single mitoK$_{ATP}$ channel from cardiac mitochondria were studied. The channel activity was measured after reconstituting purified cardiac mitochondrial inner membrane preparations into planar lipid bilayers. We provide direct evidence that there is a vectorial pH regulation of mitoK$_{ATP}$ channels. Moreover, our results suggest that the mitoK$_{ATP}$ channel is regulated by multiple phosphorylation events.

Materials and methods

Materials

L-α-Phosphatidyl-choline (asolectin; from soybean, type II-S), *n*-decane, AMP-PNP (adenosine-5′-(b,g-imido)triphosphate), and protease (Subtilisin A) from *Bacillus licheniformis* were obtained from Sigma-Aldrich, Germany. Percoll® was obtained from Fluka BioChemika. HMR 1098 (1-[5-[2-(5-chloro-o-anisamido)ethyl]-2-methoxyphenylsulfonyl]-3-methylthiourea) and BMS 191095 (4-[(4-chloro-phenyl)-(1H-imidazol-2-ylmethyl)-amino]-6-isocyano-2,2-dimethyl-chroman-3-ol) were obtained from Bristol-Myers Squibb Company.

Isolation of mitochondria

Bovine heart mitochondria were isolated at 4 °C as described previously (Zhang et al. 2001). A fragment of bovine heart muscle was minced in an isolation buffer (200 mM mannitol, 50 mM sucrose, 5 mM KH_2PO_4, 5 mM MOPS, 0.1% BSA, 1 mM EGTA, pH 7.15) and homogenized with 25 U protease/gram tissue using a Teflon pestle. The homogenate was then centrifuged at 8,000×*g*, for 10 min to remove the protease. The pellet was resuspended in the isolation buffer and centrifuged again at 700×*g* for 10 min to remove cellular debris. The supernatant was then centrifuged at 8,000×*g*, for 10 min at 4 °C to pellet the mitochondria. The mitochondria were then washed and suspended in isolation buffer without EGTA (200 mM mannitol, 50 mM sucrose, 5 mM KH_2PO_4, 5 mM MOPS, 0.1% BSA, pH 7.15). The suspension was loaded on top of a Percoll solution (30% Percoll, 0.25 M sucrose, 1 mM EDTA, 10 mM Hepes, pH 7.4) and centrifuged at 35,000×*g*, for 30 min. The mitochondrial fraction was then collected and washed twice, with isolation buffer that lacked EGTA, and resuspended at 10–20 mg of protein/ml.

Submitochondrial particle (SMP) preparation

Freshly prepared mitochondria were sonicated (Branson 250 W) 8×15 s and centrifuged at 16,000×*g* for 15 min to pellet unbroken mitochondria. The supernatant was again centrifuged at 140,000×*g*, for 35 min, and the SMPs were resuspended in the isolation buffer without EGTA and BSA (200 mM mannitol, 50 mM sucrose, 5 mM KH_2PO_4, 5 mM MOPS, pH 7.15) at about 5 mg of protein/ml.

Planar lipid bilayer (PLB) measurements

PLB measurements were performed as previously described (Bednarczyk et al. 2004, 2005; Hordejuk et al. 2004; Kwiatkowska et al. 2007). Lipid bilayers were formed in a 250 μm diameter hole drilled in a Delrin cup that had two separated chambers (*cis* and *trans* 1 ml internal volume). The chambers contained a pH 7.2 solution of 50/150 or 150/150 mM KCl (*cis*/*trans*), and 20 mM Tris–HCl. The outline of the aperture was coated with a lipid solution and dried under N_2 prior to bilayer formation to improve membrane stability. The PLBs were painted using asolectin in *n*-decane at a final concentration of 25 mg lipid per millilitre. Bovine heart SMPs (about 5 mg of protein/ml, 1–5 μl depending on reconstitution) were added to the *trans* compartment (Fig. 1d). Incorporation of the mitoK$_{ATP}$ channel into the PLB was usually observed within a few minutes. Based on the pharmacological properties, reproducibility of the channel orientation in the PLB was ~99% (Bednarczyk et al. 2005). The studied compounds were added to the *cis* or *trans* compartments. All measurements were carried out at 24 °C. Formation and thinning of the bilayer was monitored by capacitance measurements and optical observations. Final accepted capacitance values ranged from 110 to 180 pF. The electrical connections were made of Ag/AgCl electrodes and agar salt bridges

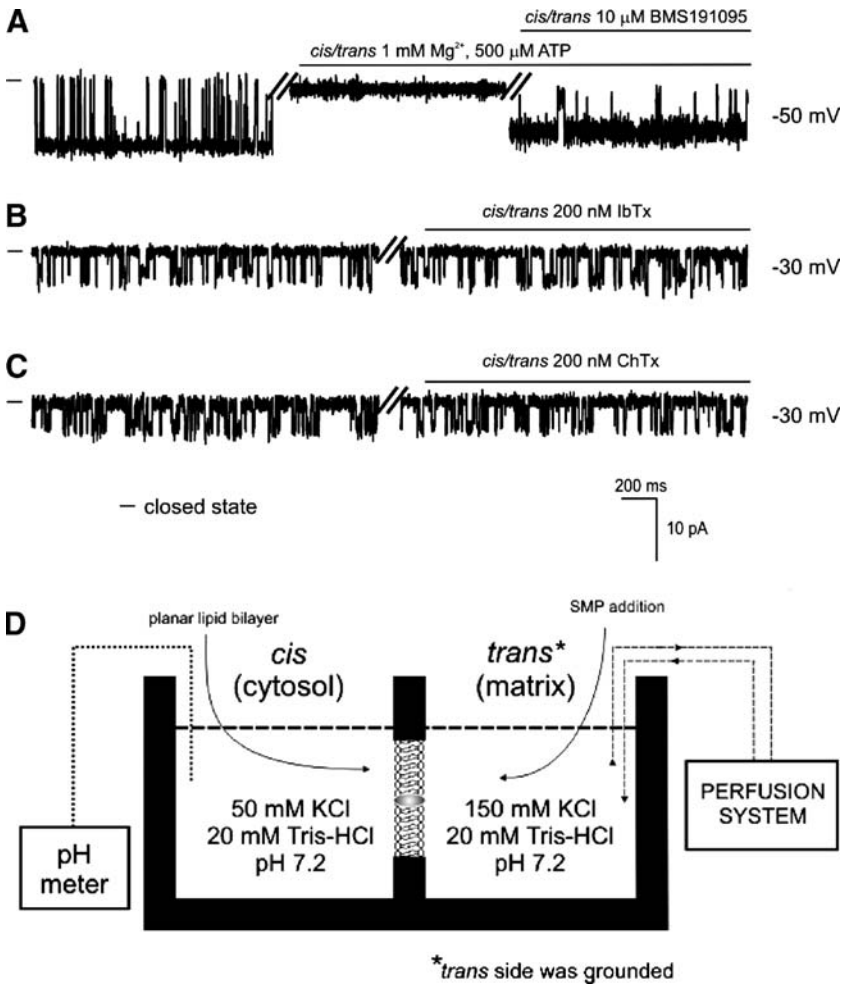

Fig. 1 Effects of the ATP/Mg^{2+} complex, BMS 191095, IbTx, and ChTx on the activity of the mitoK$_{ATP}$ channel. **a** Single-channel recordings in a 50/150 mM KCl (*cis*/*trans*) gradient solution under control conditions after adding 1 mM Mg^{2+} together with 500 μM ATP, and 10 μM BMS 191095 at −50 mV. The channel is inhibited by the ATP/Mg^{2+} complex and activated by BMS 191095, which is seen to be a very potent activator of the mitoK$_{ATP}$ channel. **b** Single-channel recordings in a 50/150 mM KCl (*cis*/*trans*) gradient solution under control conditions after adding 200 nM IbTx at −30 mV. **c** Single-channel recordings in a gradient 50/150 mM KCl (*cis*/*trans*) solution under control conditions after adding 200 nM ChTx at −30 mV. ChTx and IbTx are inhibitors of the high-conductance Ca^{2+}-activated potassium channels from plasma and inner mitochondrial membranes. These activators do not change the activity of the mitoK$_{ATP}$ channel. *Solid line* indicates the closed state of the channel. Recordings were low-pass filtered at 500 Hz. All drugs were added parallel to the *cis* and *trans* compartments. **D.** Compartments configured as *cis* and *trans* were used in the experiments. Inner mitochondrial membranes were reconstituted into planar lipid bilayers as described in "Material and methods"

(3 M KCl) to minimize liquid junction potentials. A voltage was applied to the *cis* compartment of the chamber while the *trans* compartment was grounded, and the current was measured using a bilayer membrane amplifier (BLM-120, BioLogic). The pH value was altered using 0.5 M KOH or 0.5 M HCl after the studied compounds were added to the *cis* or *trans* compartments. The amount of acid or base used was calibrated before experiments. To remove the solution from the *cis* or *trans* compartments, a perfusion system was used. The pH value was measured during experiments using a standard pH meter with a small tip pH electrode (EPI-2, Elmetron).

Data analysis

Measurements of the current output were digitized at a sampling rate of 100 kHz and transferred to a computer for off-line analysis with Chart v5.2 (PowerLab ADInstruments) and pCLAMP8.1. Signals were filtered at 500 Hz. The channel recordings reported here are representative of the most frequently observed conductances under the given conditions. The probability of a channel opening, P(open), was calculated with an automatic interval setting. The lifetimes of an open (τ_{open}) and closed (τ_{closed}) channel were calculated from a logarithmic binning mode using the Marquardt-LSQ fitting method to order one without weighting. n denotes number of experiments, and N denotes the number of events. τ_{open}, τ_{closed}, and P(open) were calculated from 60 s segments of continuous recordings, each with $N \geq 1{,}000$ events. Data from the experiments are reported as a mean value or a mean±SD (standard deviation).

Results

The mitoK$_{ATP}$ channel from bovine heart

The inner mitochondrial membrane (submitochondrial particles, SMPs) was reconstituted into planar lipid bilayers, and the current characteristics for mitochondrial ATP-regulated potassium channel (mitoK$_{ATP}$ channel) were observed (n=60). The single-channel current–time traces were usually recorded at −30 and −50 mV in 50/150 mM KCl (*cis*/*trans*) gradient conditions. The mean conductance of the channel was 103±9 pS in a 150 mM KCl symmetric solution (Bednarczyk et al. 2004). Most experiments were terminated by the addition of 150 μM 5-hydroxydecanoic acid (5-HD; *cis*/*trans*), a selective mitoK$_{ATP}$ channel inhibitor. After adding 5-HD, full activity inhibition of the channel was always observed (data not shown).

We have previously described the basic pharmacological profile of the cardiac mitoK$_{ATP}$ channel in earlier papers (Bednarczyk et al. 2004, 2005). The cardiac mitoK$_{ATP}$ channel was inhibited by adding 1 mM Mg^{2+} together with 500 μM ATP, and the effect was reversed by adding 30 μM diazoxide, a potassium channel opener. Potassium channel inhibitors, such as 150 μmol/l 5-HD and 50 μM glibenclamide, inhibited channel activity, but 100 μM HMR 1098, an inhibitor specific for plasma membrane K$_{ATP}$ channels, had no effect on the channel activity.

In this paper, we showed that the addition of 1 mM Mg^{2+} together with 500 μM ATP (*cis*/*trans*) inhibits the channel activity (n=10). Furthermore, the effect is reversible by adding the potassium channel opener BMS 191095 (*cis*/*trans*; n=6) at a concentration of 10 μM (Fig. 1a). Because of the relatively high-conductance of the observed potassium channel (~103 pS), we examined the possibility that the measured activity is due to a Ca^{2+}-activated potassium channel. Specifically, we studied substances known to modulate the activity of high-conductance Ca^{2+}-activated potassium channels. Adding either 200 nM iberiotoxin (IbTx; *cis*/*trans*; n=5) or 200 nM chrybdotoxin (ChTx; *cis*/*trans*; n=3) was found to have no influence on the measured channel activity (Fig. 1b, c, respectively).

pH modulation of the mitoK$_{ATP}$ channel activity

Figure 2a shows current–time traces of the mitoK$_{ATP}$ channel in a 50/150 mM KCl (*cis*/*trans*) gradient solution at −30 mV under control conditions, after changing pH from 7.2 to 8.2, and after perfusion to pH 7.2. A pH of 8.2 in the *trans* compartment changed the channel conductance, the open probability, and the mean open and closed dwell time distributions. The open probability [P(open)] of the mitoK$_{ATP}$ channel was increased from 0.13±0.04 to 0.66±0.09 after changing the pH from 7.2 to 8.2 (left-hand panel of Fig. 2b). The right-hand panel of Fig. 2b shows that P(open) of the mitoK$_{ATP}$ channel was not changed by alkaline conditions in the *cis* compartment, remaining at about 0.16. Figure 3a shows the current–voltage relationship for the single-channel opening at different voltages under gradient conditions after changing the pH from 7.2 to 8.2 in the *trans* compartment. In both situations, the reversal potential measured in the 50/150 mM KCl gradient solution was about 25 mV, which proves that the channel permeability P_K/P_{Cl} was not altered (Bednarczyk et al. 2005). Additionally, the conductance of the mitoK$_{ATP}$ channel increased from 110±8 pS in pH 7.2 to 145±5 pS under pH 8.2 conditions, calculated at −30 mV in 50/150 mM KCl (*cis*/*trans*) gradient solutions. Figure 3b, c shows the mean open and closed dwell time distributions of the mitoK$_{ATP}$ channel in 50/150 mM KCl (*cis*/*trans*) gradient solutions at −30 mV. Upon alkalizing the trans compartment, the mean open dwell time increased from 9.2±3.1 to 24.1±4.5 ms, and the mean closed dwell time

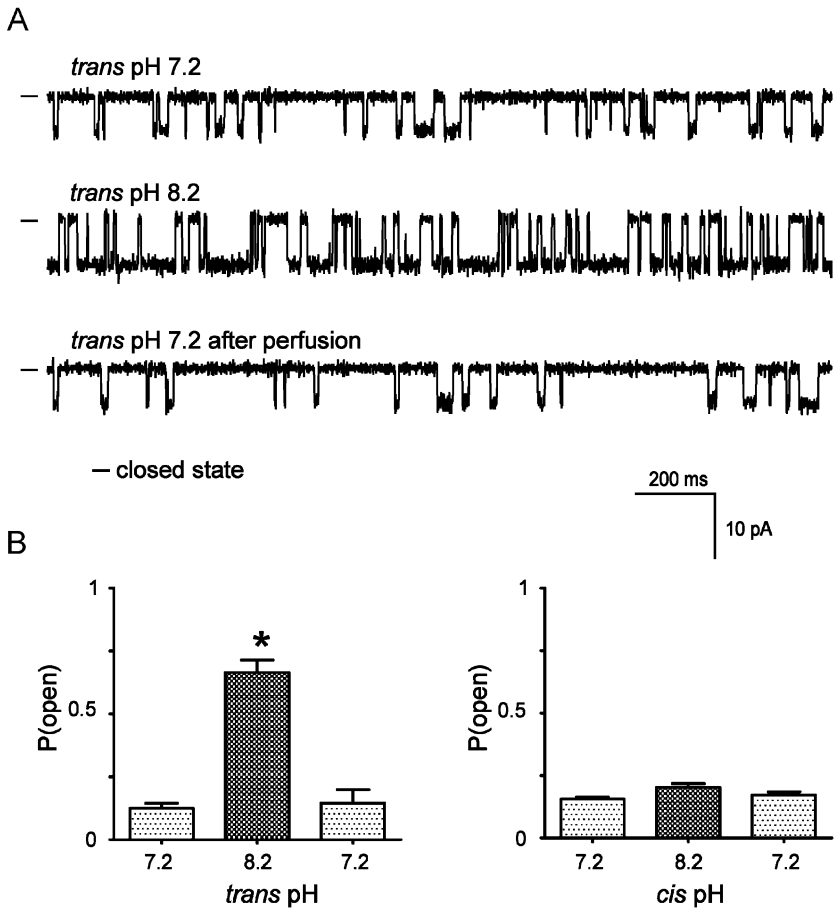

Fig. 2 Effects of alkaline pH on the single-channel activity of the mitoK$_{ATP}$ channel. **a** Single-channel recordings in a 50/150 mM KCl (*cis*/*trans*) gradient solution at −30 mV under control conditions after increasing the pH from 7.2 to 8.2, and after perfusion. The pH was changed only in the *trans* compartment. *Solid line* indicates the closed state of the channel. Recordings were low-pass filtered at 500 Hz. **b** The open probability [*P*(open)] of the mitoK$_{ATP}$ channel in a 50/150 mM KCl (*cis*/*trans*) gradient solution at −30 mV under control conditions, after increasing the pH from 7.2 to 8.2, and after perfusion in the *trans* compartment. The *right panel* shows *P*(open) of the mitoK$_{ATP}$ channels in a 50/150 mM KCl (*cis*/*trans*) gradient solution at −30 mV under control conditions, after increasing the pH from 7.2 to 8.2, and after perfusion in the *cis* compartment. The results are presented as mean±SD. *$P<0.001$ vs. control

decreased from 67.1±10.5 ms to 13.8±3.7 ms. All biophysical parameters of the mitoK$_{ATP}$ channel were restored after perfusion to pH 7.2 control conditions.

Additionally, the activity of the mitoK$_{ATP}$ channel was studied after acidifying the sample to pH 6.2 in both the *cis* and *trans* compartments. Figure 4a shows the single-channel recordings in 50/150 mM KCl (*cis*/*trans*) gradient solutions at −30 mV under control conditions after changing the pH from 7.2 to 6.2, and after perfusing the *cis* compartments from 6.2 to 7.2 ($n=7$). The open probability decreased from 0.19±0.03 to 0.08±0.02 upon acidification of the *cis* compartment. This effect was reversed by perfusion, but the open probability increased to 0.43±0.11 compared to the control (Fig. 4c). Figure 4b illustrates the activity changes of the channel after acidification of the *trans* compartment, but in this case, we did not observe typical opening of the mitoK$_{ATP}$ channel after perfusion ($n=12$). However, in only one experiment, one with a short incubation time at a lower pH, perfusion did reverse control activity of the mitoK$_{ATP}$ channel.

Nucleotide regulation of the mitoK$_{ATP}$ channel activity

We also examined the effect of the ATP/Mg^{2+} complex, sole ATP, and non-hydrolysable ATP analog on the single-channel activity. Figure 5a shows single-channel recordings in 50/150 mM KCl (*cis*/*trans*) gradient solutions at −30 mV under control conditions and after the addition of 1 mM Mg^{2+} together with 500 μM ATP to the *cis* compartment. The presence of the ATP/Mg^{2+} complex inhibits the channel activity within 10 min, but only in the *cis* compartment ($n=10$). The channel activity was not influenced by 1 mM Mg^{2+} together with 500 μM ATP ($n=6$), or by 500 μM ATP ($n=4$) added to the *trans* side (data not shown). Interestingly, the channel activity was not affected by adding 500 μM ATP without the presence of magnesium

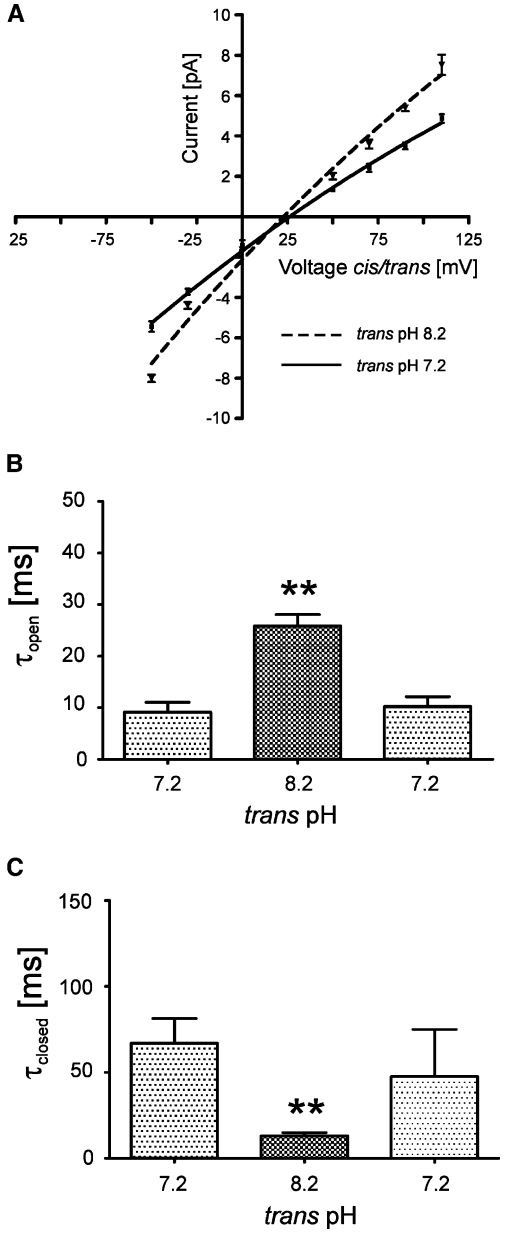

Fig. 3 Effects of alkaline pH on the current amplitude, and on the open and closed dwell times of the mitoK$_{ATP}$ channel. **a** Current–voltage characteristics of single-channel events in a 50/150 mM KCl (*cis/trans*) gradient solution under control conditions and increasing the pH from 7.2 to 8.2 in the *trans* compartment. The *solid line* represents data taken at pH 7.2, and the *dashed line* represents data at pH 8.2 in the *trans* compartment. **b** Open (*left panel*) and closed (*right panel*) dwell time distributions of the mitoK$_{ATP}$ channel in a 50/150 mM KCl (*cis/trans*) gradient solution at −30 mV under control condition, after increasing the pH from 7.2 to 8.2, and after perfusion in the *trans* compartment. All results are presented as mean±SD. $^{**}P<0.05$ vs. control

ions to the *cis* compartment (n=4; Fig. 5b). In this case, we investigated the influence of non-hydrolysable ATP analogs, such as AMP-PNP. Channel activity did not appear inhibited after adding 1 mM Mg^{2+} together with 500 μM AMP-PNP to the *cis* (n=10) and *trans* (n=4) compartments. Figure 5c shows single-channel recordings in 50/150 mM KCl (*cis/trans*) gradient solutions at −30 mV under control conditions and after the addition of 1 mM Mg^{2+} together with 500 μM AMP-PNP to the *cis* compartment. All experiments were done within 10 minutes under the same control conditions.

Interestingly, the mitoK$_{ATP}$ channel reconstituted into a planar lipid bilayer retained the properties of "run-down" activity and ATP/Mg^{2+} dependent recovery. In a solution without ATP/Mg^{2+}, the channel activity spontaneously decreased (spontaneous "run-down"). The mitoK$_{ATP}$ channel activity could be recovered by adding 1 mM Mg^{2+} together with 500 μM ATP to both *cis* and *trans* compartments for ~1 min., followed by perfusion with ATP-free solution, first in the *trans* compartment, and then in the *cis* compartment (Fig. 5d; n=5). After this procedure, the channel activity was stable. Surprisingly, changing the sequence of perfusion (first the *cis*, then the *trans* compartment) could not recover the activity of the mitoK$_{ATP}$ channel (data not shown; n=3).

Discussion

The primary function of cardiac mitochondria is to synthesize ATP. This process is based on transferring protons from the mitochondrial matrix to the cytosol, which establishes a potential gradient (negative with respect to cytosol) across the inner mitochondrial membrane. This mitochondrial potential gradient, along with the pH gradient, provides the driving force for proton transport through F_1F_0-ATP synthases to generate ATP in the mitochondrial matrix. This is followed by transport of ATP from the matrix to the cytosol. This mechanism is affected by cardiac ischemia, which is accompanied by an intracellular acidification that changes the ATP level in the cytosol. This simplified description of mitochondrial function underlines the importance of pH and ATP as potential regulators of the mitochondrial inner membrane integrity. Hence, in the present paper, we studied (with the use of the planar lipid bilayer technique) the regulation of a mitoK$_{ATP}$ channel by pH and ATP (Fig. 6).

Early papers on this topic demonstrated channel inhibition of mitoK$_{ATP}$ channels by ATP, identifying the phenomenon in the liver and heart mitochondria (Paucek et al. 1992; Inoue et al. 1991). In this paper, we show the existence of additional modes of regulation of the mitoK$_{ATP}$ channel by ATP.

Pharmacology of the mitoK$_{ATP}$ channels applied on tissue/cellular/mitochondrial systems is difficult to establish due to different side effects of channel inhibitors or potassium channel openers (Szewczyk et al. 2006). Despite this complication, the single-channel activity, with the use of patch clamp or planar lipid bilayer techniques, can be

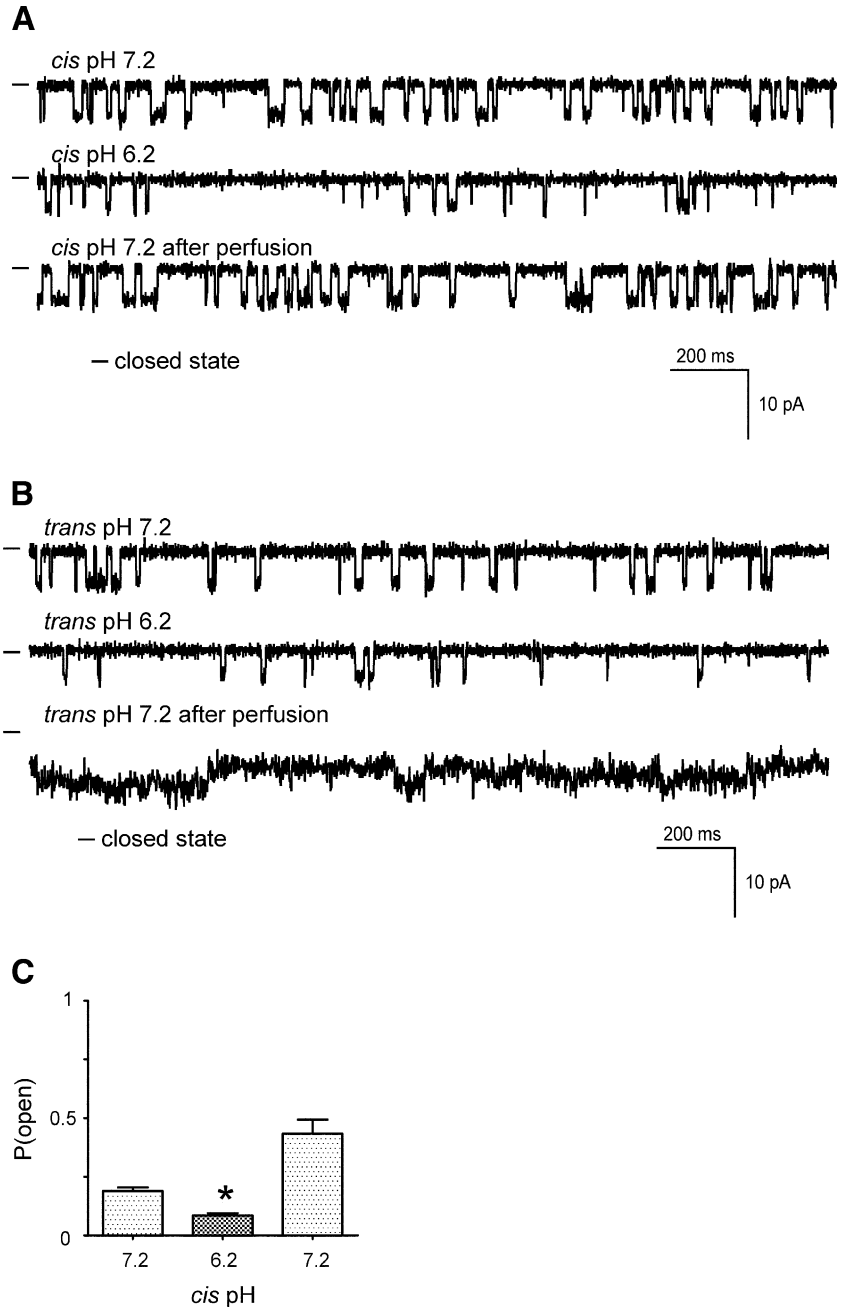

Fig. 4 Effects of acidification on the single mitoK$_{ATP}$ channel activity. **a** Single-channel recordings in a 50/150 mM KCl (*cis*/*trans*) gradient solution at −30 mV under control conditions, after decreasing the pH from 7.2 to 6.2, and after perfusion. The pH was changed only in the *cis* compartment. **b** Single-channel recordings in a 50/150 mM KCl (*cis*/*trans*) gradient solution at −30 mV under control conditions, after decreasing the pH from 7.2 to 6.2, and after perfusion. The pH was changed only in the *trans* compartment. *Solid line* indicates the closed state of the channel. Recordings were low-pass filtered at 500 Hz. **c** The open probability [*P*(open)] of the mitoK$_{ATP}$ channel in a 50/150 mM KCl (*cis*/*trans*) gradient solution at −30 mV under control conditions, after decreasing the pH from 7.2 to 6.2, and after perfusion in the *cis* compartment. The results are presented as mean± SD. **P*<0.05 vs. control

described as follows, supporting the claim that mitoK$_{ATP}$ channel activity is measured:

- The channel has to be blocked by ATP/Mg^{2+} (Paucek et al. 1992; Inoue et al. 1991; Zhang et al. 2001; Jaburek et al. 1998).
- The ATP/Mg^{2+}-inhibited channel should be reactivated by potassium channel openers, such as diazoxide, BMS 191095, or GDP (Garlid et al. 1996; Jaburek et al. 1998).
- The channel should be blocked by 5-HD and glibenclamide (Paucek et al. 1992; Inoue et al. 1991; Zhang et al. 2001; Nakae et al. 2003; Jaburek et al. 1998).

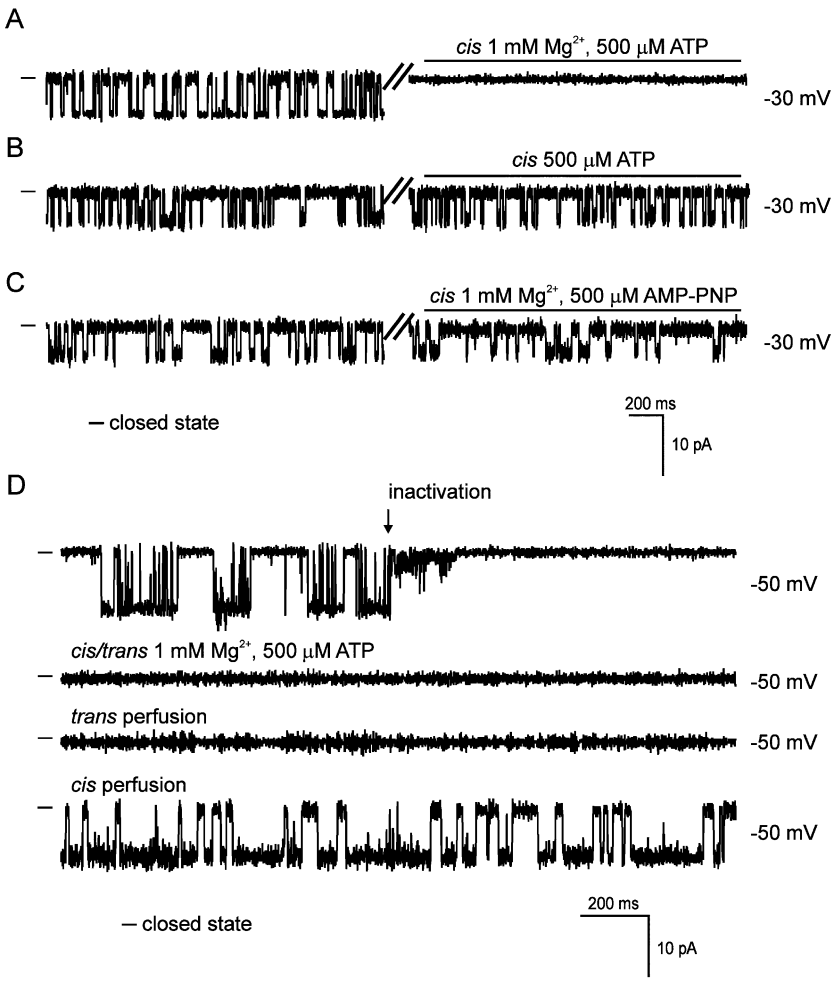

Fig. 5 Effects of ATP/Mg^{2+}, ATP, and AMP-PNP/Mg^{2+} on the activity of the mitoK$_{ATP}$ channel. **a** Single-channel recordings in a 50/150 mM KCl (*cis*/*trans*) gradient solution at −30 mV under control conditions after adding 1 mM Mg^{2+} together with 500 μM ATP to the *cis* compartment. **b** Single-channel recordings in a 50/150 mM KCl (*cis*/*trans*) gradient solution at −30 mV under control conditions after adding 500 μM sole ATP to the *cis* compartment. **c** Single-channel recordings in a 50/150 mM KCl (*cis*/*trans*) gradient solution at −30 mV under control conditions after adding 1 mM Mg^{2+} together with 500 μM AMP-PNP to the *cis* compartment. **d** Single-channel recordings in a 50/150 mmol/L KCl (*cis*/*trans*) gradient solution at −50 mV under control condition, after adding 1 mM Mg^{2+} together with 500 μM ATP to the *cis*/*trans* compartment, and after perfusing first the *trans* compartment followed by the *cis* compartment. *Solid line* indicates the closed state of the channel. Recordings were low-pass filtered at 500 Hz

- The channel activity should not be affected by the plasma membrane ATP-regulated potassium inhibitor HMR1098 (Zhang et al. 2001; Sato et al. 2000).

We have previously shown that reconstituting the inner mitochondrial membrane from bovine heart allows measurements of the mitoK$_{ATP}$ channel activity with the above properties (Bednarczyk et al. 2004, 2005). For the first time, we show in this work the effect of the potassium channel opener BMS 191095 on a single mitoK$_{ATP}$ channel. Previously, the properties of BMS 191095 as a mitochondrial channel opener were based on experiments using isolated mitochondria or perfused heart (Grover et al. 2001; Busija et al. 2005).

The planar lipid bilayer (PLB) technique allows easy access to both sides of the reconstituted channel. Previous studies (Bednarczyk et al. 2004, 2005) and experiments on isolated mitochondria showing mitoK$_{ATP}$ channel inhibition by ATP from the cytosolic side (Yarov-Yarovoy et al. 1997) suggest that the *cis* compartment of the PLB chamber represents the cytosolic side of an inner mitochondrial membrane. Meanwhile, the *trans* compartment represents the mitochondrial matrix (Fig. 1).

Under physiological conditions, mitochondria generate and maintain an alkaline matrix pH, which is a result of proton transport by the electron transport (Abad et al. 2004). Our observation suggests that an alkaline matrix pH affects channel gating and conductance, resulting in an

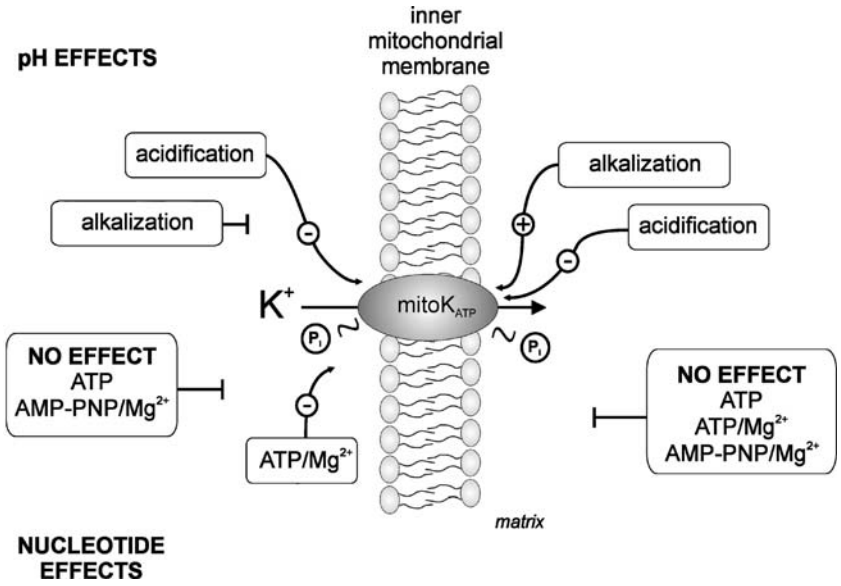

Fig. 6 Interaction of the nucleotides and effects of pH on the activity of the mitoK$_{ATP}$ channel. The scheme summarizes the results demonstrated in this paper. ⊕ indicates an activation, and ⊖ represents an inhibition of the mitoK$_{ATP}$ channel after the addition of various substances or pH changes. Phosphorylation of the channel is marked with a Ⓟ. An ⊣ indicates that no changes of the channel activity occurred upon treatment

increased macroscopic K^+ flux via mitoK$_{ATP}$ channels. These results may support the idea that the electrophoretic potassium influx into functional mitochondria enables the formation of ΔpH by partly compensating for the charge transfer due to the proton pumping. Similar observations were described with the use of potassium channel openers and rat liver mitochondria (Czyz et al. 1995). To summarize, an increased flux of potassium via mitoK$_{ATP}$ channels into the mitochondrial matrix gives rise to a sufficient ΔpH. Logically, this kind of "alkaline" stimulation of the mitoK$_{ATP}$ channels is observed from the matrix side *but not* from the cytosolic side.

Ischemia results in cardiomyocyte acidification, followed by a recovery of pH during reperfusion (Jahangir et al. 1994). In our studies, we observed that acidification inhibited the mitoK$_{ATP}$ channel activity. This effect was not caused by channel run-down because the channel activity recovered upon treatment with a neutral pH medium (i.e., without the presence of the ATP/Mg^{2+} complex).

Despite the fact that the molecular identity of the mitoK$_{ATP}$ channel is not clear, there were some indications suggesting functional similarity between mitochondrial channels and plasma membrane ATP-regulated potassium channels (Suzuki et al. 1997; Zhou et al. 1999). Hence, it is important to mention that the K$_{ATP}$ channels are regulated by protons (Wu et al. 2002; Wang et al. 2003; Manning Fox et al. 2006).

Initial observations of mitoK$_{ATP}$ channels suggested that this protein is blocked by the ATP/Mg^{2+} complex but not by ATP alone (Paucek et al. 1992; Inoue et al. 1991). Surprisingly, we were not able to observe a dose-dependent single-channel inhibition by the ATP/Mg^{2+} complex. Moreover, when ATP was replaced with a non-hydrolysable ATP analogue, 5′-adenylylimidodiphosphate (AMP-PNP), no inhibition in the presence of Mg^{2+} was observed. This suggests that a phosphorylation event is needed to inhibit mitoK$_{ATP}$ channels. Our results investigating ATP inhibition are not consistent with previous studies using proteoliposomes (Paucek et al. 1992) or with single-channel studies (Zhang et al. 2001; Jiang et al. 2006). These discrepancies cannot be readily explained, but they may be due to a difference in properties between mitoK$_{ATP}$ channels present in bovine and rat cardiac mitochondria. These observations raise the general question as to what extent changes of the channel activity is caused by channel phosphorylation. Recently, it was shown that the potassium channel opener diazoxide changed the abundance of phosphoprotein in rat ventricular myocytes (Li et al. 2006).

Exposure of cardiac myocytes to a protein kinase C activator (phorbol 12-myristate 13-acetate) potentiated and accelerated the effect of the diazoxide (Sato et al. 1998). The role of the ε isoform of protein kinase C in the mechanism of preconditioning in isolated heart has been reported (Ohnuma et al. 2002). Recently, it was shown that the mitoK$_{ATP}$ channel forms functional association with protein kinase C (ε isoform; Jaburek et al. 2006). It was also postulated that potassium channel openers diazoxide may activate a mitoK$_{ATP}$ channel via protein kinase C activation, rather than by directly interacting with channel proteins (Kim et al. 2006). Functional coupling of protein

kinase G and mitoK$_{ATP}$ has also been investigated (Costa et al. 2005).

The mitoK$_{ATP}$ channels undergo a "run-down" similar to plasma membrane K$_{ATP}$ channels (Xie et al. 1999). We have shown here that the channels could be reactivated by a brief incubation of the planar lipid bilayer in the presence of ATP/Mg^{2+} complex. Interestingly, in order for the channel to reactivate, ATP/Mg^{2+} needed to be present on both sides of the membrane. Incubation from only the matrix or cytosolic side was not sufficient to reactivate the mitoK$_{ATP}$ channel. These phenomena are subject to further investigations to clarify whether this process is due to channel protein phosphorylation or membrane lipid phosphorylation, as described for mitoK$_{ATP}$ channels and their regulation by phosphatidylinositol (Xie et al. 1999).

In summary, the present results suggest a signaling pathway by which pH regulates the cardiac mitoK$_{ATP}$ channel. Our study also confirms that phosphorylation events play an important role for the mitoK$_{ATP}$ channel activity. Further studies should investigate whether pharmacological cardioprotective drugs (potassium channel openers) cause an acidosis-induced blockage of the mitoK$_{ATP}$ channel, and they should investigate whether the mitoK$_{ATP}$ channel undergoes a "run-down" under severe intracellular acidosis. Additionally, more studies are needed to identify the molecular identity of protein kinases involved in the mitoK$_{ATP}$ channel activity. This should help answer the question of how ATP and protons interact to regulate channel activity, as well as clarify whether there is cross-talk between these signaling pathways.

The results from our study suggest that the cardiac mitoK$_{ATP}$ channel is regulated by pH and phosphorylation events.

Acknowledgements This study was supported in part by the Polish State Committee for Scientific Research grant No. 6P04A01019, by the Ministry of Science and Higher Education grant No. 301-053-31/1676, and by the Polish Mitochondrial Network MitoNet.pl.

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