

# The human mitochondrial $K_{ATP}$ channel is modulated by calcium and nitric oxide: a patch-clamp approach

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Received 29 October 2003; received in revised form 12 January 2004; accepted 12 January 2004

Available online 26 January 2004

## Abstract

ATP-sensitive potassium channels of the inner mitochondrial membrane ( $mtK_{ATP}$ ) are blocked by ATP. They are suggested to be involved in protective mechanisms such as ischemic preconditioning (IPC). Here we identify this channel type for the first time in a human cell line (Jurkat cells). Vesicles of the inner mitochondrial membrane (mitoplasts) were prepared by hypoosmotic shock. Single-channel currents were measured by means of the patch-clamp technique. We identified an outward-rectifying channel with a slope conductance of 15 and 82 pS at negative and positive potentials, respectively. The block by 5-hydroxydecanoic acid and inhibition by ATP characterize this channel as the  $mtK_{ATP}$  channel. ATP also increased the frequency of events within the burst. This effect was modulated by the  $Ca^{2+}$ -bath concentration. We also show that the human  $mtK_{ATP}$  channel is a direct target for nitric oxide that blocked the channel activity. Although the molecular structure of this channel type is still unknown, its characterization as an outward-rectifying channel and modulation by calcium ions and nitric oxide may help to elucidate its functional significance, which possibly implicates a role in cell survival after IPC.

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**Keywords:** Apoptosis; Oxidative stress; Ischemic preconditioning; Protection; Diazoxide; 5-Hydroxydecanoate

## 1. Introduction

$K_{ATP}$  channels are well known from the plasma membranes of various tissue types across different animal species, (for review see Ref. [1]) including heart [2], skeletal muscle [3], brain [4], kidney [5,6], and pancreatic  $\beta$ -cells [7]. Twelve years ago, Inoue et al. [8] measured single-channel currents of an ATP-sensitive K-channel also in the inner membrane of rat liver cell mitochondria. This mitochondrial counterpart of the plasma  $K_{ATP}$  channel, the  $mtK_{ATP}$  channel, allows the transport of potassium into the mitochondrial matrix under the control of intracellular ATP (for review, see Ref. [9]). These channels link the cellular energetic state to the membrane potential.  $mtK_{ATP}$  channels are blocked by

ATP, ADP, 5-hydroxydecanoate (5-HD) [10,11], palmitoyl- or oleyl-CoA [12], and glibenclamide [13]. Activation can be achieved by GTP or GDP application. In addition, the synthetic agents diazoxide and cromakalim were identified to be  $mtK_{ATP}$  channel activators whereby the  $mtK_{ATP}$  channels are even 2000 times more sensitive to diazoxide than plasma membrane  $K_{ATP}$  channels [14].

There is evidence that the  $mtK_{ATP}$  channels play an important role in the response to hypoxic cell injury, since they are involved in ischemic preconditioning (IPC) as first described by Murry et al. [15]. IPC is a phenomenon that leads to increased cellular survival when a noxious stimulus is preceded by a brief period of ischemia. It was shown that  $mtK_{ATP}$  channel opening by diazoxide can mimic IPC protection by inducing cardioprotection. The additional presence of the  $mtK_{ATP}$  blocker 5-HD abolishes this effect [16] (for review, see [Ref. 17]). Korge et al. [18] have shown that under conditions of altered ion homeostasis (that occurs during ischemia), opening of  $mtK_{ATP}$  channels leads to a reduction of mitochondrial  $Ca^{2+}$ -over-

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loading. This, in turn, prevents activation of the permeability transition pore (PTP) and the loss of cytochrome *c* from the mitochondrial intermembrane space. In agreement with these findings, the mitochondrial apoptotic pathway in cardiac cells is inhibited by the  $\text{mtK}_{\text{ATP}}$  channel activator diazoxide whereas this effect is abolished by the blockers 5-HD and glibenclamide [19]. Recent investigations have shown that the  $\text{mtK}_{\text{ATP}}$  channel might play a dual role in cellular protection. In contrast, Liu et al. [20] reported a protective effect against focal ischemic brain injury by  $\text{mtK}_{\text{ATP}}$  channel inhibition using 5-HD administration prior to the onset of ischemia. Although it was shown that an opening of the  $\text{mtK}_{\text{ATP}}$  channel reduces mitochondrial membrane potential only by 2–4 mV [21], indicating that this channel is probably rare, it appears to have a considerable influence on cell survival.

Mitochondria are a prime target for nitric oxide (NO) which is, among others, a blocker of cytochrome oxidase (for review, see Ref. [22]). NO-mediated inhibition of mitochondrial respiration has become of particular interest since the mitochondrion-associated NO synthesis may produce NO locally—either by one of the conventional NOS attached to mitochondria or by a specific  $\text{mtNOS}$  and thus regulate the rate of cellular respiration [23,24]. NO was also reported to dissipate the mitochondrial membrane potential [25] and to induce  $\text{Ca}^{2+}$  release by PTP activation [26]. The data concerning the influence of NO on the  $\text{mtK}_{\text{ATP}}$  channel are rather controversial. Both the antiapoptotic effect due to activation of the  $\text{mtK}_{\text{ATP}}$  channel by NO [27] and the abolishment of the diazoxide-induced protection by a nitric oxide synthase inhibitor [16] were observed. Since the protective effect during IPC, which is also characterized by an increased NO-production, is attributed in part to an opening of the  $\text{mtK}_{\text{ATP}}$  channel, it is of great interest to explore its modulation by NO.

Moreover, despite the data that characterize functional and pharmacological traits of the  $\text{mtK}_{\text{ATP}}$  channel, there is still a lack of information on its electrophysiological characteristics at the single-channel level. Inoue et al. [8] recorded a reduction of the  $\text{mtK}_{\text{ATP}}$  channel open probability in the presence of ATP, glibenclamide and 4-aminopyridine. Hence, our aim was to prove that in human T-lymphocytes (Jurkat cell line) the here investigated  $\text{mtK}^+$  channel displays main characteristics of the  $\text{mtK}_{\text{ATP}}$  channel, e.g. block by ATP and inhibition by the selective blocker 5-HD. We also studied if the  $\text{mtK}_{\text{ATP}}$  channel is modulated by  $\text{Ca}^{2+}$  and NO, since these regulatory molecules play crucial roles in cellular survival after IPC.

## 2. Methods

### 2.1. Cell cultures

The human T-lymphocytes (Jurkat cell line) were cultured in RPMI or Dulbecco's modified Eagles medium

(DMEM) supplemented by 10% fetal calf serum (PAA Laboratories, Cölbe, Germany), 100 IU/ml penicillin, 0.1 mg/ml streptomycin, 2 mM glutamine and 1% nonessential amino acids (PAA Laboratories). No differences between the cells cultured in either medium were observed. Cells were grown in 250-ml tissue culture flasks and kept in an incubator at 37 °C, 6.5%  $\text{CO}_2$  and 100% humidity. The cells were fed and reseeded every third day.

### 2.2. Mitoplast preparation

All procedures were performed at 4 °C. The cells were harvested by centrifugation at  $290 \times g$  for 10 min, resuspended in the solution for the mitochondria isolation (250 mM sucrose, 5 mM HEPES and 0.1% BSA, pH=7.2) and homogenized using a tight 2-ml glass potter. One fast ( $6700 \times g$ , 10 min) and one slow centrifugation ( $500 \times g$ , 10 min) step were performed to separate the fraction of purified mitochondria. BSA and sucrose were removed by two further fast centrifugation steps ( $6700 \times g$ , 10 min) in a solution containing 150 mM KCl and 10 mM HEPES (pH=7.2). Mitoplasts were obtained by hypoosmotic shock: the outer membrane was ruptured during a 1-min incubation of the mitochondria in a solution containing 5 mM HEPES, and, depending on the experimental conditions, either 100 or 200  $\mu\text{M}$   $\text{CaCl}_2$  (pH=7.2) [28]. Isotonicity was restored by the addition of a hypertonic medium containing 750 mM KCl, 30 mM HEPES, and 100 or 200  $\mu\text{M}$  of  $\text{CaCl}_2$  (pH=7.2). Mitoplasts were kept on ice for a maximum of 36 h.

### 2.3. Chemicals

KCl and  $\text{CaCl}_2$  were purchased from Merck (Darmstadt, Germany); HEPES,  $\text{K}_2\text{ATP}$ , 5-hydroxydecanoic acid (5-HD), diazoxide, methyl pyruvate, bovine serum albumin (BSA) and sucrose were from Sigma (Deisenhofen, Germany). The concentrations of free  $\text{Ca}^{2+}$  were calculated by means of the software 'bound and determined' [29]. NO gas was purchased from Air Liquid (Paris, France).

### 2.4. NO solution

For application of authentic NO, the control patch bath solution was first deoxygenated by 20-min ventilation with nitrogen gas in a sealed flask. NO stock solution was prepared by saturating the content of one of the flasks with pure NO gas. The concentration of the stock solution was 1–3 mM as measured by a chemiluminescence NO detector (Nitric Oxide Analyzer, Sievers Instruments). Dilutions to 1  $\mu\text{M}$  were made by transferring aliquots of the NO-stocks in gas-tight 200- $\mu\text{l}$  glass syringes to other sealed flasks containing deoxygenated buffer and then transferred into gas-tight 2-ml Hamilton glass syringes that were mounted onto a syringe pump whereby

the flow was set to 50  $\mu\text{l}/\text{min}$ . The patches were exposed to pulses of NO-containing medium by moving the patch into a wide glass pipette that was connected to the filled Hamilton syringes.

### 2.5. Patch-clamp recording

For patch-clamp experiments borosilicate glass pipettes (Clark, Pangbourne, UK) were fabricated in two steps to yield a resistance of 8–15 M $\Omega$ . Bath and pipette solution were symmetric and contained 150 mM KCl, 10 mM HEPES and 100 (or 200)  $\mu\text{M}$   $\text{CaCl}_2$  (pH=7.2). Free-floating mitoplasts were caught by the pipette that was moved by an electrically driven micromanipulator. Occasionally, also mitoplasts attached to the bottom of the Petri dish were used. Gigaseals (0.6–10 G $\Omega$ ) formed spontaneously or by applying gentle suction. Experiments were performed at a temperature of 25–27  $^{\circ}\text{C}$  in the mitoplast-attached mode (matrix side out). Solutions with test substances were applied by moving the pipette tip into the flow of a peristaltic-pump driven “sewer-pipe”. Ion selectivity was tested in a solution of 130 mM NaCl, 20 mM KCl, and 10 mM K-HEPES (pH=7.2). Currents through the ion channels were amplified by an EPC 7-amplifier (HEKA, Lambrecht, Germany), filtered at a corner frequency of 1

kHz (4-pole Bessel), and sampled at 4 kHz. The current was recorded in segments of 1-min duration.

### 2.6. Data analysis

The software pCLAMP8.2 (Axon, Foster City, CA, USA) was used for data analysis. Gaussian distribution functions were fit to the amplitude histograms in order to determine the ion channel amplitude and the open probability. The current amplitude of the single channel was calculated as the difference between the peak values of the corresponding histogram (Fig. 1). To characterize the mitochondrial channel kinetics, the frequency of the channel events was analyzed. An event was considered if the signal crossed a defined threshold two times. The threshold was set to half value of the channel amplitude. The frequency of events was calculated as the number of events per burst. The interval between two bursts was defined as any closed state that lasted longer than 20 ms.

Data are presented as mean  $\pm$  S.E. The significance of differences of the mean values between multiple groups was examined using ANOVA. The Student's *t* test was used to evaluate the statistical significance of differences between the two groups.  $P < 0.05$  was considered statistically significant.

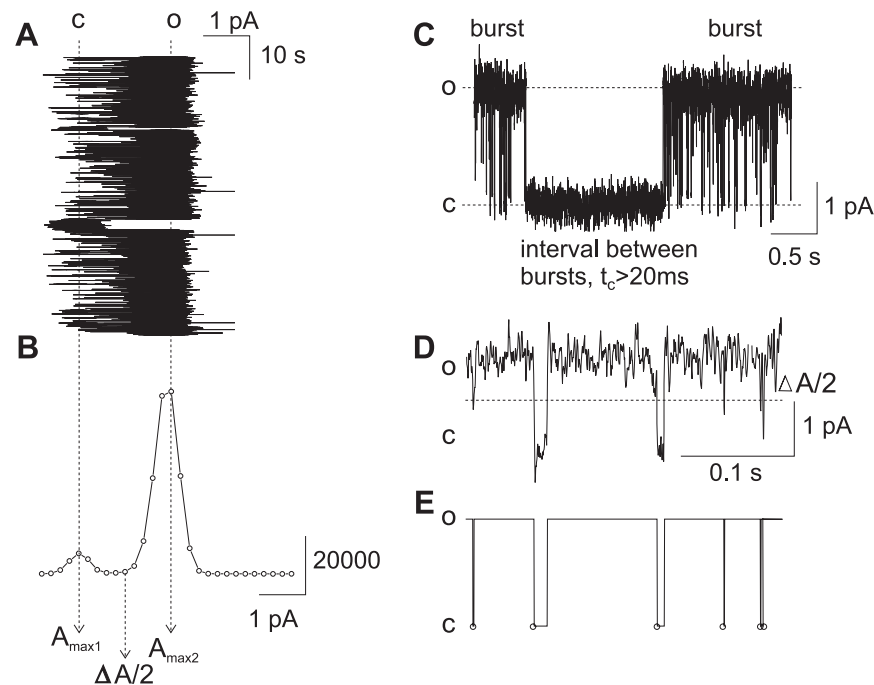


Fig. 1. Analysis of the channel burst frequency. (A) Original record from a mitoplast patch containing the active channel (60 s duration). The dotted lines indicate closed (c) and open (o) state. (B) Amplitude histogram calculated from the data shown in A. The two maxima correspond to the average level of the closed ( $A_{\text{max}1}$ ) and opened ( $A_{\text{max}2}$ ) state. Their difference ( $\Delta A$ ) is the channel amplitude. The amplitude threshold is defined as the half value of the channel amplitude plus average level of the closed state ( $(\Delta A/2) + A_{\text{max}1}$ ). (C) Definition of the interval between two bursts. Any closed state that lasted longer than 20 ms was considered as an interval between bursts.  $t_c$  is the duration of the closed state. (D) Time resolution of the signal. An event was considered as such when the channel closed and opened, i.e. the signal crossed the threshold ( $\Delta A/2$ ) twice. (E) Original data (C) were filtered by equalizing the values exceeding the threshold to the average open level ( $A_{\text{max}1}$ ) and, correspondingly, all the values below the threshold were set to the averaged closed level ( $A_{\text{max}2}$ ). The bottom trace shows filtered data which allow to characterize the burst activity.

### 3. Results

The characterized ATP-sensitive channel was present in 4.6% of the patches of the inner mitochondrial membrane of human T-lymphocytes.

#### 3.1. *i/E* curve characteristics

In symmetrical solutions with 150 mM  $K^+$  we detected a weakly outward rectifying channel with a single-channel conductance of 15 pS at negative (determined as the regression between  $-80$  and  $-20$  mV) and 82 pS at positive (determined as the regression between  $20$  and  $80$  mV) holding potentials (Fig. 2A and B). To test for the

ion selectivity of the channel, we changed the bath solution (matrix side of the recorded channel) to a solution of which 84% of the  $K^+$  ions were replaced by  $Na^+$  ions. Fig. 2C shows the resulting outward part of an *i/E* curve with a single-channel conductance reversibly reduced by 43%. A set of four experiments showed a mean reduction of *i* from  $1.86 \pm 0.05$  to  $1.16 \pm 0.20$  pA at  $40$  mV (Fig. 2D). This value returned to  $1.77 \pm 0.10$  pA upon wash in KCl.

#### 3.2. ATP reduces the open probability ( $P_o$ ) of the channel

Under control conditions ( $E=40$  mV) in an ATP-free solution the channel stayed more open than closed

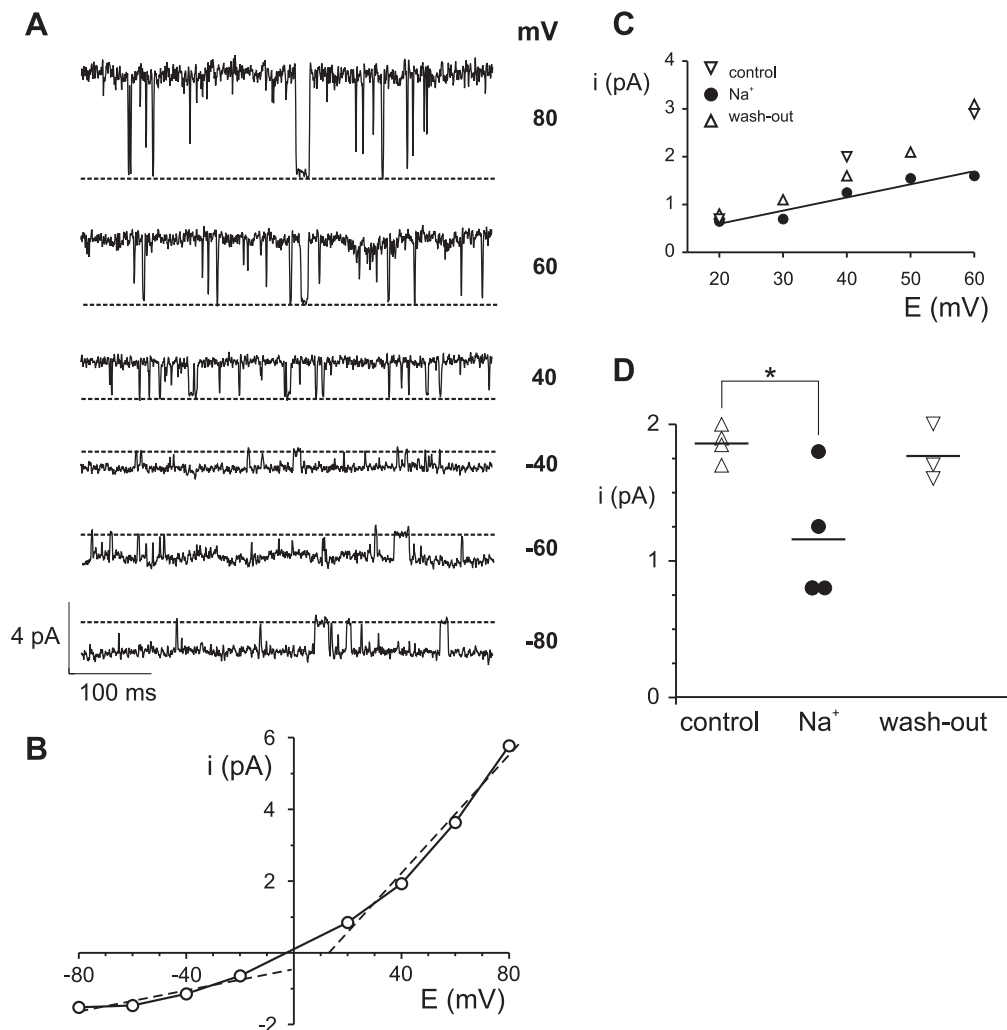


Fig. 2. (A) Representative currents of the channel at various membrane potentials recordings were taken after ATP application ( $0.5$  mM) during the wash-out part ( $200 \mu M Ca^{2+}$ ) of the experiment. Dotted lines indicate the current levels of the closed states. (B) Slope conductances were calculated by means of a straight line fit. The slope conductances were found to be  $82$  pS at positive and  $14$  pS at negative potential. The patch mode is mitoplast-attached or matrix side-out. The pipette and bath solutions contained  $150$  mM  $K^+$ . The data reveal an outward-rectification of the mtK<sub>ATP</sub> channel. Same experiment as in A. (C) Conductance of the mtK<sub>ATP</sub> channel from a representative experiment in a  $Na^+$ -rich solution ( $130$  mM NaCl and  $20$  mM KCl) at various potentials. The shown straight line reflects the linear regression that was calculated for five potentials. (D) Conductance of the mtK<sub>ATP</sub> channel in a  $Na^+$ -rich solution at  $40$  mV. Open symbols indicate measurements before (triangle, tip upward) and after (triangle, tip downward)  $Na^+$  application, filled circles indicate recordings during  $Na^+$  application.

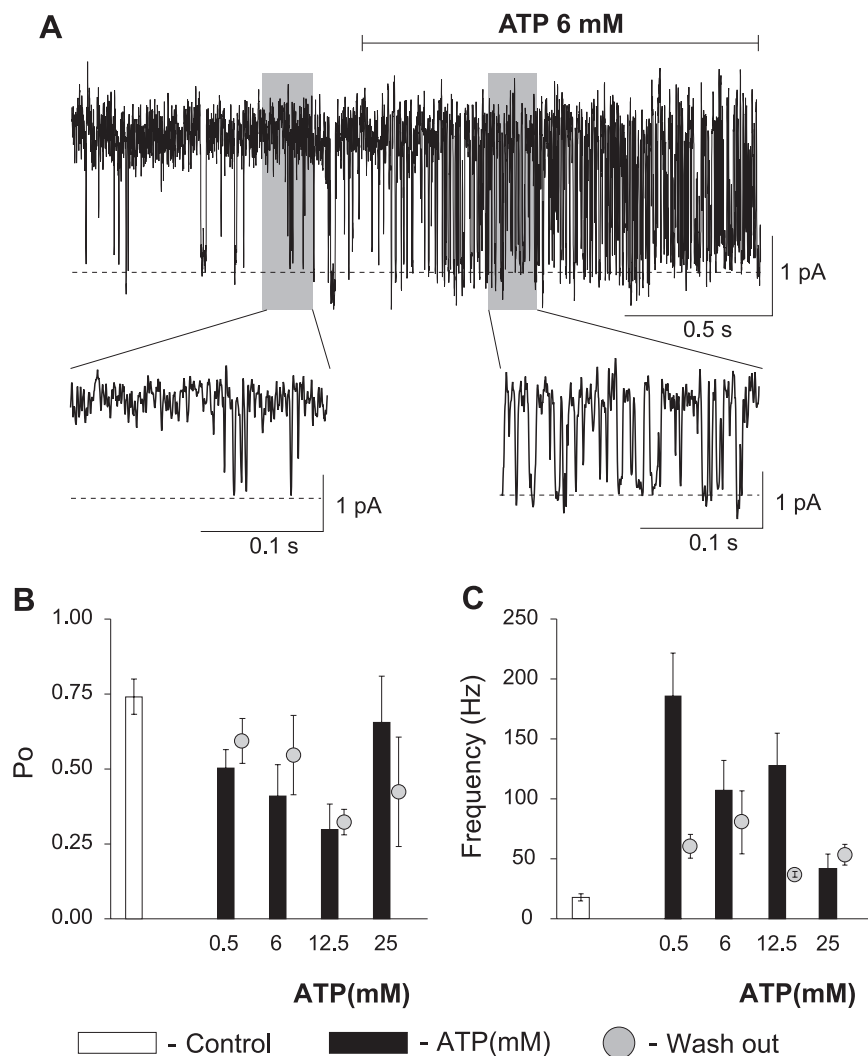


Fig. 3. (A) Representative trace of  $\text{mtK}_{\text{ATP}}$  channel activity in the absence and presence of ATP. Current trace ( $E=40$  mV) shows switching from ATP-free to ATP-containing bath solution and extended in the time parts of the signal. ATP-application results in an increase of the frequency of events. The dotted line indicates closed state of the channel. (B) Open probability ( $P_o$ ) is reduced when the  $\text{mtK}_{\text{ATP}}$  channel is exposed to ATP (ANOVA,  $P<0.05$ ,  $n=12$ ). Under control conditions (empty bar) the  $\text{mtK}_{\text{ATP}}$  channel is mostly open. An increase in ATP bath concentration (between 0.5 and 12.5 mM) leads to a reduction of  $P_o$ . A slight tendency to reversibility was observed after returning from 0.5 and 6 mM ATP, respectively. 12.5 mM ATP causes an irreversible reduction of  $P_o$ . 25 mM ATP does not have a significant effect on  $P_o$ , but during the wash-out phase a significant reduction of  $P_o$  was observed ( $t$  test  $P<0.05$ ,  $n=3$ ). (C) The frequency of events of the  $\text{mtK}_{\text{ATP}}$  channel is increased in the presence of ATP (ANOVA,  $P<0.001$ ,  $n=11$ ). The strongest increase was observed in the presence of the lowest ATP concentration used (0.5 mM). All measurements were done at 40 mV.

( $P_o=0.74 \pm 0.05$ ,  $n=12$ ). Adding ATP reduced  $P_o$  significantly with the exception of the highest concentration of 25 mM where  $P_o$  increased again causing a biphasic dose–response relation (Fig. 3B). The strongest effect was observed after the application of 12.5 mM ATP ( $P_o=0.30 \pm 0.09$ ,  $n=4$ ). The ATP effect on  $P_o$  was not reversible. At the lower concentrations (0.5 and 6 mM ATP), a mild  $P_o$  recovery was observed but it was insignificant. Acute application of 25 mM ATP did not significantly reduce  $P_o$ . However, during the wash-out phase,  $P_o$  continued to decline to  $0.43 \pm 0.18$  ( $n=3$ ,  $P<0.05$ ) (Fig. 3B). Since the channel was partially  $\text{K}^+$  selective and since physiological concentrations of ATP

were able to change its activity, we will refer to it as the “mitochondrial ATP-sensitive potassium ( $\text{mtK}_{\text{ATP}}$ ) channel”.

Table 1

The frequency of events of the  $\text{mtK}_{\text{ATP}}$  channel does not depend on  $\text{Ca}^{2+}$ , when the  $\text{Ca}^{2+}$  concentration was changed during ATP application

ATP (mM)	$\text{Ca}^{2+}$ ( $\mu\text{M}$ )	Frequency (Hz)	Number of experiments
0	200	$17.7 \pm 2.9$	11
25	8	$42.0 \pm 12.0$	3
0.5	123	$185.8 \pm 36.0$	3
0.5	7	$121.5 \pm 33.0$	3



### 3.3. ATP changes the kinetics of the $mtK_{ATP}$ channel

Under control conditions (40 mV), very short closures with a frequency of  $17.7 \pm 2.9$  Hz ( $n=11$ ) were regularly observed. After adding 0.5 mM ATP to the bath solution, the frequency of the events increased considerably to  $185.8 \pm 35.9$  Hz (Fig. 3A and C). The effect of ATP on frequency was determined to be reversely proportional to the ATP concentration in the bath. In the presence of 25 mM ATP the frequency was  $42.0 \pm 12.0$  Hz and did not differ significantly as compared to the control.

### 3.4. Free $Ca^{2+}$ does not influence the $mtK_{ATP}$ channel kinetics during ATP application

The biphasic effect on the frequency of events in the burst indicates the existence of at least one more parameter in our system that influences  $mtK_{ATP}$  kinetics. Apart from ATP, only the concentration of unbound  $Ca^{2+}$  ions was changed as ATP binds  $Ca^{2+}$  ions in the solution. Therefore, we checked whether  $Ca^{2+}$  controls the frequency of events in the presence of ATP in  $Mg^{2+}$ -free solutions.

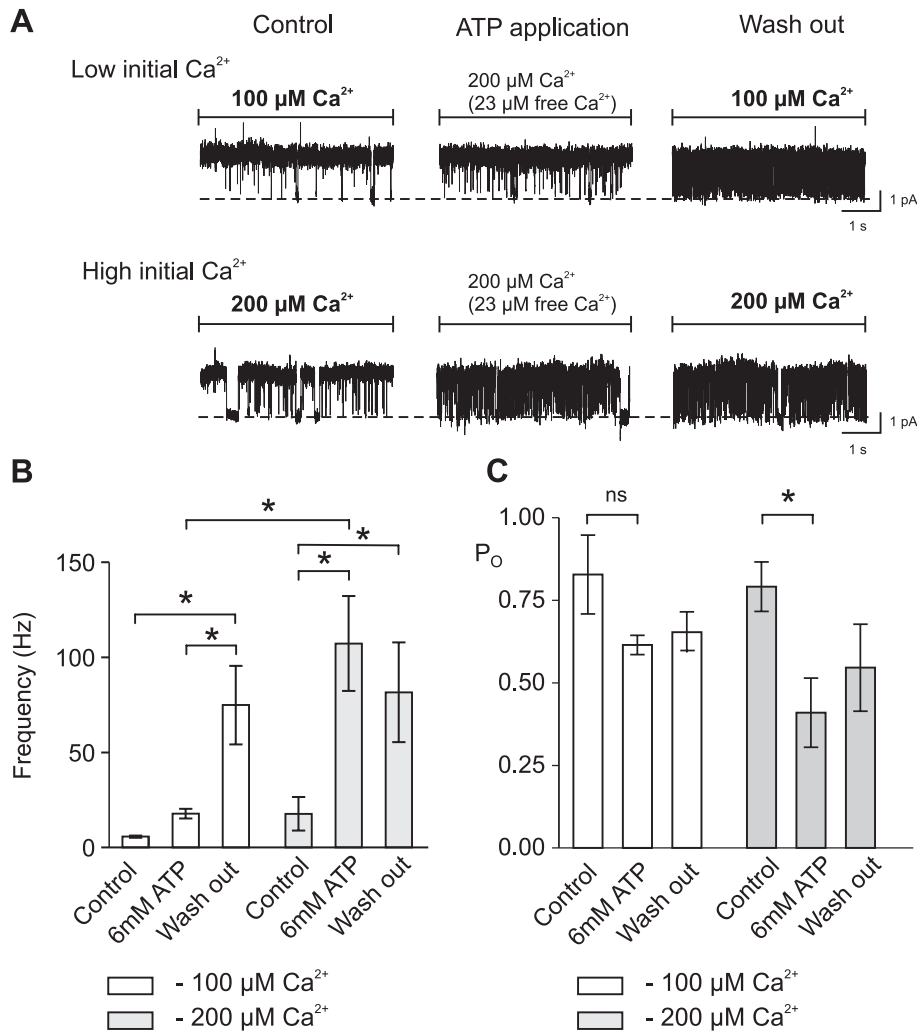


Fig. 4. The  $mtK_{ATP}$  channel kinetics during ATP application depend on the initial  $Ca^{2+}$  concentration. (A) Two representative experiments with three records are shown. Upper trace: the initial free calcium concentration was kept at a low level (100 μM). Application of 6 mM ATP that reduces the free calcium  $Ca^{2+}$  concentration to 23 μM did not change the burst frequency. When ATP was removed during wash-out (and the  $Ca^{2+}$  concentration was therefore restored to 100 μM), an increase of the burst frequency was observed. Lower trace: The initial free  $Ca^{2+}$  concentration was kept at a high level (200 μM). Application of 6 mM ATP that reduced the  $Ca^{2+}$  concentration to 23 μM resulted in an increase in frequency of events. The effect was not reversible during wash-out. (B) Summary of the frequency analysis. The application of 100 μM of  $Ca^{2+}$  at the onset of the experiment did not increase the burst frequency during the subsequent addition of ATP. In contrast, exposure of the channel to the initially high  $Ca^{2+}$  concentration of 200 μM caused a significant sixfold increase in the frequency. However, when ATP was removed, during wash-out, both groups displayed an irreversible increase of the frequency of events. (C)  $P_o$  is presented as a function of initial  $Ca^{2+}$  concentration. There was a similar pattern of  $P_o$  reduction between the groups with low and high initial calcium concentration. No significant change of  $P_o$  after 6 mM ATP application was observed when the initial  $Ca^{2+}$  concentration was 100 μM ( $P_{o,control}=0.83 \pm 0.12$ ,  $P_{o,ATP}=0.61 \pm 0.03$ ,  $n=3$ ). When the initial calcium was 200 μM, the subsequent addition of 6 mM ATP significantly reduced  $P_o$  ( $P_{o,control}=0.80 \pm 0.08$ ,  $P_{o,ATP}=0.41 \pm 0.10$ ,  $n=3$ ,  $P<0.05$ ). All measurements were done at 40 mV.

Under control conditions the concentration of the free calcium ions was 200  $\mu\text{M}$ . When 25 mM ATP was added to the solution, a reduction of the concentration of free calcium ions from 200 to 8  $\mu\text{M}$  occurred (Brooks and Storey, 1992) due to the binding of  $\text{Ca}^{2+}$  to ATP. This condition did not drastically increase the frequency as compared to the controls. When 0.5 mM ATP was added to the bath with 200  $\mu\text{M}$   $\text{Ca}^{2+}$ , the concentration of the free  $\text{Ca}^{2+}$  did not drop much and the frequency was significantly increased. If a high concentration of free  $\text{Ca}^{2+}$  ions is responsible for the increased frequency in ATP containing solution, then a reduced concentration of free calcium ions (to 7  $\mu\text{M}$ ) in the presence of 0.5 mM ATP (similar to a  $\text{Ca}^{2+}$  level of 8  $\mu\text{M}$  that results from the addition of 25 mM ATP) should not lead to an increase of the frequency and it should be similar to that observed with 25 mM ATP. In contrast, the results showed a significant increase of the frequency to a value differing only nonsignificantly from the value at 0.5 mM ATP and at high  $\text{Ca}^{2+}$  concentration (for summarized data, see Table 1). Therefore, the changes of the concentration of free  $\text{Ca}^{2+}$  during ATP application cannot explain the modified kinetics.

### 3.5. The $\text{mtK}_{\text{ATP}}$ channel response to ATP depends on the initial amount of unbound $\text{Ca}^{2+}$ in the system

Since the amount of free  $\text{Ca}^{2+}$  during ATP application was not responsible for the changed frequency of events, we tested if the initial concentration of unbound  $\text{Ca}^{2+}$  in the bath (before ATP application) could modulate the response of the  $\text{mtK}_{\text{ATP}}$  channel to ATP. For this purpose, the initial concentration of  $\text{Ca}^{2+}$  was reduced from 200 to 100  $\mu\text{M}$ . The concentration of  $\text{Ca}^{2+}$  during ATP application was kept as in the standard experiments; i.e. 200  $\mu\text{M}$   $\text{CaCl}_2$  that corresponds to 23  $\mu\text{M}$  of free  $\text{Ca}^{2+}$  in the presence of 6 mM ATP (Fig. 4A).

The frequency of events under control conditions in the presence of low (100  $\mu\text{M}$ ) and high (200  $\mu\text{M}$ ) free  $\text{Ca}^{2+}$  was not significantly different ( $5.8 \pm 0.6$  and  $17.7 \pm 8.8$  Hz, respectively,  $n=3$ ). Application of 6 mM ATP significantly changed the frequency only if the channel was exposed to the high concentration, i.e. 200  $\mu\text{M}$   $\text{Ca}^{2+}$  initially. In the experiments with low (100  $\mu\text{M}$ ) initial concentration of  $\text{Ca}^{2+}$ , an increased frequency of events was not observed during the ATP application but during the wash-out phase (Fig. 4B). The open probability was also dependent on the initial  $\text{Ca}^{2+}$  concentration. In the presence of low initial  $\text{Ca}^{2+}$ ,  $P_o$  was not significantly reduced by 6 mM ATP (Fig. 4C).

### 3.6. Pharmacological characterization of the $\text{mtK}_{\text{ATP}}$ channel

5-HD is a selective blocking agent of the  $\text{mtK}_{\text{ATP}}$  channel [10]. Applying 1 mM 5-HD to the inner side of the human  $\text{mtK}_{\text{ATP}}$  channel induced an irreversible time-dependent block.  $P_o$  was reduced by an average of 51.2% ( $n=3$ , Fig. 5A).

To test if the low rate (4.6%) of detecting a channel event is due to the low channel density or to a general blocked (or inactive) state of the channel, we applied diazoxide, a selective activator of  $\text{mtK}_{\text{ATP}}$ . Application of diazoxide at concentrations of 50, 100, and 500  $\mu\text{M}$  to a patch without any channel activity did not lead to a clear activation of the  $\text{mtK}_{\text{ATP}}$  channel. 50  $\mu\text{M}$  diazoxide could activate an  $\text{mtK}_{\text{ATP}}$  channel in only one out of seven experiments performed ( $P_{o\text{control}}=0$ ;  $P_{o\text{diazoxide}}=0.24 \pm 0.08$ ;  $P_{o\text{wash-out}}=0.02$ ). In six additional experiments, the diazoxide-induced activity had a more noise-like appearance with much shorter events and a large variability of the amplitude (up to 10 pA at 40 mV). 50  $\mu\text{M}$  diazoxide in the bath increased the  $P_o$  of this type of activity from 0 to  $0.63 \pm 0.22$  ( $n=4$ ) after 15 min of perfusion. After adding 100  $\mu\text{M}$  of diazoxide to the bath, this

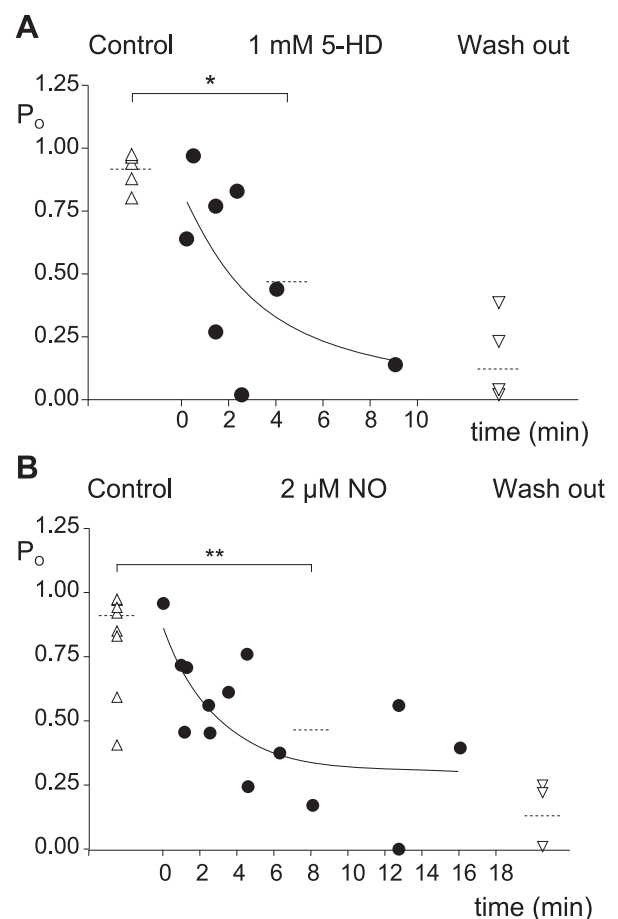


Fig. 5. (A) Effect of 5-HD on  $P_o$  of the  $\text{mtK}_{\text{ATP}}$  channel. Perfusion with 1 mM 5-HD (selective blocker of the  $\text{mtK}_{\text{ATP}}$  channel) led to an irreversible decrease of  $P_o$  ( $t$  test,  $P=0.034$ ). Open symbols indicate measurements before (triangle, tip upward) and after (triangle, tip downward) application of 5-HD; filled circles indicate recordings during 5-HD application. All measurements were done at 40 mV. Dotted lines represent the mean of the experimental groups. (B) Effect of 2  $\mu\text{M}$  nitric oxide on  $P_o$  of the  $\text{mtK}_{\text{ATP}}$  channel. Perfusion with 2  $\mu\text{M}$  nitric oxide irreversibly decreased  $P_o$  ( $t$  test,  $P=0.003$ ). Open symbols indicate measurements before (triangle, tip up) and after (triangle, tip down) application of NO, filled circles indicate recordings during NO application. All measurements were done at 40 mV. Dotted lines represent the mean of the experimental groups.

effect appeared after 2 min; application of 500  $\mu\text{M}$  led to loss of the patch. These effects were irreversible during the wash-out phase.

### 3.7. Effect of NO on the $\text{mtK}_{\text{ATP}}$ channel

We applied NO to six patches containing active  $\text{mtK}_{\text{ATP}}$  channels. Using data from three independent experiments, Fig. 5B demonstrates that after adding 2  $\mu\text{M}$  NO,  $P_o$  time-dependently declined. This irreversible effect was also observed in three additional independent experiments at different depolarizing potentials ( $-30$ ,  $-60$  and  $-80$  mV) and in the presence of 1  $\mu\text{M}$  NO (data not shown).

## 4. Discussion

The  $\text{mtK}_{\text{ATP}}$  channel was characterized in mitoplasts obtained from human T-lymphocyte cultures. We show here that under control conditions this channel stays permanently open with regularly occurring short closures at a frequency of 17.7 Hz. Kinetics look similar to the  $\text{mtK}_{\text{ATP}}$  channel found in rat liver mitochondria [8] and to the reconstituted bovine myocardial  $\text{mtK}_{\text{ATP}}$  channel [30].

### 4.1. Conductance

The  $\text{mtK}_{\text{ATP}}$  channel displayed an outward-rectifying property with a slope conductance of 15 pS at negative and 82 pS at positive voltage. These values are rough estimates as the  $i/E$  curve was bent by rectification. Our measured values differ from the first descriptions of the  $\text{mtK}_{\text{ATP}}$  channel in mitochondria. The mean slope conductance of  $\text{mtK}_{\text{ATP}}$  channels reconstituted from bovine ventricular myocardium was 56 pS at negative potential and showed inward-rectifying characteristics in symmetrical 150 mM  $\text{K}^+$  solutions [30]. Measurements of rat liver  $\text{mtK}_{\text{ATP}}$  channel resulted in a single-channel conductance of 9 pS only [8]. They were obtained, however, at low  $\text{K}^+$  concentration (33.3 mM  $\text{K}^+$  and 66.7 mM  $\text{Na}^+$  bath and 100 mM  $\text{K}^+$  pipette solution) and the channel showed a tendency towards outward rectification. Such differences may indicate species- or tissue-dependent properties of the  $\text{mtK}_{\text{ATP}}$  channel. The finding that the human  $\text{mtK}_{\text{ATP}}$  channel has rectifying properties provides more insight into the possible physiological functions of this channel. A consequence is that at a physiological membrane potential of approximately  $-180$  mV, the channel should express minimal ion conductance.

### 4.2. ATP effects on the $\text{mtK}_{\text{ATP}}$ channel

A very detailed description of the ATP blocking effect on the  $\text{mtK}_{\text{ATP}}$  channel was given in previous reports at whole mitochondria (for review see Ref. [1]). Our results show

additionally that ATP not only blocks the activity ( $P_o$ ), but also modulates the burst frequency of the channel. In the presence of ATP,  $P_o$  is reduced but not completely abolished. The effect of ATP on  $P_o$  has a biphasic character, i.e. the highest ATP amount used did not induce any significant change, but ATP at a lower concentration (12.5 mM) reduced  $P_o$  down to 0.3. The effect of ATP on the burst frequency has a similar biphasic character, i.e. increase in burst frequency from 17.7 up to 187.0 Hz in the presence of 0.5 mM ATP and only a slight change to 42.0 Hz caused by 25 mM ATP.

### 4.3. $\text{Ca}^{2+}$ modulation of the $\text{mtK}_{\text{ATP}}$ channel

The biphasic effect of ATP on both  $P_o$  and burst frequency suggests the existence of an additional factor in our experimental system that modulates the  $\text{mtK}_{\text{ATP}}$  channel. It has been shown before that plasma membrane  $\text{K}_{\text{ATP}}$  channels can be affected by cytosolic free  $\text{Ca}^{2+}$  [31,32]. Also, it is well known that ATP binds  $\text{Ca}^{2+}$  and consequently reduces the free  $\text{Ca}^{2+}$  concentration. Therefore, we investigated if free  $\text{Ca}^{2+}$  may also modulate the  $\text{K}_{\text{ATP}}$  channel in human mitochondria.

We found that in ATP-free solution a change in the bath  $\text{Ca}^{2+}$  concentration alone influenced neither the  $P_o$  nor the burst frequency of the  $\text{mtK}_{\text{ATP}}$  channel. Only both factors together, the presence of ATP and the initial calcium concentration (before ATP application), modulated the behavior of the  $\text{mtK}_{\text{ATP}}$  channel. It suggests that the  $\text{mtK}_{\text{ATP}}$  channel couples the energy state, the mitochondrial membrane potential, and the cellular/mitochondrial changes in free calcium.

A hypothetical mechanism of the  $\text{mtK}_{\text{ATP}}$  channel modulation by  $\text{Ca}^{2+}$  and ATP that fits our observations is presented in Fig. 6. This model is based on the following assumptions: (i) the  $\text{mtK}_{\text{ATP}}$  channel has two sites where  $\text{Ca}^{2+}$  and ATP can bind irreversibly, (ii) increased burst frequency and following  $P_o$  reduction are due to the  $\text{ATP}-\text{Ca}_x$  and  $\text{ATP}_y-\text{Ca}$  complex formation ( $x, y$  are the number of bound ions,  $x, y \geq 2$ ).

Once the  $\text{Ca}^{2+}$  concentration is increased over a certain threshold value,  $\text{Ca}^{2+}$  can be irreversibly bound to the  $\text{mtK}_{\text{ATP}}$  channel. In ATP containing solutions this makes a site available for the  $\text{ATP}-\text{Ca}_x$  complex formation and thereby causes the block of the  $\text{mtK}_{\text{ATP}}$  channel. A similar situation occurs when ATP irreversibly binds to the channel and, with an increased  $\text{Ca}^{2+}$  concentration, the  $\text{ATP}_y-\text{Ca}$  complex can block the  $\text{mtK}_{\text{ATP}}$  channel. Thus, if the channel is exposed to ATP and high  $\text{Ca}^{2+}$  ( $>100 \mu\text{M}$ ), two sites exist at which a block can occur.

However, although the presented model of the  $\text{mtK}_{\text{ATP}}$  channel modulation explains our findings, we cannot exclude the possibility that  $\text{ATP}-\text{Ca}_x/\text{ATP}_y-\text{Ca}$  binds to other allosteric sites of  $\text{mtK}_{\text{ATP}}$  channel and causes conformational changes and inhibits channel activity. Further experiments are needed to verify the proposed model.



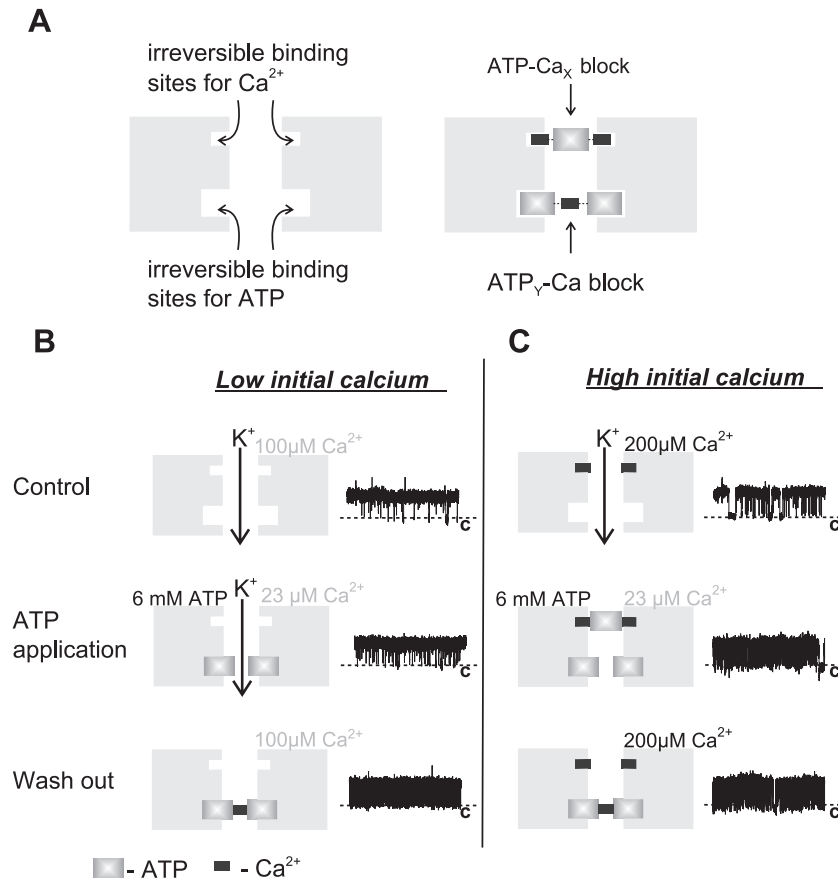


Fig. 6. A hypothetical model of the  $\text{mtK}_{\text{ATP}}$  channel regulation by calcium and ATP is presented. (A) The hypothesis is based on the following assumptions; (i) the  $\text{mtK}_{\text{ATP}}$  channel has two sites where  $\text{Ca}^{2+}$  and ATP can bind irreversibly. (ii) The block of the channel and increased burst frequency are due to the ATP- $\text{Ca}_x$  and the ATP- $\text{Ca}_y$  complex formation. (B) (Low initial calcium) Under control conditions the free calcium concentration is lower than a certain threshold and calcium is not bound to the channel. Therefore, both binding sites are free and the channel is mostly open. Subsequent addition of ATP reduces the free calcium concentration to 23  $\mu\text{M}$  and simultaneously ATP-binding sites of the channel become irreversibly occupied. However, the channel still remains open. During the wash-out phase ATP is absent, hence  $\text{Ca}^{2+}$  is restored to the level of 100  $\mu\text{M}$ . Free  $\text{Ca}^{2+}$  may bind to the ATP molecules that are attached to the binding sites in the channel. Now, the ATP- $\text{Ca}_y$  complex is formed and blocks the channel. (C) (High initial calcium) When the concentration of free  $\text{Ca}^{2+}$  is higher than a certain threshold (e.g. >100  $\mu\text{M}$ ),  $\text{Ca}^{2+}$  will irreversibly bind to the  $\text{mtK}_{\text{ATP}}$  channel. This process does not change the channel behavior. Only when ATP is added, the channel is blocked due to ATP- $\text{Ca}_x$  complex formation. Also, similar to the situation with low initial calcium, ATP will irreversibly bind to the  $\text{mtK}_{\text{ATP}}$  channel. During the wash-out phase the ATP- $\text{Ca}_x$  complex will be removed (due to the absence of ATP). A simultaneous increase of the concentration of free calcium ions leads to the ATP- $\text{Ca}_y$  complex formation and the inhibition of the  $\text{mtK}_{\text{ATP}}$  channel.

#### 4.4. Low $\text{mtK}_{\text{ATP}}$ channel density or irreversible block?

The probability that a patch contains a  $\text{mtK}_{\text{ATP}}$  channel was very low (approximately 5 patches of 100 trials). This rate of success was much lower than that in rat liver cells (approximately one in three patches) observed in Ref. [8]. Possible reasons for the differences in success rate are: (i) differences in the channel density between species and (ii) different experimental conditions that led to an inactivation of the channel. We attempted to test the second possibility that patches may contain silent  $\text{mtK}_{\text{ATP}}$  channels due to an unknown blocking mechanism by applying diazoxide, the selective activator of  $\text{mtK}_{\text{ATP}}$  channel. Only one of seven experiments showed  $\text{mtK}_{\text{ATP}}$  channel activation by 50  $\mu\text{M}$  diazoxide. It was reported that plasma  $\text{K}_{\text{ATP}}$  channel could be blocked by  $\text{Ca}^{2+}$  and reactivated by Mg-ATP in rat

myocytes [33], whereas the  $\text{Ca}^{2+}$  block in frog skeletal muscle was found to be irreversible [32]. An attempt to test for  $\text{Ca}^{2+}$  induction of an irreversible block was impossible under our experimental conditions because the gigaseal is unstable in a  $\text{Ca}^{2+}$ -free solution. Therefore, the question whether the density of  $\text{mtK}_{\text{ATP}}$  channel is very low in Jurkat cells or if our preparation procedure led to an irreversible block of the majority of channels remains undetermined.

#### 4.5. Nitric oxide blocks the $\text{mtK}_{\text{ATP}}$ channel

Our results show a significant block of the  $\text{mtK}_{\text{ATP}}$  channel in the presence of a physiological amount of NO. As these measurements were done at the single-channel level, they prove that NO provokes alterations of the  $\text{mtK}_{\text{ATP}}$  channel behavior.

However, this result contradicts with the observation of inhibition of a protective effect induced by diazoxide (mtK<sub>ATP</sub> channel activator) in the presence of L-NAME (NOS inhibitor) [16]. Also, Sasaki et al. [27] showed the activating effect of NO on mtK<sub>ATP</sub> channel. This discrepancy may be explained by the possible presence of NO in different redox states (NO<sup>+</sup> and NO) when the donor (e.g. SNAP) is applied. Also, formation of peroxynitrite (ONOO<sup>-</sup>) and other reactive nitrogen species cannot be excluded in a system that comprises energized mitochondria, i.e. that involves an active respiratory chain that may leak superoxide. In contrast to Ref. [27], we have tested the direct effect of authentic NO on the mtK<sub>ATP</sub>. It is shown for other investigations that NO has a dual effect dependent on the used concentration [34]. Hence, the discrepancy in the observed effect of NO on the mtK<sub>ATP</sub> channel activity may be due to experimental conditions that comprise a wide range of NO concentrations. Thus, we provide evidence that besides an effect on the respiratory chain, the mtK<sub>ATP</sub> channel is a second direct target for NO.

Taken together, we demonstrated the existence of a mtK<sub>ATP</sub> channel in human tumor cell line. This channel is influenced not only by known modulators such as ATP and 5-HD but also Ca<sup>2+</sup> and NO affect its state and its kinetics. Thus, even though the molecular structure of the mtK<sub>ATP</sub> channel is still unknown, our results provide insight into the understanding of human mtK<sub>ATP</sub> channel function.

## Acknowledgements

We are very grateful to Dr. K. Winkler-Stuck for discussions, Dipl. Ing. M. Schindler for support in data processing, and appreciate expert technical assistance by C. Hühne, K. Kaiser, J. Witzke and Y. Yamamoto. This work was supported by the NBL3-Partnerprojekt5 FKZ 01ZZ0107, BMFT, and Deutsche Parkinson Vereinigung.

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