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

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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.

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 β-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 mtK_{ATP} channel activator diazoxide whereas this effect is abolished by the blockers 5-HD and glibenclamide [19]. Recent investigations have shown that the mtK_{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 mtK_{ATP} channel inhibition using 5-HD administration prior to the onset of ischemia. Although it was shown that an opening of the mtK_{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 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 Ca^{2+} release by PTP activation [26]. The data concerning the influence of NO on the mtK_{ATP} channel are rather controversial. Both the antiapoptotic effect due to activation of the mtK_{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 mtK_{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 mtK_{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 mtK_{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 mtK^{+} channel displays main characteristics of the mtK_{ATP} channel, e.g. block by ATP and inhibition by the selective blocker 5-HD. We also studied if the mtK_{ATP} channel is modulated by 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% 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 × *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 × *g*, 10 min) and one slow centrifugation (500 × *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 × *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 μM $CaCl_2$ (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 μM of $CaCl_2$ (pH = 7.2). Mitoplasts were kept on ice for a maximum of 36 h.

2.3. Chemicals

KCl and $CaCl_2$ were purchased from Merck (Darmstadt, Germany); HEPES, K_2ATP, 5-hydroxydecanoic acid (5-HD), diazoxide, methyl pyruvate, bovine serum albumin (BSA) and sucrose were from Sigma (Deisenhofen, Germany). The concentrations of free 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 μM were made by transferring aliquots of the NO-stocks in gas-tight 200-μl 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 μl/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 MΩ. Bath and pipette solution were symmetric and contained 150 mM KCl, 10 mM HEPES and 100 (or 200) μM $CaCl_2$ (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 GΩ) formed spontaneously or by applying gentle suction. Experiments were performed at a temperature of 25–27 °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 then 20 ms.

Data are presented as mean ± 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.

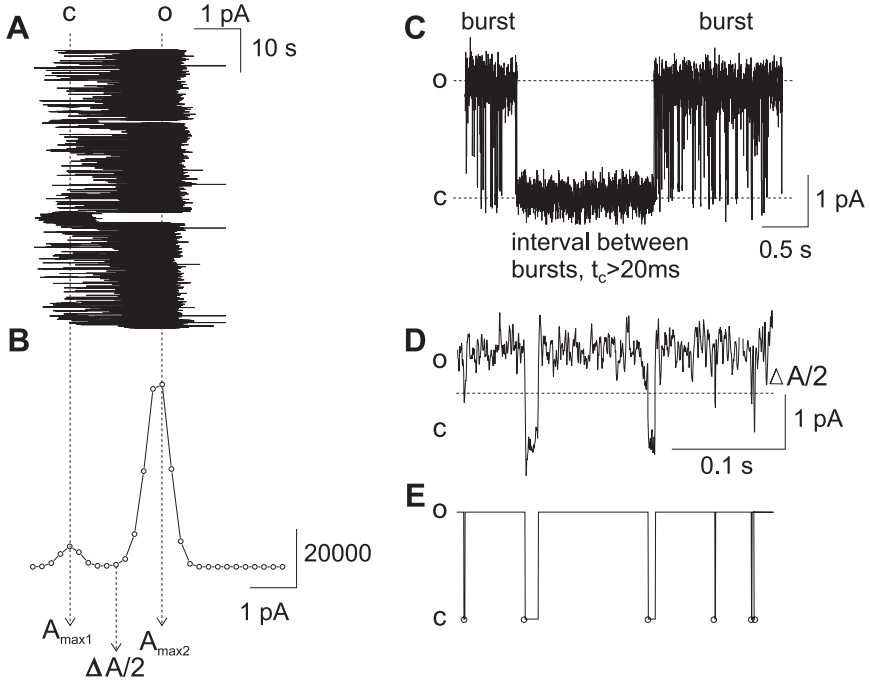

Fig. 1. Analysis of the channel burst frequency. (A) Original record from a mitoplast patch containing the active channel (60 s duration). The dotted lines indicate closed (c) and open (o) state. (B) Amplitude histogram calculated from the data shown in A. The two maxima correspond to the average level of the closed (A_{max1}) and opened (A_{max2}) state. Their difference (ΔA) 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_{max1}). (C) Definition of the interval between two bursts. Any closed state that lasted longer then 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_{max1}) and, correspondingly, all the values below the threshold were set to the averaged closed level (A_{max2}). 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 ± 0.05 to 1.16 ± 0.20 pA at 40 mV (Fig. 2D). This value returned to 1.77 ± 0.10 pA upon wash in KCl.

3.2. ATP reduces the open probability (Po) of the channel

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

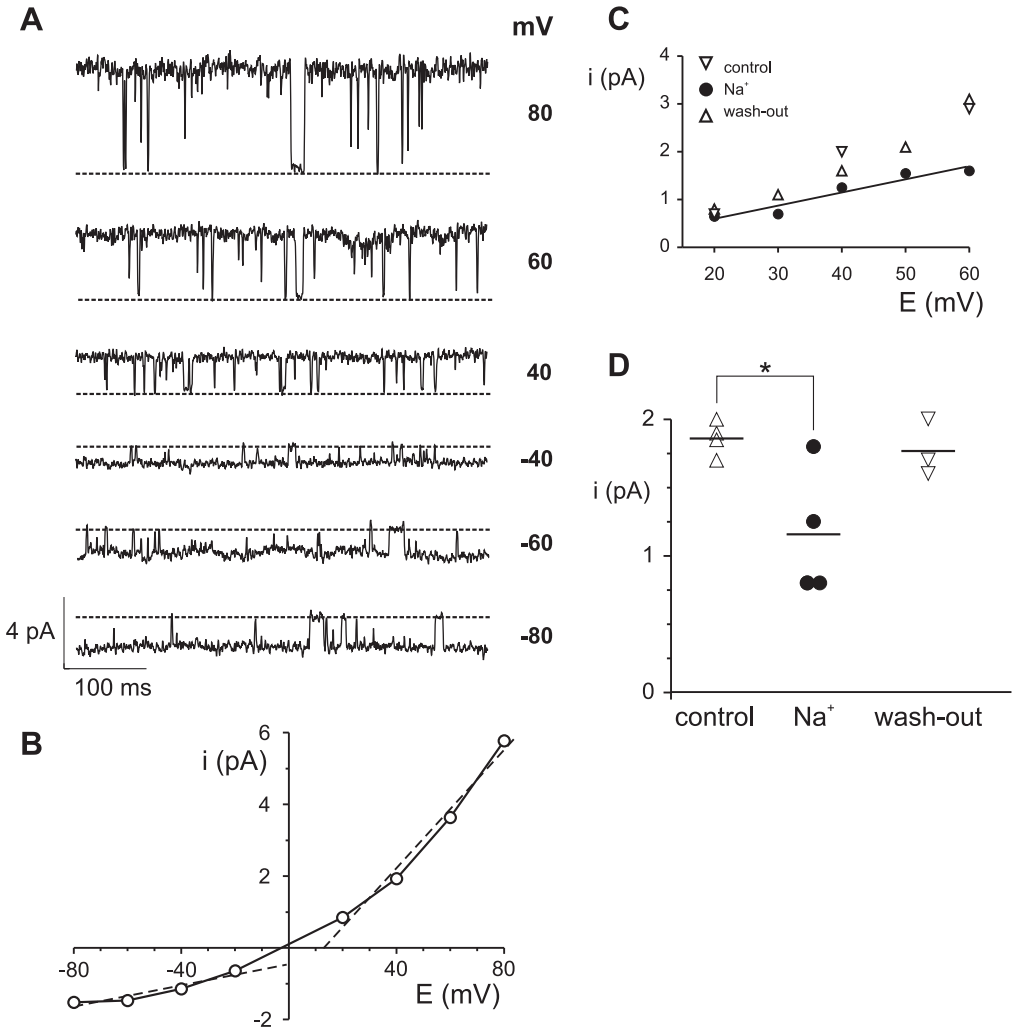

Fig. 2. (A) Representative currents of the channel at various membrane potentials recordings were taken after ATP application (0.5 mM) during the wash-out part (200 μM Ca^{2+}) of the experiment. Dotted lines indicate the current levels of the closed states. (B) Slope conductances were calculated by 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_{ATP} channel. Same experiment as in A. (C) Conductance of the mtK_{ATP} 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_{ATP} 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.

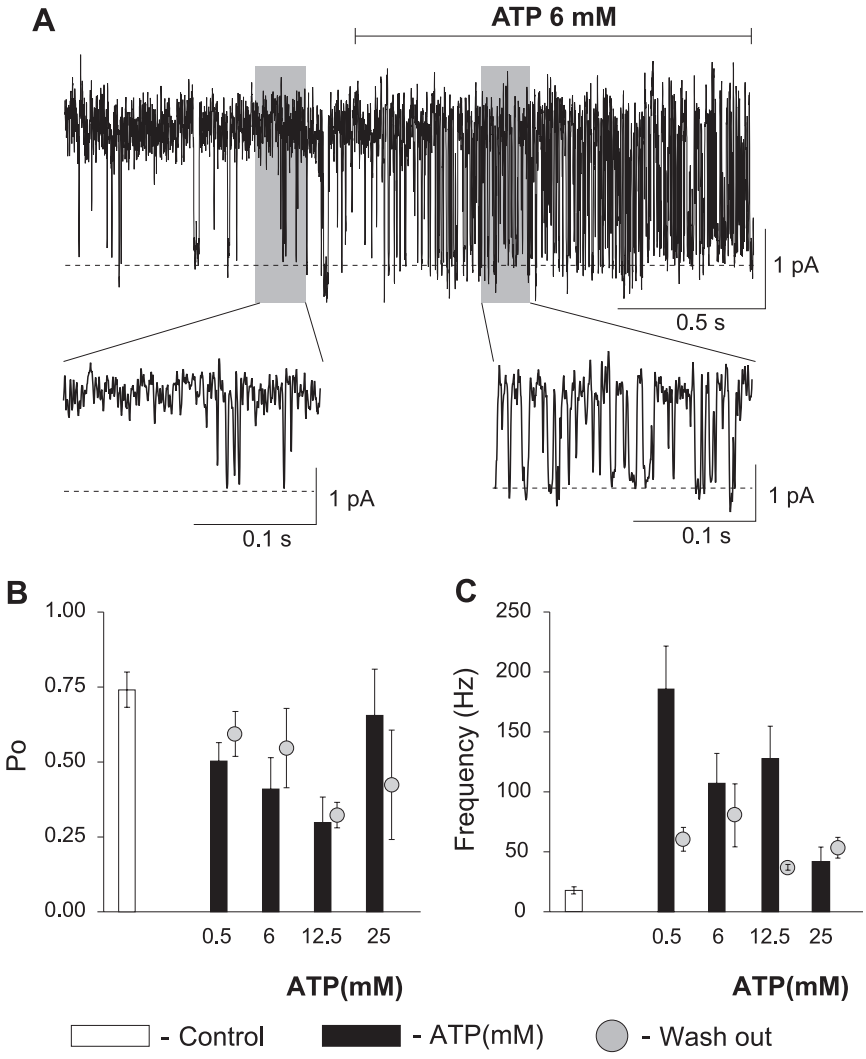

Fig. 3. (A) Representative trace of mtK_{ATP} channel activity in the absence and presence of ATP. Current trace ($E = 40$ mV) shows switching from ATP-free to ATP-containing bath solution and extended in the time parts of the signal. ATP-application results in an increase of the frequency of events. The dotted line indicates closed state of the channel. (B) Open probability (Po) is reduced when the mtK_{ATP} channel is exposed to ATP (ANOVA, $P < 0.05$, $n = 12$). Under control conditions (empty bar) the mtK_{ATP} channel is mostly open. An increase in ATP bath concentration (between 0.5 and 12.5 mM) leads to a reduction of Po. 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 Po. 25 mM ATP does not have a significant effect on Po, but during the wash-out phase a significant reduction of Po was observed (t test $P < 0.05$, $n = 3$). (C) The frequency of events of the mtK_{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.

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

Table 1
The frequency of events of the mtK_{ATP} channel does not depend on Ca^{2+}, when the Ca^{2+} concentration was changed during ATP application

ATP (mM)	Ca^{2+} (μM)	Frequency (Hz)	Number of experiments
0	200	17.7 ± 2.9	11
25	8	42.0 ± 12.0	3
0.5	123	185.8 ± 36.0	3
0.5	7	121.5 ± 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 ± 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 ± 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 ± 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.

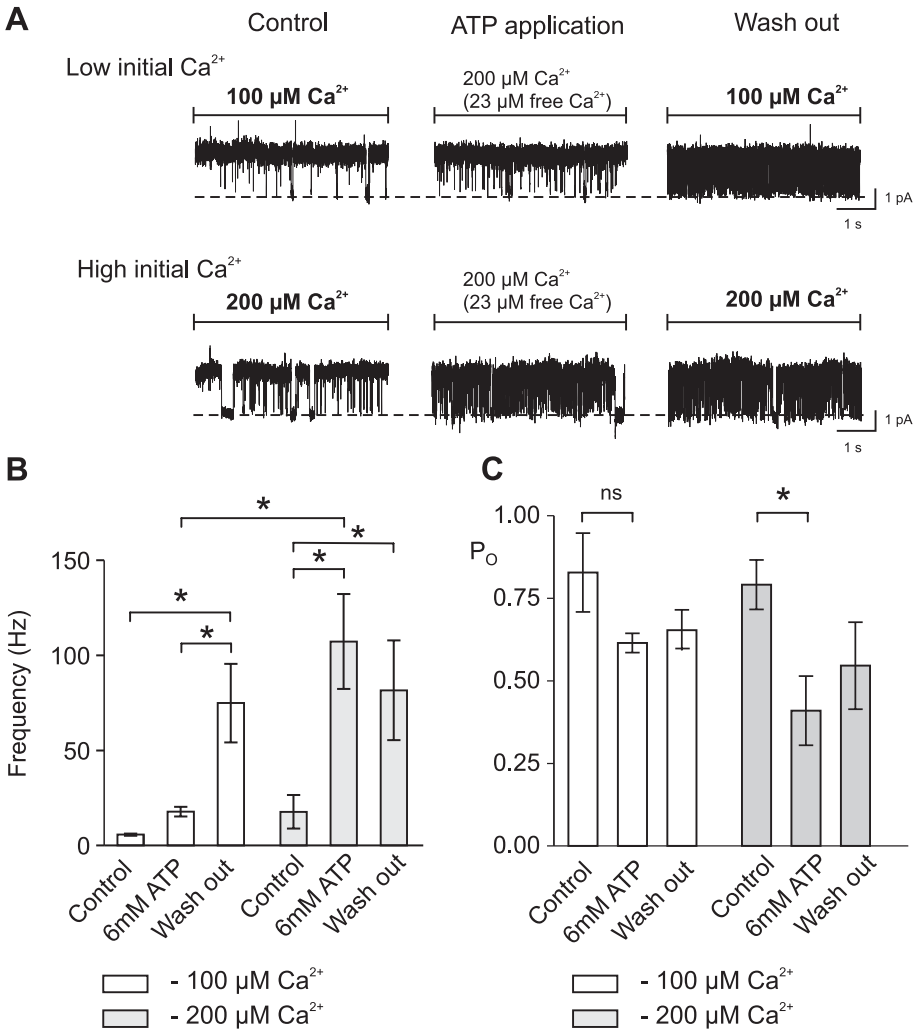

Fig. 4. The mtK_{ATP} channel kinetics during ATP application depend on the initial Ca^{2+} concentration. (A) Two representative experiments with three records are shown. Upper trace: the initial free calcium concentration was kept at a low level (100 µM). Application of 6 mM ATP that reduces the free 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) Po is presented as a function of initial Ca^{2+} concentration. There was a similar pattern of Po reduction between the groups with low and high initial calcium concentration. No significant change of Po after 6 mM ATP application was observed when the initial Ca^{2+} concentration was 100 µM ($Po_{control} = 0.83 \pm 0.12$, $Po_{ATP} = 0.61 \pm 0.03$, $n = 3$). When the initial calcium was 200 µM, the subsequent addition of 6 mM ATP significantly reduced Po ($Po_{control} = 0.80 \pm 0.08$, $Po_{ATP} = 0.41 \pm 0.10$, $n = 3$, $P <= 0.05$). All measurements were done at 40 mV.

Under control conditions the concentration of the free calcium ions was 200 μM. When 25 mM ATP was added to the solution, a reduction of the concentration of free calcium ions from 200 to 8 μM occurred (Brooks and Storey, 1992) due to the binding of 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 μM Ca^{2+}, the concentration of the free Ca^{2+} did not drop much and the frequency was significantly increased. If a high concentration of free Ca^{2+} ions is responsible for the increased frequency in ATP containing solution, then a reduced concentration of free calcium ions (to 7 μM) in the presence of 0.5 mM ATP (similar to a Ca^{2+} level of 8 μM 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 Ca^{2+} concentration (for summarized data, see Table 1). Therefore, the changes of the concentration of free Ca^{2+} during ATP application cannot explain the modified kinetics.

3.5. The mtK_{ATP} channel response to ATP depends on the initial amount of unbound Ca^{2+} in the system

Since the amount of free Ca^{2+} during ATP application was not responsible for the changed frequency of events, we tested if the initial concentration of unbound Ca^{2+} in the bath (before ATP application) could modulate the response of the mtK_{ATP} channel to ATP. For this purpose, the initial concentration of Ca^{2+} was reduced from 200 to 100 μM. The concentration of Ca^{2+} during ATP application was kept as in the standard experiments; i.e. 200 μM $CaCl_2$ that corresponds to 23 μM of free Ca^{2+} in the presence of 6 mM ATP (Fig. 4A).

The frequency of events under control conditions in the presence of low (100 μM) and high (200 μM) free Ca^{2+} was not significantly different (5.8 ± 0.6 and 17.7 ± 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 μM Ca^{2+} initially. In the experiments with low (100 μM) initial concentration of 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 Ca^{2+} concentration. In the presence of low initial Ca^{2+}, Po was not significantly reduced by 6 mM ATP (Fig. 4C).

3.6. Pharmacological characterization of the mtK_{ATP} channel

5-HD is a selective blocking agent of the mtK_{ATP} channel [10]. Applying 1 mM 5-HD to the inner side of the human mtK_{ATP} channel induced an irreversible time-dependent block. Po 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 mtK_{ATP}. Application of diazoxide at concentrations of 50, 100, and 500 μM to a patch without any channel activity did not lead to a clear activation of the mtK_{ATP} channel. 50 μM diazoxide could activate an mtK_{ATP} channel in only one out of seven experiments performed ($Po_{control} = 0$; $Po_{diazoxide} = 0.24 \pm 0.08$; $Po_{wash\text{-}out} = 0.02$). 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 μM diazoxide in the bath increased the Po of this type of activity from 0 to 0.63 ± 0.22 ($n = 4$) after 15 min of perfusion. After adding 100 μM of diazoxide to the bath, this

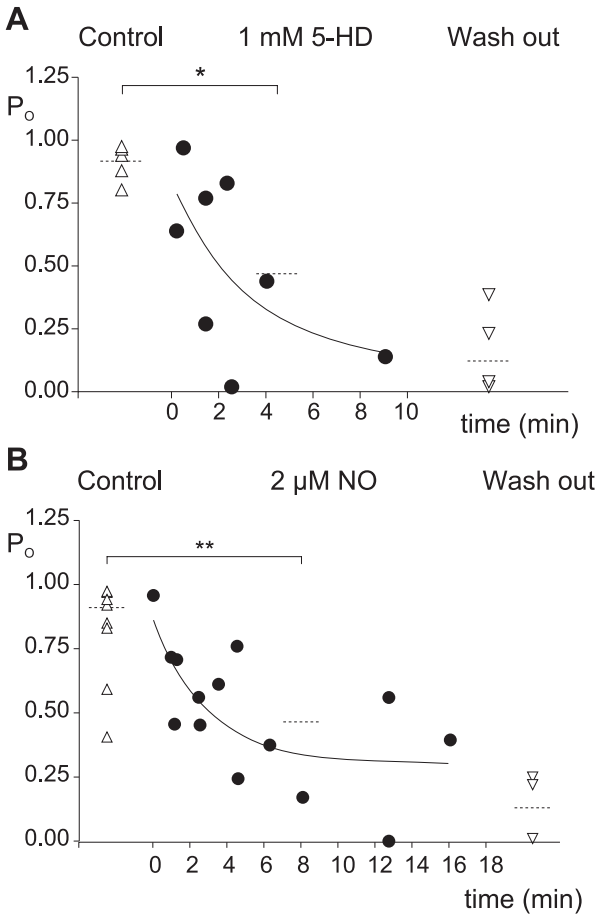

Fig. 5. (A) Effect of 5-HD on Po of the mtK_{ATP} channel. Perfusion with 1 mM 5-HD (selective blocker of the mtK_{ATP} channel) led to an irreversible decrease of Po (*t* test, $P = 0.034$). Open symbols indicate measurements before (triangle, tip upward) and after (triangle, tip downward) application 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 μM nitric oxide on Po of the mtK_{ATP} channel. Perfusion with 2 μM NO irreversibly decreased Po (*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 μM led to loss of the patch. These effects were irreversible during the washout phase.

3.7. Effect of NO on the mtK_{ATP} channel

We applied NO to six patches containing active mtK_{ATP} channels. Using data from three independent experiments, Fig. 5B demonstrates that after adding 2 μM NO, Po 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 μM NO (data not shown).

4. Discussion

The mtK_{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 mtK_{ATP} channel found in rat liver mitochondria [8] and to the reconstituted bovine myocardial mtK_{ATP} channel [30].

4.1. Conductance

The mtK_{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 mtK_{ATP} channel in mitochondria. The mean slope conductance of mtK_{ATP} channels reconstituted from bovine ventricular myocardium was 56 pS at negative potential and showed inward-rectifying characteristics in symmetrical 150 mM K^+ solutions [30]. Measurements of rat liver mtK_{ATP} channel resulted in a single-channel conductance of 9 pS only [8]. They were obtained, however, at low K^+ concentration (33.3 mM K^+ and 66.7 mM Na^+ bath and 100 mM K^+ pipette solution) and the channel showed a tendency towards outward rectification. Such differences may indicate species- or tissue-dependent properties of the mtK_{ATP} channel. The finding that the human mtK_{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 mtK_{ATP} channel

A very detailed description of the ATP blocking effect on the mtK_{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, Po is reduced but not completely abolished. The effect of ATP on Po 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 Po 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. Ca^{2+} modulation of the mtK_{ATP} channel

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

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

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

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

However, although the presented model of the mtK_{ATP} channel modulation explains our findings, we cannot exclude the possibility that ATP–Ca_x/ATP_y–Ca binds to other allosteric sites of mtK_{ATP} channel and causes conformational changes and inhibits channel activity. Further experiments are needed to verify the proposed model.

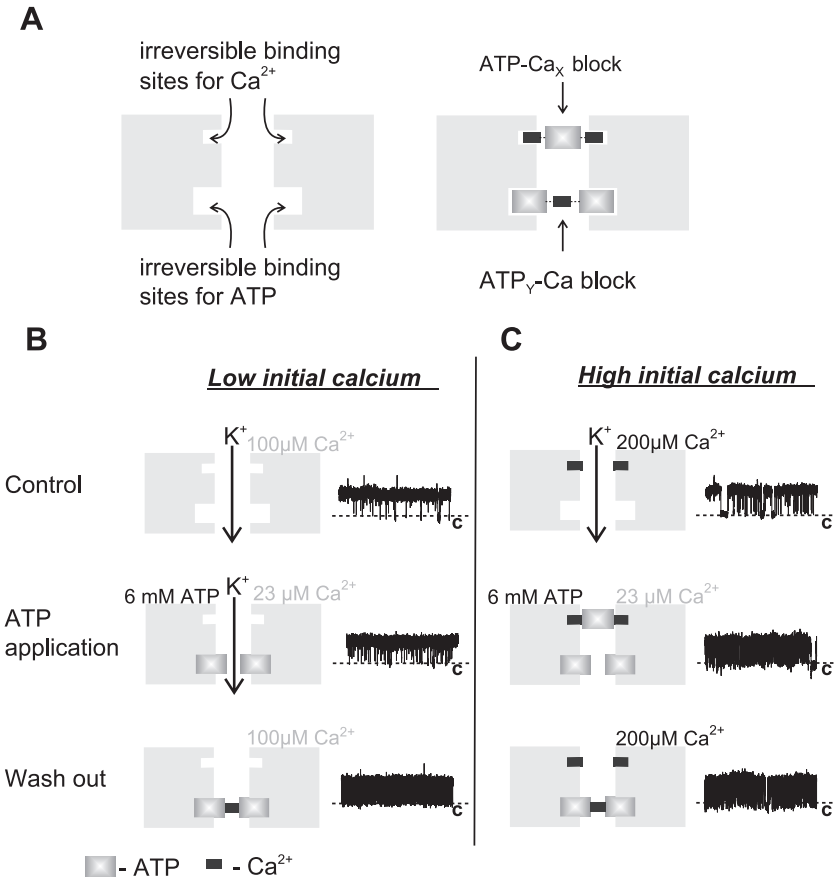

Fig. 6. A hypothetical model of the mtK_{ATP} channel regulation by calcium and ATP is presented. (A) The hypothesis is based on the following assumptions; (i) the mtK_{ATP} channel has two sites where Ca^{2+} and ATP can bind irreversibly. (ii) The block of the channel and increased burst frequency are due to the ATP–Ca_x and the ATP_y–Ca 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 μM 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 Ca^{2+} is restored to the level of 100 μM. Free Ca^{2+} may bind to the ATP molecules that are attached to the binding sites in the channel. Now, the ATP_y–Ca complex is formed and blocks the channel. (C) (High initial calcium) When the concentration of free Ca^{2+} is higher than a certain threshold (e.g. >100 μM), Ca^{2+} will irreversibly bind to the mtK_{ATP} channel. This process does not change the channel behavior. Only when ATP is added, the channel is blocked due to ATP–Ca_x complex formation. Also, similar to the situation with low initial calcium, ATP will irreversibly bind to the mtK_{ATP} channel. During the wash-out phase the ATP–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_y–Ca complex formation and the inhibition of the mtK_{ATP} channel.

4.4. Low mtK_{ATP} channel density or irreversible block?

The probability that a patch contains a mtK_{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 mtK_{ATP} channels due to an unknown blocking mechanism by applying diazoxide, the selective activator of mtK_{ATP} channel. Only one of seven experiments showed mtK_{ATP} channel activation by 50 μM diazoxide. It was reported that plasma K_{ATP} channel could be blocked by Ca^{2+} and reactivated by Mg-ATP in rat myocytes [33], whereas the Ca^{2+} block in frog skeletal muscle was found to be irreversible [32]. An attempt to test for Ca^{2+} induction of an irreversible block was impossible under our experimental conditions because the gigaseal is unstable in a Ca^{2+}-free solution. Therefore, the question whether the density of mtK_{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 mtK_{ATP} channel

Our results show a significant block of the mtK_{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 mtK_{ATP} channel behavior.

However, this result contradicts with the observation of inhibition of a protective effect induced by diazoxide (mtK_{ATP} channel activator) in the presence of L-NAME (NOS inhibitor) [16]. Also, Sasaki et al. [27] showed the activating effect of NO on mtK_{ATP} channel. This discrepancy may be explained by the possible presence of NO in different redox states (NO^+ and NO) when the donor (e.g. SNAP) is applied. Also, formation of peroxynitrite ($ONOO^-$) 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_{ATP}. 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_{ATP} 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_{ATP} channel is a second direct target for NO.

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

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