The human mitochondrial \( K_{\text{ATP}} \) 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 (mt\( K_{\text{ATP}} \)) 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 mt\( K_{\text{ATP}} \) 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 mt\( K_{\text{ATP}} \) 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

\( K_{\text{ATP}} \) 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 \( K_{\text{ATP}} \) channel, the mt\( K_{\text{ATP}} \) 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. mt\( K_{\text{ATP}} \) 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 mt\( K_{\text{ATP}} \) channel activators whereby the mt\( K_{\text{ATP}} \) channels are even 2000 times more sensitive to diazoxide than plasma membrane \( K_{\text{ATP}} \) channels [14].

There is evidence that the mt\( K_{\text{ATP}} \) 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 mt\( K_{\text{ATP}} \) channel opening by diazoxide can mimic IPC protection by inducing cardioprotection. The additional presence of the mt\( K_{\text{ATP}} \) 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 mt\( K_{\text{ATP}} \) channels leads to a reduction of mitochondrial Ca\(^{2+}\)-over-
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<sub>ATP</sub> channel activator diazoxide whereas this effect is abolished by the blockers 5-HD and glibenclamide [19]. Recent investigations have shown that the mtK<sub>ATP</sub> 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<sub>ATP</sub> channel inhibition using 5-HD administration prior to the onset of ischemia. Although it was shown that an opening of the mtK<sub>ATP</sub> 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<sup>2+</sup> release by PTP activation [26]. The data concerning the influence of NO on the mtK<sub>ATP</sub> channel are rather controversial. Both the antiapoptotic effect due to activation of the mtK<sub>ATP</sub> 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<sub>ATP</sub> 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<sub>ATP</sub> 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<sub>ATP</sub> 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<sup>+</sup> channel displays main characteristics of the mtK<sub>ATP</sub> channel, e.g. block by ATP and inhibition by the selective blocker 5-HD. We also studied if the mtK<sub>ATP</sub> channel is modulated by Ca<sup>2+</sup> 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, Colbe, 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<sub>2</sub> and 100% humidity. The cells were fed and reseded 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 hypotonic 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<sub>2</sub> (pH = 7.2) [20]. Isotonicity was restored by the addition of a hypertonic medium containing 750 mM KCl, 30 mM HEPES, and 100 or 200 µM of CaCl<sub>2</sub> (pH = 7.2). Mitoplasts were kept on ice for a maximum of 36 h.

2.3. Chemicals

KCl and CaCl<sub>2</sub> were purchased from Merck (Darmstadt, Germany); HEPES, K<sub>2</sub>ATP, 5-hydroxydecanoic acid (5-HD), diazoxide, methyl pyruvate, bovine serum albumin (BSA) and sucrose were from Sigma (Deisenhofen, Germany). The concentrations of free Ca<sup>2+</sup> 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 CaCl₂ (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 than 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 open (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 (ΔA/2 + A_max1). (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 (ΔA/2) twice. (E) Original data (C) were filtered by equalizing the values exceeding the threshold to the average open level (A_max2) and, correspondingly, all the values below the threshold were set to the averaged closed level (A_max1). 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.

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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 Ca²⁺) 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 mtKATP channel. Same experiment as in A. (C) Conductance of the mtKATP 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 mtKATP 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.
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 ± 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 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 mtKATP 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.

Table 1

The frequency of events of the mtKATP channel does not depend on Ca²⁺, when the Ca²⁺ concentration was changed during ATP application

<table>
<thead>
<tr>
<th>ATP (mM)</th>
<th>Ca²⁺ (µM)</th>
<th>Frequency (Hz)</th>
<th>Number of experiments</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>200</td>
<td>17.7 ± 2.9</td>
<td>11</td>
</tr>
<tr>
<td>25</td>
<td>8</td>
<td>42.0 ± 12.0</td>
<td>3</td>
</tr>
<tr>
<td>0.5</td>
<td>123</td>
<td>185.8 ± 36.0</td>
<td>3</td>
</tr>
<tr>
<td>0.5</td>
<td>7</td>
<td>121.5 ± 33.0</td>
<td>3</td>
</tr>
</tbody>
</table>
3.3. ATP changes the kinetics of the mtK<sub>ATP</sub> 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<sup>2+</sup> does not influence the mtK<sub>ATP</sub> 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<sub>ATP</sub> kinetics. Apart from ATP, only the concentration of unbound Ca<sup>2+</sup> ions was changed as ATP binds Ca<sup>2+</sup> ions in the solution. Therefore, we checked whether Ca<sup>2+</sup> controls the frequency of events in the presence of ATP in Mg<sup>2+</sup>-free solutions.

![Diagram](image)

Fig. 4. The mtK<sub>ATP</sub> channel kinetics during ATP application depend on the initial Ca<sup>2+</sup> 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<sup>2+</sup> concentration to 23 μM did not change the burst frequency. When ATP was removed during wash-out (and the Ca<sup>2+</sup> concentration was therefore restored to 100 μM), an increase of the burst frequency was observed. Lower trace: The initial free Ca<sup>2+</sup> concentration was kept at a high level (200 μM). Application of 6 mM ATP that reduced the Ca<sup>2+</sup> 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<sup>2+</sup> 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<sup>2+</sup> 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<sup>2+</sup> 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<sup>2+</sup> concentration was 100 μM (Po<sub>control</sub> = 0.83 ± 0.12, Po<sub>ATP</sub> = 0.61 ± 0.03, n = 3). When the initial calcium was 200 μM, the subsequent addition of 6 mM ATP significantly reduced Po (Po<sub>control</sub> = 0.80 ± 0.08, Po<sub>ATP</sub> = 0.41 ± 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 to 200 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 8 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<sub>ATP</sub> 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<sub>ATP</sub> 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<sub>ATP</sub> channel

5-HD is a selective blocking agent of the mtK<sub>ATP</sub> channel [10]. Applying 1 mM 5-HD to the inner side of the human mtK<sub>ATP</sub> 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<sub>ATP</sub>. 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<sub>ATP</sub> channel. 50 μM diazoxide could activate an mtK<sub>ATP</sub> channel in only one out of seven experiments performed (Po<sub>control</sub> = 0; Po<sub>diazoxide</sub> = 0.24 ± 0.08; Po<sub>wash-out</sub> = 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...
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 mtKATP channel

We applied NO to six patches containing active mtKATP 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 mtKATP 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 mtKATP channel found in rat liver mitochondria [8] and to the reconstituted bovine myocardial mtKATP channel [30].

4.1. Conductance

The mtKATP 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 mtKATP channel in mitochondria. The mean slope conductance of mtKATP 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 mtKATP 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 mtKATP channel. The finding that the human mtKATP 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 mtKATP channel

A very detailed description of the ATP blocking effect on the mtKATP 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 (P0), 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. Ca2+ modulation of the mtKATP 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 mtKATP channel. It has been shown before that plasma membrane KATP channels can be affected by cytosolic free Ca2+ [31,32]. Also, it is well known that ATP binds Ca2+ and consequently reduces the free Ca2+ concentration. Therefore, we investigated if free Ca2+ may also modulate the KATP channel in human mitochondria.

We found that in ATP-free solution a change in the bath Ca2+ concentration alone influenced neither the Po nor the burst frequency of the mtKATP channel. Only two factors together, the presence of ATP and the initial calcium concentration (before ATP application), modulated the behavior of the mtKATP channel. It suggests that the mtKATP channel couples the energy state, the mitochondrial membrane potential, and the cellular/mitochondrial changes in free calcium.

A hypothetical mechanism of the mtKATP channel modulation by Ca2+ and ATP that fits our observations is presented in Fig. 6. This model is based on the following assumptions: (i) the mtKATP channel has two sites where Ca2+ and ATP can bind irreversibly, (ii) increased burst frequency and following Po reduction are due to the ATP–Ca complex formation, and (iii) ATP binding to the channel causes conformational changes and inhibits channel activity. Further experiments are needed to verify the proposed model.
4.4. Low mtK<sub>ATP</sub> channel density or irreversible block?

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

Our results show a significant block of the mtK<sub>ATP</sub> 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<sub>ATP</sub> channel behavior.
However, this result contradicts with the observation of inhibition of a protective effect induced by diazoxide (mtK\textsubscript{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\textsubscript{ATP} channel. This discrepancy may be explained by the possible presence of NO in different redox states (NO\textsuperscript{+} and NO) when the donor (e.g. SNAP) is applied. Also, formation of peroxynitrite (ONOO\textsuperscript{-}) 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\textsubscript{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\textsubscript{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\textsubscript{ATP} channel is a second direct target for NO.

Taken together, we demonstrated the existence of a mtK\textsubscript{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\textsuperscript{2+} and NO affect its state and its kinetics. Thus, even though the molecular structure of the mtK\textsubscript{ATP} channel is still unknown, our results provide insight into the understanding of human mtK\textsubscript{ATP} channel function.

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