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Ion conductance pathways in potato tuber (*Solanum tuberosum*) inner mitochondrial membrane

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ABSTRACT

Single-ion channel activities were measured after reconstitution of potato tuber mitochondrial inner membranes into planar lipid bilayers. In addition to the recently described large-conductance Ca^{2+}-activated potassium channel activity (Koszela-Piotrowska et al., 2009), the following mitochondrial ion conductance pathways were recorded: (i) an ATP-regulated potassium channel (mitoK$_{ATP}$ channel) activity with a conductance of 164 ± 8 pS, (ii) a large-conductance Ca^{2+}-insensitive iberiotoxin-sensitive potassium channel activity with a conductance of 312 pS ± 23, and (iii) a chloride 4,4′-diisothiocyanostilbene-2,2′-disulfonic acid (DIDS)-inhibited channel activity with a conductance of 117 pS ± 4. In isolated non-phosphorylating potato tuber mitochondria, individual and combined potassium channel activities caused significant (up to 14 mV) but not collapsing K^+-influx-induced membrane potential depolarisation. Under phosphorylating conditions, the coupling parameters were unchanged in the presence of high K^+ level, indicating that plant K^+ channels function as energy-dissipating systems that are not able to divert energy from oxidative phosphorylation. A potato tuber K^+ channel that is ATP-, 5-hydroxydecanonic acid-, glybenclamide-inhibited and diazoxide-stimulated caused low cation flux, modestly decreasing membrane potential (up to a few mV) and increasing respiration in non-phosphorylating mitochondria. Immunological analysis with antibodies raised against the mammalian plasma membrane ATP-regulated K^+ channel identified a pore-forming subunit of the Kir-like family in potato tuber mitochondrial inner membrane. These results suggest that a mitoK$_{ATP}$ channel similar to that of mammalian mitochondria is present in potato tuber mitochondria.

1. Introduction

The mitochondrial inner membrane has an intrinsically low permeability to ions that prevents energy dissipation and depolarisation of the mitochondrial membrane potential ($\Delta\Psi$). However, ion channels (anion, monovalent and divalent cation channels) exist in the inner membrane of mitochondria and their conductance is tightly controlled. The channels have profound effects on mitochondrial metabolism and the efficiency of oxidative phosphorylation (ATP synthesis). Ions pass through the inner mitochondrial membrane in a precisely regulated manner to control many processes, from respiration and mitochondrial morphology to cell proliferation and cell death (for recent reviews see [1–4]).

The most abundant cellular cation is potassium, and its transport via potassium channels seems to be an important mechanism controlling the integrity of inner mitochondrial membranes. The electrogenic transport of K^+ into the mitochondrial matrix is strictly ion channel-dependent and resembles plasma membrane ion channel activity. Mitochondrial volume homeostasis depends on monovalent cation transport across the inner membrane, which couples electrophoretic K^+ influx through potassium channels to K^+ extrusion through a K^+/H^+ exchanger. Mammalian mitochondrial inner membrane potassium channels include ATP-regulated potassium channels (mitoK$_{ATP}$ channels), voltage-dependent potassium channels (mitoKv1.3 channels), twin-pore TASK-3 potassium channels and two types of Ca^{2+}-activated potassium channels: large-conductance (mitoBK$_{Ca}$ channels) and intermediate-conductance channels [1,2,5]. These channels have basic biophysical and biochemical properties

Abbreviations: BK$_{Ca}$ channel, plasma membrane large-conductance Ca^{2+}-sensitive potassium channel; DIDS, 4,4′-diisothiocyanostilbene-2,2′-disulfonic acid; 5-HD, 5-hydroxydecanonic acid; IbTx, iberiotoxin; mitoK$_{ATP}$ channel, mitochondrial ATP-regulated potassium channel; mitoBK$_{Ca}$ channel, mitochondrial large-conductance Ca^{2+}-activated potassium channel; mitoBK channel, mitochondrial large-conductance Ca^{2+}-insensitive potassium channel; K$_{ATP}$ channel, plasma membrane ATP-regulated potassium channel; mitoCl, mitochondrial chloride channel; BSA, bovine serum albumin; MCC, multiple conductance channel; PLM, planar lipid membrane; *P* (open), channel open probability; SMP, submitochondrial particles; TPP^+, tetraphenylphosphonium; $\Delta\Psi$, mitochondrial membrane potential; $\Delta\mu H^+$, electrochemical proton gradient

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similar to those of some types of potassium channels present in the plasma membrane of various mammalian cells. Investigations of the pharmacological profile and immunoreactivity with specific antibodies have suggested that the mammalian mitoK$_{ATP}$ channel belongs to the inward rectifier K^+ channel family Kir6.x [6]. The mitoK$_{ATP}$ channel has also been described in the mitochondria of eukaryotic microorganisms, such as the amoeboid protist *Acanthamoeba castellanii* [7]. The existence of a potassium import inhibited by ATP in plant mitochondria was first shown for durum wheat and pea mitochondria [8–10]. In these mitochondria, the sensitivity of mitochondrial swelling and $\Delta\Psi$ to potassium channel modulators is similar to that previously described for the mammalian mitoK$_{ATP}$ channel. The plant mitoK$_{ATP}$ channel is activated by diazoxide and GTP in durum wheat and pea mitochondria, and its activity is inhibited by glibenclamide and 5-hydroxydecanonic acid (5-HD) only in pea mitochondria. Moreover, in pea stem mitochondria, the mitoK$_{ATP}$ channel appears to be stimulated by cyclosporin A and regulated by dithiol-disulfide interconversion, H_2O_2 and NO [9,11]. The primary function of the mitoK$_{ATP}$ channel is to allow K^+ transport into the mitochondrial matrix. The physiological function of the plant mitoK$_{ATP}$ channel remains unclear. This channel is proposed to be involved in the regulation of mitochondrial volume, in programmed cell death and in the prevention of ROS formation [8–12]. The other plant mitochondrial potassium channel, the mitoBK$_{Ca}$ channel, was previously studied mainly in mammalian mitochondria, but it has been recently described using electrophysiological measurements in a reconstituted system [13]. Furthermore, bioenergetic (mitochondrial respiration and $\Delta\Psi$) measurements were made in isolated potato tuber mitochondria with the use of substances known to modulate mitoBK$_{Ca}$ channels in mammalian mitochondria [13].

While mitochondrial ion channels have been intensively studied in a variety of mammalian cells, less attention has been devoted to plant mitochondrial ion channels [2,3]. The aim of this study was to measure the conductance of ion channels functioning in the plant mitochondrial inner membrane using a planar lipid membrane (PLM) reconstituted system. In contrast to the low K^+ flux through the mammalian and protist mitoK$_{ATP}$ channels [7,14], swelling and $\Delta\Psi$ (with safranin as a probe) studies with isolated plant mitochondria (including potato tuber mitochondria) have previously shown that K^+ present at a high concentration can be imported at high rates, thereby strongly reducing or even collapsing $\Delta\Psi$ [8]. Therefore, our aim was to verify this finding by functional analysis in isolated potato tuber mitochondria of the influence of mitoK$_{ATP}$ channel activity on mitochondrial $\Delta\Psi$ and respiration. Additionally, the electrophysiological properties of a single plant mitoK$_{ATP}$ channel in a reconstituted system were characterised and the channel was identified immunologically.

2. Materials and methods

2.1. Chemicals

L-α-Phosphatidylcholine (asolectin) and *n*-decane for PLM method were obtained from Sigma-Aldrich. All other chemicals (mostly from Sigma-Aldrich) were of the highest purity commercially available.

2.2. Isolation of mitochondria and preparation of the inner mitochondrial membrane fraction

Solanum tuberosum (cultivar Bryza) tubers were purchased from the local supermarket. Mitochondria were isolated and purified on a self-generating Percoll gradient as described earlier [13].

To obtain submitochondrial particles (SMP), which are mitochondrial-inner-membrane-enriched fractions, mitochondria were isolated with media containing potassium salts [13]. A plasma membrane-enriched fraction was also prepared [13].

2.3. Planar lipid membrane (PLM) measurements

The experiments were performed with potato tuber SMP as described previously [13]. In brief, PLMs were formed in a 250-μm-diameter hole drilled in a Delrin cup (Warner Instruments), which separates the two chambers (*cis* and *trans*, each with a 1 ml internal volume). The chambers contained 50/450 mM KCl (*trans*/*cis*) and 20 mM Tris–HCl solution, pH 7.2. The outline of the aperture was coated with a lipid solution and N_2 dried prior to bilayer formation to improve membrane stability. PLMs were painted using asolectin in *n*-decane at a final concentration of 25 mg of lipid/ml. SMP (approx. 3 μg of protein/ml, 0.5–1.5 μl/reconstitution) were added to the *trans* compartment (see Fig. 1A). Measurements were carried out at room temperature (24–25 °C). Formation and thinning of the bilayer were monitored by capacitance measurements and optical observations. Final accepted capacitance values ranged from 120 to 230 pF. Electrical connections were created using Ag/AgCl electrodes and agar salt bridges (3 M KCl) to minimise liquid junction potentials. Voltage was applied to the *cis* compartment of the chamber and the *trans* compartment was grounded. The current was measured using a bilayer membrane amplifier (BLM-120, BioLogic).

2.4. Electrophysiological data analysis

Signals were low-pass-filtered at 500 Hz. The current was digitised at a sampling rate of 100 kHz (A/D converter PowerLab 2/20, ADInstruments) and transferred to a PC for off-line analysis by Chart version 5.5.5 (PowerLab, ADInstruments) and pCLAMP10.2 (Axon Instruments). The pCLAMP10.2 software package was used for data processing. The channel recordings presented are representative of the most frequently observed conductance values under the given conditions. Ion conductance was calculated from a nonlinear curve of the points in the current–voltage relationship using the GraphPad Prism4 program. The channel open probability [*P* (open)] was calculated with an automatic interval setting. The channel open (τ_{open}) and closed (τ_{closed}) lifetimes were calculated without additional filtering by using a Clampfit 10.2 program. *n* denotes the number of experiments and *N* the number of events. τ_{open}, τ_{closed} and *P* (open) were calculated from segments of continuous recordings lasting 60s and with $N \geq 1000$ events. Data from the experiments are reported as the mean value ± SD. Clampfit 10.2 was used to determine single channel current amplitude histograms (pClamp software, Molecular Devices). The permeability ratios for K^+ (P_K) and Cl^- (P_{Cl}) were calculated according to the Goldman-Hodgkin-Katz equation [7,15].

2.5. Mitochondrial oxygen consumption and membrane potential measurements

Oxygen uptake was measured polarographically with a Clarck type oxygen electrode in 3 ml of standard incubation medium (25 °C) consisting of 0.3 M mannitol, 3 mM NaH_2PO_4, 1 mM $MgCl_2$, 1.5 mM EGTA, 10 mM Tris/HCl, 10 mM MES, pH 6.8 and 0.1% BSA. Changes in the composition of the incubation medium are described in the figure legends. In all experiments, the osmolarity of incubation medium was kept constant by adjusting the mannitol concentration. Measurements were performed with 0.8–1 mg of mitochondrial protein in the presence of 1.5 mM benzohydroxymate (an inhibitor of alternative oxidase). Exogenous NADH (1.5 mM) plus rotenone (2 μM) was used as a respiratory substrate. Under non-phosphorylating (State 4) conditions, measurements were performed in the presence of 1.8 μM carboxyatractyloside (an inhibitor of ATP/ADP antiporters) and 0.5 μM oligomycin (an inhibitor of F_1F_0-ATP synthase). Phosphorylating (State 3) respiration measurements were performed in the presence of 150 μM (pulse) ADP. Only high quality mitochondria preparations, i.e., with an ADP/O value of around 1.25 and a respiratory

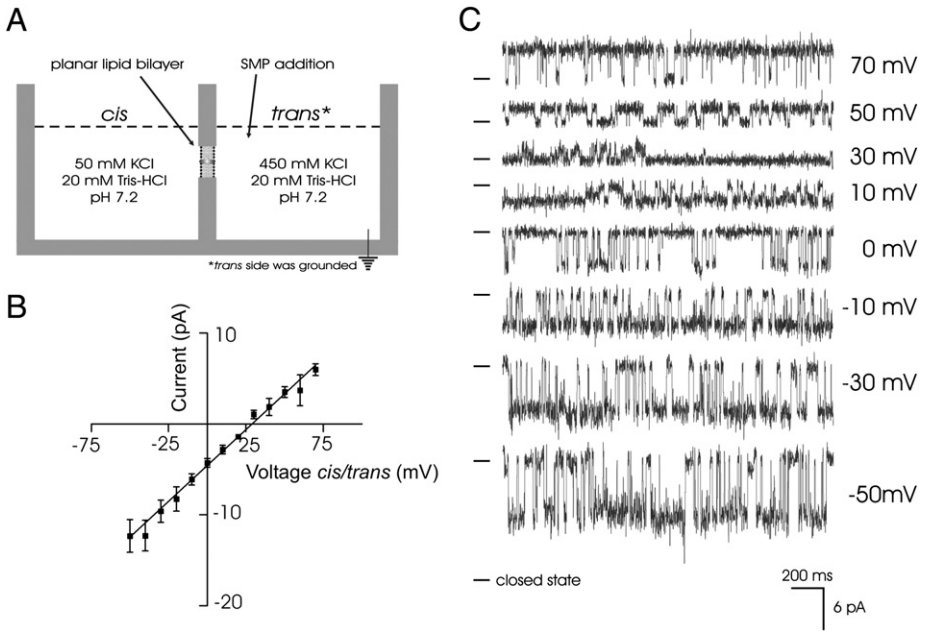

Fig. 1. Single-channel recordings of the potato tuber mitoK$_{ATP}$ channel in planar lipid bilayers. *A*, Schematic configuration of the *cis* and *trans* compartments used in the experiments. *B*, Current–voltage (I/V) characteristics of single-channel events in 50/450 mM KCl (*cis*/*trans*) gradient solutions ($n = 9$, mean ± SD) at different voltages. *C*, Single channel current-time recordings at different voltages. "–" indicates current at the closed state of the channel. Each example is representative of nine measurements.

control ratio of around 2.5–3, were used in all experiments. O_2 uptake values are given in nmol $O_2 \times min^{-1} \times mg^{-1}$ protein.

The mitochondrial membrane potential ($\Delta\Psi$) was measured simultaneously with oxygen uptake using a tetraphenylphosphonium (TPP^+)-specific electrode [13]. Measurements were performed in the presence of 6 μM TPP^+. To calculate the $\Delta\Psi$ value, the matrix volume of potato tuber mitochondria was assumed to be $2.0\ \mu l \times mg^{-1}$ protein. The calculation assumes that the TPP^+ distribution between mitochondria and medium followed the Nernst equation. The values of $\Delta\Psi$ were corrected for TPP^+ binding using apparent external and internal partition coefficients of TPP [16]. The correction shifted calculated $\Delta\Psi$ values to lower values (approx. 30-mV shift), but it did not influence changes in the resulting membrane potential (relative changes). Values of $\Delta\Psi$ are given in mV.

2.6. SDS-PAGE and immunoblotting

Samples of isolated mitochondrial proteins, SMP or the plasma membrane-enriched fraction were solubilised in sample buffer containing 2% (w/v) SDS, 50 mM Tris/Cl (pH 6.8), 10% glycerol, 0.004% (w/v) bromophenol blue and 8% mercaptoethanol and then boiled for 4 min. Proteins were separated in 12.5% SDS-polyacrylamide gels and then electrotransferred to a nitrocellulose membrane. Membranes were then hybridised with anti-Kir6.1 and anti-Kir6.2 antibodies (Santa Cruz Biotechnology) at dilutions of 1:250 in the presence or absence of blocking peptide. Cross reactivity was also checked with antibodies raised against a plant plasma membrane H^+-ATPase (at a dilution of 1:1000). Protein detection was achieved with secondary antibodies linked to horseradish peroxidase (at a dilution of 1:20000) and the ECL chemiluminescence system.

3. Results

3.1. Electrophysiological identification of potato tuber inner mitochondrial membrane ion channels in planar lipid bilayers

In our previous work, we established the existence of the mitochondrial large-conductance Ca^{2+}-activated potassium channel (mitoBK$_{Ca}$ channel) in potato tuber mitochondrial inner membrane [13]. In the present study, using the same PLM method, we measured the ion conductance of other monovalent ion channels functioning in the potato tuber mitochondrial inner membrane. The inner mitochondrial membrane-enriched SMP fractions from six different preparations were used. Before adding the SMP suspension, the quality of the lipid bilayers was checked at 0 mV and ±50 mV to confirm the absence of channel-like activity. From 10 to 20 min after adding SMP vesicles to the *trans*-bilayer chamber, a positive or negative current at 0 mV was routinely observed. At 0 mV, in the 450/50 mM KCl (*trans*/*cis*) gradient solutions used in this study, a current carried by cations flowing from the *trans* (ground) compartment to the *cis* (active electrode) compartment is negative and plotted downwards. Conversely, a current carried by anions flowing the same direction is positive and plotted upwards.

In PLM measurements, a variety of channel activities ($n = 120$) were observed, both cation- and anion-selective. The frequency of incorporation of potato tuber mitochondrial inner membrane channels with identifiable properties was determined. Approximately 48% of the incorporated channels behaved like large-conductance Ca^{2+}-activated potassium channels (mitoBK$_{Ca}$ channels), 20%—large-conductance Ca^{2+}-insensitive potassium channels (mitoBK channels), 18%—ATP-regulated potassium channels (mitoK$_{ATP}$ channels), and 14%—mitochondria chloride channels (mitoCl channels).

3.1.1. The mitochondrial ATP-regulated potassium channel (mitoK$_{ATP}$ channel)

Fig. 1 shows representative current-time traces (Fig. 1C) and current–voltage relationship (Fig. 1B) for a potato tuber mitoK$_{ATP}$ channel in 50/450 mM KCl (*cis*/*trans*) gradient. The current was measured as a function of applied potential at 20-mV intervals ranging from +70 mV to −50 mV (Fig. 1C). The calculated ion conductance was equal to 164 ± 8 pS ($n = 9$). The reversal potential, i.e., the potential at which the current reversed direction, was calculated from curve fitting to the experimental data (Fig. 1B) and was equal to +27.5 mV. This indicated that the examined ion channel was cation-selective. Additionally, the calculated permeability ratio (P_K /P_{Cl}) was equal to 4.2. All incorporated mitoK$_{ATP}$ channels were active at both positive and negative voltages ($n = 21$) (Fig. 1C).

Fig. 2 demonstrates the effect of 1 mM ATP (in the presence of 1 mM $MgCl_2$) on the studied K^+ ion channel. Fig. 2A shows the single-channel recordings in the 50/450 mM KCl (*cis*/*trans*) gradient solutions at 0 mV before and after the addition of 1 mM ATP to both chambers (cis/trans). ATP caused complete inhibition of the channel. As shown in Fig. 2B, the inhibition is clearly depicted in amplitude histograms fitted with superimposed Gaussian curves and calculated from the same experiment. The channel open probability (P) at 0 mV potential decreased from 0.57 ± 0.11 in control recordings to 0.096 ± 0.095 after addition of 1 mM ATP ($n = 4$, $p = 0.0057$, unpaired t test) (Fig. 2C).

3.1.2. The mitochondrial large-conductance calcium-insensitive potassium channel (mitoBK channel)

Among the large-conductance K^+ channel activities recorded in PLMs, we have focused on the Ca^{2+}-insensitive channels. Single-channel current-time traces were recorded at different voltages in the asymmetrical 50/450 mM KCl (*cis*/*trans*) condition ($n = 25$) (Fig. 3A). The channel conductance determined from a nonlinear curve of applied voltages from +70 mV to −30 mV was equal to 312 ± 23 pS ($n = 4$) (Fig. 3B). The mean reversal potential of +31 mV calculated from curve fitting to the experimental data and the calculated permeability ratio (P_K / P_{Cl}) of 5.3 prove that the examined channel is selective for cations. Similar to the mitoK$_{ATP}$ channel described above, all incorporated large-conductance channels were active at both positive and negative voltages. However, at 0 mV and only at negative potentials, the open probability of the channel was slightly reduced. Occasionally, sub-states could be observed.

Compared to a recently described potato tuber mitochondria large-conductance Ca^{2+}-activated potassium channel (the mitoBK$_{Ca}$ channel) [13] with conductance from 502 to 615 pS, the large-conductance channels we studied in this work were not sensitive to Ca^{2+}. After addition of 300 μM Ca^{2+} (*cis*/*trans*), no increase in the channel open probability was observed ($n = 5$, data not shown). However, similarly to the mitoBK$_{Ca}$ channel, the mitoBK Ca^{2+}-insensitive channel was inhibited by IbTx, a known inhibitor of plasma-membrane and mitochondrial BK$_{Ca}$ channels. Fig. 3C presents the inhibitory effect of IbTx on the potato tuber mitoBK Ca^{2+}-insensitive channel of approx. 312 pS conductance. 400 nM IbTx caused almost complete transition of the channel into a closed state (Fig. 3C). The channel open probability (P) at 0 mV potential decreased from 0.84 ± 0.10 to 0.33 ± 0.13 after addition of 200 nM IbTx ($p = 0.0051$, unpaired t test) and to 0.0095 ± 0.0094 after addition of 400 nM IbTx ($p = 0.0003$, unpaired t test). Thus, IbTx blocked the channel's activity in a dose-dependent manner (Fig. 3C). The apparent concentration of IbTx that provided half maximal inhibition was approximately 150 nM. Charybdotoxin, the other K^+ channel blocker, also inhibited the potato tuber mitoBK Ca^{2+}-insensitive channel (data not shown).

3.1.3. The mitochondrial chloride channel (mitoCl channel)

The last group of potato tuber mitochondrial channels ($n = 17$) reconstituted in PLMs displayed a conductance similar to that of the

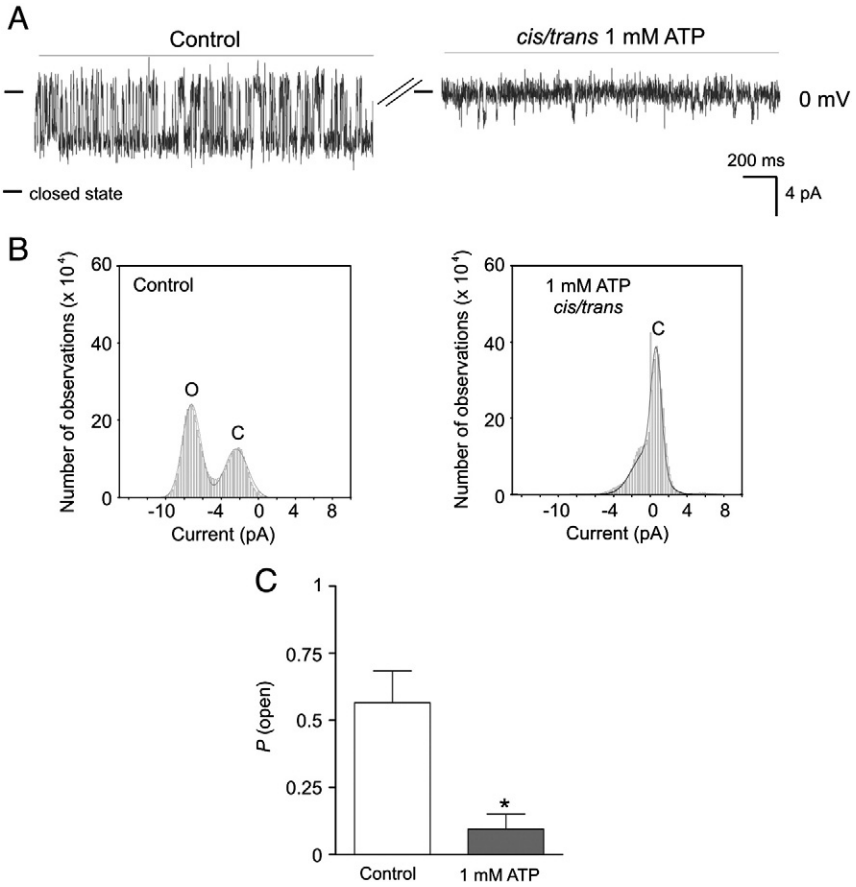

Fig. 2. Effects of ATP on the activity of the potato tuber mitoK$_{ATP}$ channel reconstituted in PLM. *A*, Examples of single-channel recordings in 50/450 mM KCl (*cis*/*trans*) gradient solutions at 0 mV under control conditions and after addition of 1 mM ATP (in the presence of 1 mM $MgCl_2$) to the *cis* and *trans* compartments ($n = 5$). "–" indicates current at the closed state of the channel. *B*, Amplitudes measured in the absence or presence of 1 mM ATP. Amplitude histograms were obtained from measuring channel openings with a total recording time of 1 min. All points shown were fitted by two Gaussian distributions. The closed state corresponds to the peak at 0 pA. O, open state; C, closed state. *C*, Channel open probability (P) at 0 mV potential. *, p value in the presence of ATP is significantly different from control value ($p < 0.01$, Student's unpaired t test). Means of four different experiments (±SD) are shown.

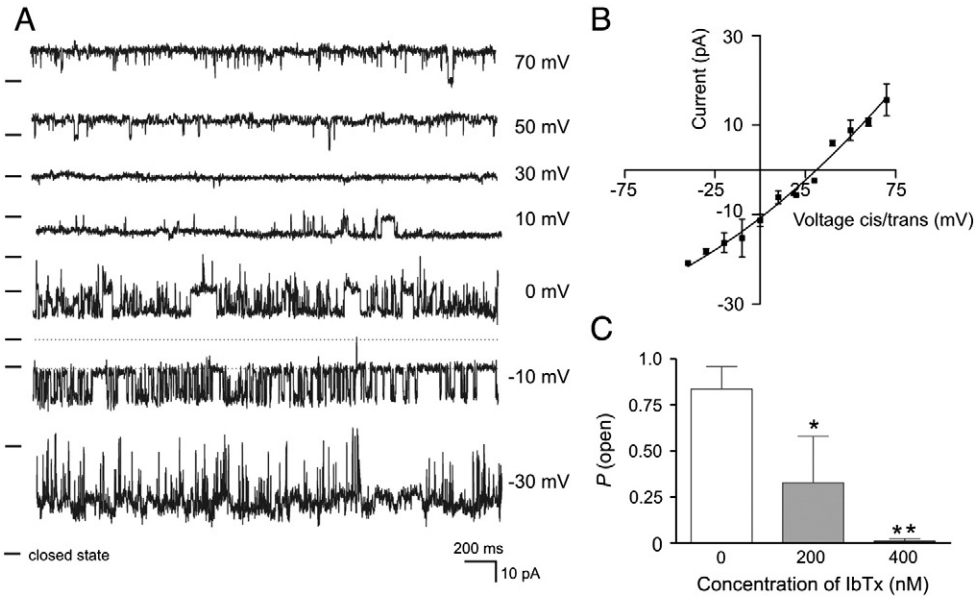

Fig. 3. Effects of IbTx on the activity of a potato tuber mitoBK Ca^{2+}-insensitive channel reconstituted in PLM. *A*, Single-channel current-time recordings in 50/450 mM KCl (*cis/trans*) gradient solutions at different voltages. "–" indicates current at the closed state of the channel. *B*, Current–voltage (I/V) relationship of single-channel events ($n=4$, mean ± SD). *C*, Channel open probability (*P*) at 0 mV potential under control conditions and after addition of 200 or 400 nM IbTx to the *cis* and *trans* compartments. * and **, *p* values in the presence of 200 or 400 nM IbTx are significantly different from control values ($p<0.01$ and $p<0.001$, respectively, Student's unpaired *t* test). Data from five experiments are presented as the mean ± SD.

mammalian mitoCl channel. Examples of single-channel recordings of the potato tuber mitoCl channel are presented in Fig. 4A. Fig. 4B shows the current–voltage relationship calculated from the mean amplitude of the channel currents at different potentials (from −50 mV to 75 mV). In 50/450 mM KCl (*cis/trans*) gradient solutions, the calculated ion conductance was equal to 117 ± 4 pS ($n=4$). The calculated reversal potential of −28 mV and the calculated permeability ratio for Cl^- and K^+ (P_{Cl}/P_K) of 4.4 indicate that this channel is mildly selective for anions. A characteristic of the channel was a flickering behaviour, i.e., bursts of frequent short openings and long openings at positive potentials. The activity of all the incorporated mitoCl channels was dependent on voltage (Fig. 4A). Channel openings were almost absent at negative potentials. These results are similar to data reported for chloride channels from rat skeletal muscle and brain mitochondria, where mitoCl channel activity was very low or absent, respectively, at negative potentials [17].

Under 50/450 mM KCl (*cis/trans*) gradient conditions, 200 μM DIDS inhibited the activity of the potato tuber mitoCl channel immediately after addition to the *cis* and *trans* compartments (Fig. 4C). As illustrated in the amplitude histograms, the open probability changed from 99% to zero after the addition of DIDS.

3.2. Contributions of potassium channels to K^+-influx-induced $\Delta\Psi$ depolarisation in potato tuber mitochondria

As described above, K^+ conductance activities of the three distinct potassium channels, i.e., the mitoBK$_{Ca}$ channel, the mitoBK Ca^{2+}-insensitive channel and the mitoK$_{ATP}$ channel, were detected with the PLM method in potato tuber mitochondrial inner membrane-enriched SMP. Our aim was to determine the influence of these three channels on K^+-influx-induced depolarisation of $\Delta\Psi$ in isolated non-phosphorylating potato tuber mitochondria. Because succinate dehydrogenase is activated by ATP, which is also an inhibitor of the mitoK$_{ATP}$ channel, addition of ATP to potato tuber mitochondria oxidising succinate led to dehydrogenase activation and thereby resulted in an increase in $\Delta\Psi$ and respiration (data not shown). As the same effect on $\Delta\Psi$ is expected with the inhibition of the mitoK$_{ATP}$ channel by ATP (accompanied by a decrease in respiration), the use of succinate as a respiratory substrate could lead to inaccuracy and overestimation of the channel's function. Moreover, it was found that in the absence of activators and inhibitors (including ATP) of K^+ channels, changes in $\Delta\Psi$ accompanied by K^+ (at given KCl concentrations) entry into mitochondria were independent of the respiratory substrate used (data not shown). Therefore, exogenous NADH was used in this study as an oxidisable substrate instead of succinate.

The total activity of the three K^+ channels, i.e., the mitoBK$_{Ca}$ channel, the mitoBK Ca^{2+}-insensitive channel and the mitoK$_{ATP}$ channel, was measured as the difference between $\Delta\Psi$ in the presence of the K^+ channel activators and $\Delta\Psi$ in the presence of the K^+ channel activators and inhibitors. When the three K^+ channels were active, high concentrations of KCl (50 and 100 mM) caused approx. 9 and 14 mV (respectively) depolarisation of State 4 $\Delta\Psi$ in exogenous NADH-oxidising mitochondria under non-phosphorylating conditions (Table 1). The activities of the two large-conductance K^+ channels, the mitoBK$_{Ca}$ channel and the mitoBK Ca^{2+}-insensitive channel, when measured separately had a similar impact on $\Delta\Psi$ depolarisation, i.e., approx. 4 and 7 mV (at 50 and 100 mM KCl, respectively). Among the potato tuber mitochondria K^+ channels we studied, the activity of the mitoK$_{ATP}$ channel had the weakest influence on State 4 $\Delta\Psi$. When this activity was measured as the difference between $\Delta\Psi$ in the presence of a mitoK$_{ATP}$ channel activator (diazoxide) and $\Delta\Psi$ in the presence of the mitoK$_{ATP}$ channel activator plus inhibitors (diazoxide and ATP), depolarisations of approx. 2 and 4 mV (at 50 and 100 mM KCl, respectively) were observed (Table 1).

Under non-phosphorylating conditions, in the presence of 100 mM KCl and when all three K^+ channels were active (in the presence of Ca^{2+} and diazoxide and in the absence of K^+ channel inhibitors), $\Delta\Psi$ decreased from approx. 186 to 173 mV (Table 1) and respiratory rate increased by a factor of 1.23 (Table 2). However, under phosphorylating conditions, State 3 respiratory rate and $\Delta\Psi$ remained constant. The changes observed under non-phosphorylating conditions were significant but not enough to disturb oxidative phosphorylation in potato tuber mitochondria. Coupling parameters, i.e., the ADP/O ratio and the respiratory control ratio were unchanged in mitochondria respiring in the absence or presence of 100 mM KCl

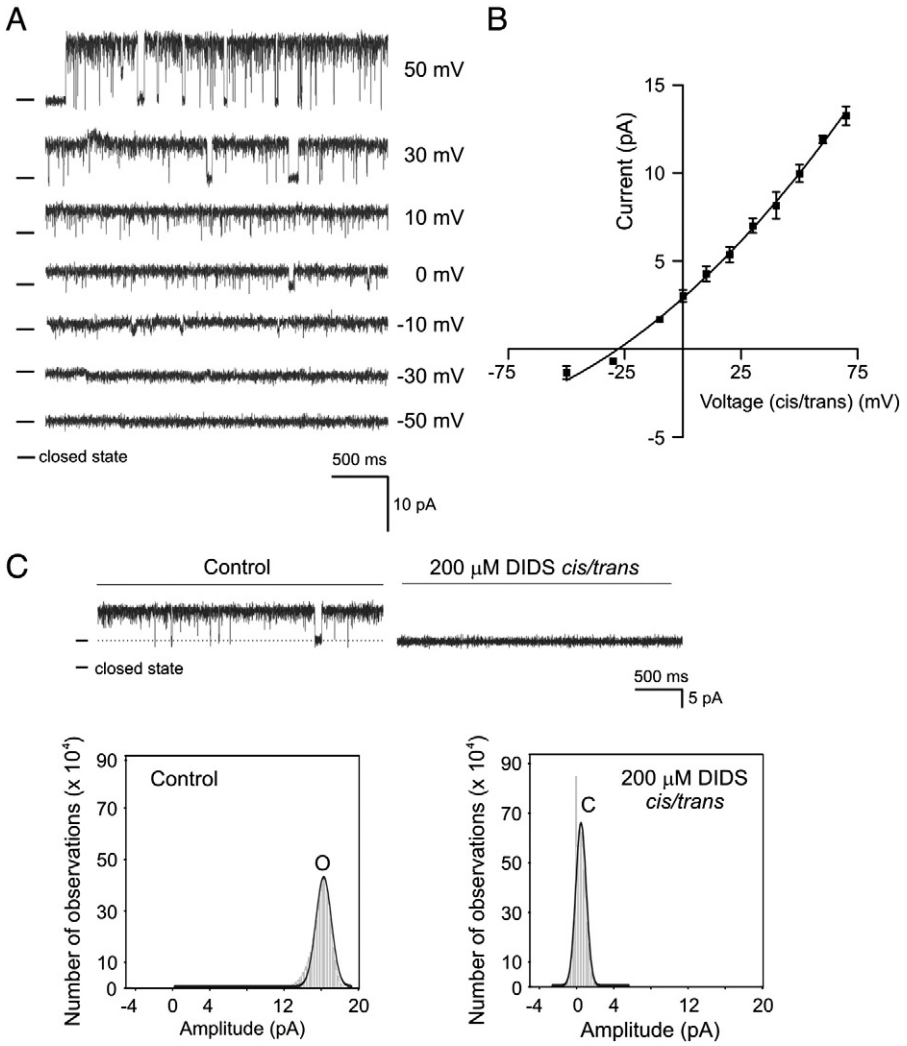

Fig. 4. Single-channel recordings of potato tuber mitoCl channels reconstituted in PLM. *A*, Single-channel current-time recordings in 50/450 mM KCl (*cis/trans*) gradient solutions at different voltages. *B*, Current–voltage (I/V) relationship of single-channel events ($n = 4$, ± SD). *C*, Example of single-channel recordings at 30 mV under control conditions and after addition of 200 μM DIDS to the *cis* and *trans* compartments ($n = 4$). *below*, amplitude histograms from a control experiment and after addition of 200 μM DIDS. Amplitude histograms were obtained from measuring channel openings with a total recording time of 1 min. All points shown were fitted by two Gaussian distributions. The closed state corresponds to the peak at 0 pA. O, open state, C, closed state. "–" indicates current at the closed state of the channel.

(at constant osmolarity). These measurements provide direct evidence against significant uncoupling as a consequence of K^+ channel opening in mitochondria of potato tubers.

3.3. Effect of mitoK$_{ATP}$ channel modulators on respiratory rate and $\Delta\Psi$ in isolated tuber mitochondria. Immunological detection of plant mitoK$_{ATP}$ channel protein

To investigate the effects of mitoK$_{ATP}$ channel modulators on the potassium permeability of isolated potato tuber mitochondria, we measured mitochondrial State 4 (non-phosphorylating) respiration and $\Delta\Psi$ in potassium-containing medium. Table 3 shows the effects of mitoK$_{ATP}$ modulators, i.e. an activator (diazoxide) and three inhibitors (ATP, 5-HD and glibenclamide), on K^+-flux-induced changes in respiratory rate and $\Delta\Psi$ when the modulators are added before or after 50 mM KCl addition. This comparison allows the mitoK$_{ATP}$ channel contribution to K^+-flux induced changes in respiration and $\Delta\Psi$ to be estimated. Under all studied conditions, diazoxide (100 μM) depolarised $\Delta\Psi$ and stimulated respiration, while ATP (1 mM), 5-HD (0.5 mM) and glibenclamide (10 μM) had the opposite effect. These results indicate that similarly to plasma membrane ATP-dependent potassium channels (K_{ATP} channels), the mitoK$_{ATP}$ channel of potato tuber mitochondria is activated by diazoxide and partially blocked by inhibitors in the following descending order: ATP>5-HD>glibenclamide. Moreover, the diazoxide-stimulated K^+ influx was inhibited by the inhibitors, and a partial reversion of mitoK$_{ATP}$ channel inhibition after addition of diazoxide was also observed (data not shown). These results also indicate that ATP has the greatest inhibitory effect on potato tuber mitoK$_{ATP}$ channel activity. We conclude that in potato tuber mitochondria, diazoxide-stimulated ATP-, 5-HD- and glibenclamide-sensitive K^+ flux exists, indicating the presence of the mitoK$_{ATP}$ channel. Under non-phosphorylating conditions, in the presence of 50 mM KCl, the activity of the mitoK$_{ATP}$ channel led to a decrease in $\Delta\Psi$ by as much as 2.3 mV and a simultaneous increase in the mitochondrial respiration rate by as much as approx. 10% (Table 3).

We investigated the effect of diazoxide and ATP on isolated respiring (with exogenous NADH) potato tuber mitochondria in incubation medium containing different monovalent cations (chloride salts) as well as their effect on those incubated in the presence of K^+ (Fig. 5) or in the absence of any monovalent cations. In medium deprived of monovalent cations, no diazoxide-induced ATP-sensitive increase in non-phosphorylating respiration or $\Delta\Psi$ depolarisation was observed (data not shown). Fig. 5 shows the influence of increasing concentrations (up to 50 mM) of KCl, NaCl, LiCl, RbCl, and CsCl on

Table 1
Contributions of different potassium channels to K^+-influx-induced $\Delta\Psi$-depolarisation in isolated potato tuber mitochondria.

	$mitoBK_{Ca}$ + mitoBK + $mitoK_{ATP}$ (i)	$mitoBK_{Ca}$ (ii)	mitoBK (iii)	$mitoK_{ATP}$ (iv)
[KCl] (mM)	K^+-influx-induced $\Delta\Psi$-depolarisation (mV)			
50	9.0 ± 0.9	4.1 ± 0.3	4.3 ± 0.2	2.2 ± 0.3
100	14.3 ± 1.3	6.8 ± 0.6	6.1 ± 0.4	3.6 ± 0.4

Mitochondria were incubated in standard incubation medium in the presence of benzohydroxamate, carboxyatractyloside, oligomycin, NADH and K^+ channel modulators (as described below). Concentrations of K^+ channel activators: 100 μM diazoxide (the $mitoK_{ATP}$ channel), 0.8 mM $CaCl_2$ (the $mitoBK_{Ca}$ channel). Concentrations of K^+ channel inhibitors: 1 mM ATP (the $mitoK_{ATP}$ channel), 1 μM IbTx (the $mitoBK_{Ca}$ and mitoBK channels). KCl (50 or 100 mM) was added during experiments. The changes in $\Delta\Psi$ induced by addition of KCl relative to the initial $\Delta\Psi$ of non-phosphorylating mitochondria (187 ± 2 mV, ±SD, $n = 3$) are shown as (i) the difference between $\Delta\Psi$ measured in the presence of the K^+ channel activators and $\Delta\Psi$ measured in the presence of both the K^+ channel activators and inhibitors; (ii) the difference between $\Delta\Psi$ measured in the presence of the $mitoBK_{Ca}$ channel activator ($CaCl_2$) and $\Delta\Psi$ measured in the presence of both the $mitoBK_{Ca}$ channel activator and inhibitor ($CaCl_2$ and IbTx); (iii) the difference between $\Delta\Psi$ measured in the absence of K^+ channel modulators and $\Delta\Psi$ measured in the presence of the mitoBK channel inhibitor (IbTx); (iv) the difference between $\Delta\Psi$ measured in the presence of the $mitoK_{ATP}$ channel activator (diazoxide) and $\Delta\Psi$ measured in the presence of both the $mitoK_{ATP}$ channel activator and inhibitor (diazoxide and ATP). The data are representative of five different mitochondrial preparations. They are time- and population-averaged values. *n*, number of measurements for the presented experiment. To avoid inaccuracy due to possible changes in osmolarity, ionic strength or TPP binding as result of the addition of KCl, relative changes in the resulting $\Delta\Psi$, not absolute values, are presented.

diazoxide-induced ATP-sensitive $\Delta\Psi$ depolarisation (i.e., the difference between $\Delta\Psi$ in the presence of 100 μM diazoxide and $\Delta\Psi$ in the presence of diazoxide and 1 mM ATP). The results indicate that the influence of potassium channel modulators (diazoxide and ATP) on isolated potato tuber mitochondria in these experiments can be significantly attributed to K^+ influx through the inner membrane.

Immunoblotting of total mitochondrial proteins, as well as of SMP, allowed immunological detection of the potato tuber $mitoK_{ATP}$ channel. For this, antibodies raised against the mammalian plasma membrane K_{ATP} channel pore (Kir6.1 and Kir6.2 subunits) were used. In potato tuber mitochondrial and SMP fractions, a protein band with a molecular mass of approx. 51 kDa was detected using both types of antibodies (Fig. 6A, B). Much stronger signals were obtained with SMP than with isolated mitochondria, proving that the detected proteins localised in the inner membrane of potato tuber mitochondria. Moreover, specific blocking peptides blocked the antibody–antigen interaction, demonstrating the specificity of the reaction in the western blot analysis. On the other hand, mitochondrial (isolated mitochondria and SMP) and plasma membrane fractions probed with antibodies to a plant plasma membrane marker (H^+-ATPase) displayed no signal in the mitochondrial fractions, indicating the absence of surface membrane contamination (Fig. 6C). Therefore, we can conclude that the potato tuber $mitoK_{ATP}$ channel may contain a pore-forming subunit of the Kir-like family.

Table 2
Effect of activity of potassium channels on respiratory rates, $\Delta\Psi$ and coupling parameters in potato tuber mitochondria.

	State 4	State 3	RCR	ADP/O	$\Delta\Psi 4$	$\Delta\Psi 3$
–KCl	73 ± 3	214 ± 10	2.9 ± 0.3	1.25 ± 0.12	186 ± 2	143 ± 4
100 mM KCl	90 ± 5*	223 ± 19	2.5 ± 0.2	1.20 ± 0.11	173 ± 2*	140 ± 4

Mitochondria were incubated in standard incubation medium in the presence of benzohydroxamate, NADH (+ rotenone) and K^+ channel activators: 100 μM diazoxide (the $mitoK_{ATP}$ channel) and 0.8 mM $CaCl_2$ (the $mitoBK_{Ca}$ channel). Where indicated 100 mM KCl was added to the incubation medium. Osmolarity was kept constant by adjusting the mannitol concentration in the medium. Comparisons were made between results of separate experiments. RCR, respiratory control ratio; $\Delta\Psi 3$, $\Delta\Psi 4$, membrane potentials under phosphorylating and non-phosphorylating conditions (respectively) presented in mV. Respiratory rates in State 3 and State 4 are given in nmol $O_2 \times min^{-1} \times mg^{-1}$ protein. Values represent the mean (±SD) for three measurements. The data are representative of three different mitochondrial preparations. They are time- and population-averaged values. *, where indicated *p* values in the presence of 100 mM KCl are significantly different from control values ($p < 0.01$, Student's unpaired *t* test).

Table 3
Effect of $mitoK_{ATP}$ modulators on respiratory rate and $\Delta\Psi$.

Effects	No modulators	Diazoxide	ATP	5-HD	Glibenclamide
	Effect of 50 mM KCl added after modulators				
State 4 respiration	×1.15	×1.2	×1.09	×1.09	×1.12
$\Delta\Psi 4$	−6.1 ± 1.1	−8.6 ± 1.1	−5.3 ± 0.4	−5.6 ± 0.3	−6.0 ± 0.7
	Effect of modulators in the presence of 50 mM KCl				
State 4 respiration	–	×1.09	×0.92	×0.96	×0.97
$\Delta\Psi 4$	–	−2.3 ± 0.2	+1.9 ± 0.2	+1.2 ± 0.1	+0.9 ± 0.1

Mitochondria were incubated in standard incubation medium in the presence of benzohydroxamate, carboxyatractylozide, oligomycin and NADH (+ rotenone) in the absence or presence of 50 mM KCl. When 50 mM KCl was used, osmolarity was kept constant by adjusting the mannitol concentration. Concentrations: 100 μM diazoxide, 500 μM 5-HD, 1 mM ATP, 10 μM glibenclamide. The factor by which the State 4 respiratory rates were changed is shown. Values (in mV) represent the mean (±SD) for four different measurements. The data are representative of three different mitochondrial preparations. They are time- and population-averaged values. To avoid inaccuracy due to possible changes in osmolarity, ionic strength or TPP binding as result of addition of KCl during experiments (upper part), all data are shown as relative changes in the resulting $\Delta\Psi$. Comparisons were made between results of separate experiments (the lower part) or between values measured before/after additions in the same experiment (the upper part).

4. Discussion

The observations described above indicate that the inner membrane of potato tuber mitochondria exhibit activities of various

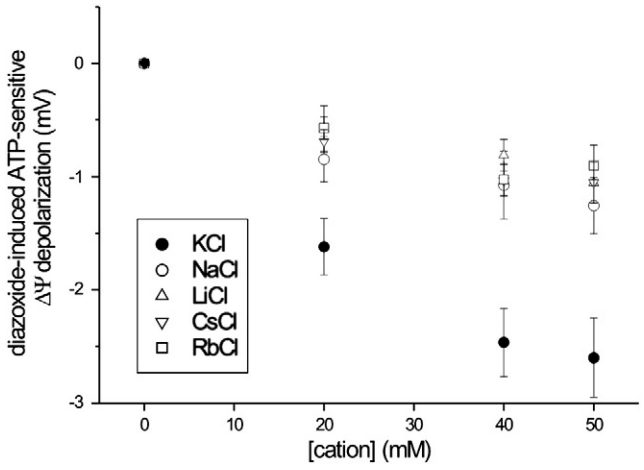

Fig. 5. Cation selectivity; influence of cations on diazoxide-induced ATP-sensitive non-phosphorylating $\Delta\Psi$. Mitochondria were incubated in standard incubation medium, except that 3 mM NaH_2PO_4 was replaced with 3 mM $TrisH_2PO_4$. Additionally, 1 μM IbTx (an inhibitor of the $mitoBK_{Ca}$ channel) was present. Increasing concentrations of KCl, NaCl, LiCl, CsCl, or RbCl (20–50 mM) were obtained by successive additions once a steady-state $\Delta\Psi$ had been established. The dilution effect of salt addition was taken into account. The change in $\Delta\Psi$ induced by a given chloride salt relative to the initial state 4 $\Delta\Psi$ (188 ± 2 mV, S.D.) is shown as the difference between $\Delta\Psi$ measured in the presence of 100 μM diazoxide and $\Delta\Psi$ measured in the presence of diazoxide and 1 mM ATP. To avoid inaccuracy due to possible changes in osmolarity, ionic strength or TPP binding as result of addition of KCl during experiments, data are shown as relative changes in the resulting $\Delta\Psi$. Values represent the mean (±SD) for three measurements. The data are representative of three different mitochondrial preparations. They are time- and population-averaged values.

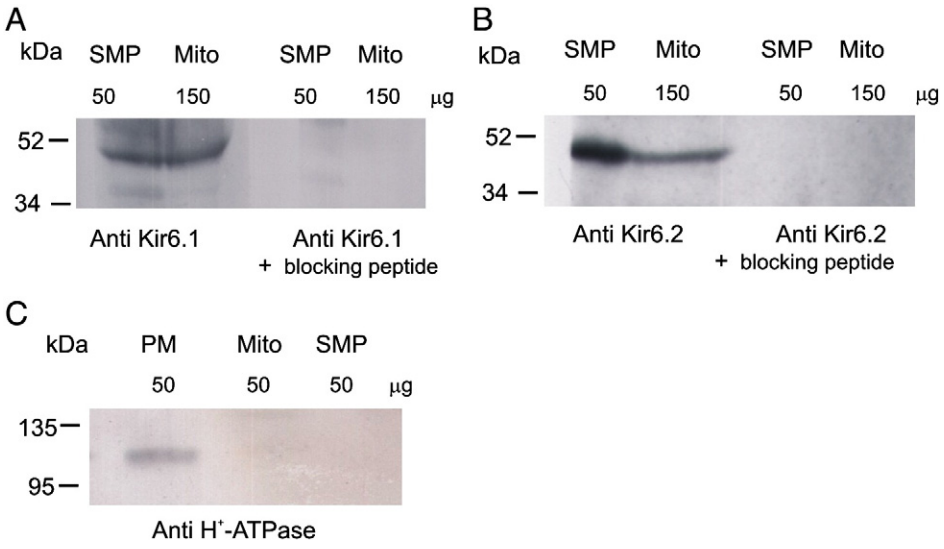

Fig. 6. Western blotting with anti-Kir6.1 (A) and anti-Kir6.2 (B) antibodies raised against the mammalian plasma-membrane K_{ATP} channel in the absence or presence of a specific blocking peptide. *C*, Detection of H^+-ATPase. Mito, mitochondria; SMP, submitochondrial particles; PM, plasma membrane-enriched fraction. Different amounts of protein were loaded into each lane as indicated. Examples of 3–7 immunoblots using samples from different preparations are shown.

monovalent ion channels: the mitoBK Ca^{2+}-insensitive channel, the mitoK$_{ATP}$ channel and the mitoCl channel. This is the first electrophysiological description of these channels in plant mitochondria. Additionally, the electrophysiological properties of the mitoBK$_{Ca}$ channel and its influence on the bioenergetics of potato tuber mitochondria were recently described [13].

Our functional studies revealed that the total activity of the three K^+ channels detected in potato tuber mitochondria, i.e., the mitoBK$_{Ca}$ channel, the mitoBK Ca-insensitive channel and the mitoK$_{ATP}$ channel, caused approx. 9 and 14 mV depolarisation of $\Delta\Psi$ under non-phosphorylating conditions at high KCl concentrations (50 and 100 mM, respectively) (Table 1). The activities of the two large-conductance K^+ channels, the mitoBK$_{Ca}$ channel and the mitoBK Ca^{2+}-insensitive channel, caused a depolarisation of $\Delta\Psi$ by a few mV at most. The weakest influence on $\Delta\Psi$ was observed for the mitoK$_{ATP}$ channel activity alone. A dissipative mechanism involving K^+ import into the mitochondrial matrix through potassium channels and a K^+/H^+ exchanger that modulates the electrochemical proton gradient ($\Delta\mu H^+$) by decreasing $\Delta\Psi$ can be thought of as an energy-dissipating system. Thus, the re-entry of protons previously ejected by the mitochondrial complexes of the respiratory chain (generating $\Delta\mu H^+$) through the potassium cycle potentially uncouples mitochondria. In this manner, potassium channels could regulate the coupling between respiration and ATP synthesis in mitochondria. However, no K^+ import-induced decrease in oxidative phosphorylation yield in potato tuber mitochondria was observed. The ADP/O ratio, which is diagnostic of ATP synthesis yield, was unaffected by the absence or presence of 100 mM KCl (at constant osmolarity) when all three potato tuber mitochondrial K^+ channels were active (Table 2). Therefore, it seems that plant K^+ channels function as an energy-dissipating system as they can decrease mitochondrial $\Delta\Psi$ but are not able to divert energy from oxidative phosphorylation. To date to our knowledge, there are no data showing a K^+ import-induced decrease in oxidative phosphorylation yield in the mitochondria of mammals or unicellular eukaryotes [3,18].

4.1. The mitoK$_{ATP}$ channel

The measurements presented in this study of the electrophysiological properties of a reconstituted single mitoK$_{ATP}$ channel in potato tuber inner mitochondrial membrane confirm the existence of this channel in plant mitochondria. Previously, the mitoK$_{ATP}$ channel was electrophysiologically characterised in mammalian and protozoan mitochondria [2,3]. The potato tuber mitoK$_{ATP}$ channel has a conductance of approx. 164 pS and a reversal potential of 27.5 mV. These electrophysiological properties as well as the kinetic open and closed states are comparable with those observed for the *A. castellanii* mitoK$_{ATP}$ channel, which is characterised by conductance that ranges from 90 to 166 pS (under conditions of 50/450 mM KCl gradient solutions) [7]. However, lower conductances of the mitoK$_{ATP}$ channel in mammals were observed in patch-clamp and PLM experiments when different gradient solutions were applied. At negative potentials in 33.3/100 mM K^+ gradient solutions (bathing/pipette solutions), the slope of the conductance currents was 9–10 pS [19]. In other patch clamp experiments in symmetrical 150 mM K^+ solutions, the human mitoK$_{ATP}$ channel with a conductance of 15 pS at negative potentials and 82 pS at positive potentials was detected [20]. Similarly, using the PLM technique with symmetrical solutions, conductances of 10 pS (in 100 mM KCl), 56 pS (150 mM KCl) and 30 pS (in 1 M KCl) were measured for mammalian mitoK$_{ATP}$ channels [21–23]. Conductance of 103 pS was observed with 50/150 mM KCl gradient [24]. Thus, in the PLM technique, experimental conditions, particularly ionic strength of buffers influence the measured channel conductance. Therefore, it cannot be excluded that under lower gradient conditions, the plant mitoK$_{ATP}$ channel could have a conductance similar to that of mammalian mitoK$_{ATP}$ channels. Similarly to mammalian and protozoan mitoK$_{ATP}$ channels [2,7], the activity of reconstituted potato tuber mitoK$_{ATP}$ channel was inhibited by ATP (Fig. 2). For the potato tuber mitoK$_{ATP}$ channel, the discrimination between K^+ and Cl^- estimated from the calculated permeability ratio (P_K/P_{Cl} of 4.2) (sect. 3.1.1) is rather weak. However, selectivity for K^+ vs. other cations is high. The mitoK$_{ATP}$ channel-mediated $\Delta\Psi$ depolarisation of isolated mitochondria was about 2.5 times higher for K^+ than for other monovalent cations (Fig. 5).

ATP-sensitive potassium import has been previously characterised in mitochondria isolated from durum wheat and pea [8–10,12]. In these mitochondria, the sensitivity of mitochondrial swelling and $\Delta\Psi$ to potassium channel modulators (diazoxide, 5-HD, glybenclamide) is similar to that previously described for the mammalian mitoK$_{ATP}$ channel. This study provided the first quantitative assessment of the plant mitoK$_{ATP}$ channel's contribution to K^+-flux induced

changes in mitochondrial respiration and $\Delta\Psi$. The respiratory rate and $\Delta\Psi$ measurements of isolated potato tuber mitochondria presented in this study indicate the existence of diazoxide-stimulated ATP-, 5-HD- and glibenclamide-inhibited K^+ flux through the mitoK$_{ATP}$ channel (Table 3). Under non-phosphorylating conditions and in the presence of 50 mM KCl, mitoK$_{ATP}$ channel activity led to a decrease in $\Delta\Psi$ by as much as 2.3 mV and to a slight increase in the mitochondrial respiration rate. In contrast to these results and also to the low K^+ flux through the mammalian mitoK$_{ATP}$ channel (for which the effect on $\Delta\Psi$ is often undetectable [18]), and through the amoeba *A. castellanii* mitoK$_{ATP}$ channel (for which the effect on $\Delta\Psi$ is slight, up to 3 mV [7]), previous swelling and $\Delta\Psi$ studies with isolated plant mitochondria (durum wheat seed, rye seed, barley seed, potato tuber and spinach leaf mitochondria) have shown that K^+ present at a high concentration (25 mM KCl) can be imported at high rates, thereby strongly reducing or even collapsing $\Delta\Psi$ [8]. However, such a strong effect on $\Delta\Psi$ (which has been measured with safranin as the fluorimetric probe) seems to be unlikely, as the same mitochondria (in the same study) were quite well coupled (revealing a high respiratory control ratio and high ADP/O values) in the presence of 10 mM KCl [8]. Moreover, many bioenergetic studies have been performed with various isolated plant mitochondria in incubation medium containing a high concentration of KCl (up to 65 mM) in which the mitochondria were well coupled [13,25–27]. This excludes a significant decrease (below the $\Delta\Psi$ value of phosphorylating mitochondria) or a collapse of $\Delta\Psi$ as a result of K^+ entry into plant mitochondria. Moreover, studies on the plant mitoK$_{ATP}$ channel based on swelling and $\Delta\Psi$ depolarisation measurements have mostly been performed with succinate as the respiratory substrate driving K^+ entry into mitochondria. No precautions regarding succinate dehydrogenase stimulation by ATP (used as a mitoK$_{ATP}$ inhibitor) were taken, which may have led to inaccuracy and overestimation of the channel's function. Changes in $\Delta\Psi$ can be attributed to ATP-inhibited K^+ entry into the matrix through the mitoK$_{ATP}$ channel only if they are independent of the respiratory substrate used and are not observed in K^+ (monovalent ion)-free media. Moreover, in the present study, we excluded inhibition of the K^+-induced mitochondrial $\Delta\Psi$ decrease (i.e., mitoK$_{ATP}$ channel activity) by exogenous NADH as claimed previously for the plant mitoK$_{ATP}$ channel [8,10]. Mitochondrial energisation by external NADH dehydrogenase present in plant mitochondria was not taken into account in these studies. Therefore, we can conclude that similarly to mammalian and protozoan mitoK$_{ATP}$ channels [2,3,14], plant mitoK$_{ATP}$ channel mediate low K^+ flux. Although they increase the inner membrane's permeability to K^+, the transport rate of the ion is so low that the overall effect on the electrical membrane potential ($\Delta\Psi$) is quite modest (2–3 mV). The low flux of K^+ across the membrane of mitochondria presumably results from a low abundance and tight regulation of the channels' activity.

In addition to the functional studies, the presence of mitoK$_{ATP}$ channel proteins in potato tuber mitochondria is indicated by their cross-reactivity with antibodies raised against human pore-forming subunits of the Kir6.0-family (Kir6.1 and Kir6.2) (Fig. 6). This suggests that the mitoK$_{ATP}$ channel present in potato tuber inner mitochondrial membrane may be structurally similar to the mammalian K_{ATP} channel. The existence of a sulfonylurea receptor with regulatory activity other than the principle pore-forming subunit of the Kir-family needs to be elucidated. The relative molecular mass of the detectable plant Kir-like subunit protein (approx. 51 kDa) is similar to that of the protein detected in mammalian mitochondria (55 kDa) [28]. The molecular identity (gene and protein sequence) of the mammalian mitoK$_{ATP}$ channel is presently unknown. Concerning plant Kir (K^+ inward rectifier) channels, the first Kir-like channel (KCO3) was identified in *Arabidopsis* by searching for K^+ channel related sequences in the genome sequence databases [29,30]. Molecularly, plant Kir-like channels are related to animal Kir channels. Previous studies of subcellular localisation and trafficking with GFP constructs indicated that plant Kir-like channels may be active at the tonoplast rather than the plasmalemma [31]. Our study indicates, for the first time, the mitochondrial (inner membrane) localisation of plant Kir-like channel and presents its electrophysiological characterisation.

4.2. The mitoBK calcium-insensitive potassium channel

The electrophysiological properties of the mitoBK calcium-insensitive potassium channel detected in the potato tuber mitochondrial inner membrane, i.e., its channel conductance (approx. 312 pS), voltage dependence and sensitivity to IbTx (Fig. 3), are similar to the properties of mitoBK$_{Ca}$ channels from mammalian mitochondria [32–34]. However, the insensitivity to Ca^{2+} of the investigated plant channel distinguishes it from mammalian mitoBK$_{Ca}$ channels. On the other hand, the potato tuber mitoBK$_{Ca}$ channel that was recently described [13] displayed channel conductance about two-fold higher (approx. 502 pS for potential from −30 mV to 20 mV and approx. 615 pS for potential from +40 mV to 90 mV) than the conductance measured in this study for the potato tuber mitoBK calcium-insensitive potassium channel (approx. 312 pS). Under the same experimental conditions (50/450 mM KCl gradient solutions in the PLM system), both the Ca^{2+}-insensitive and Ca^{2+}-sensitive large-conductance potassium channels of potato tuber mitochondria displayed similar reversal potentials (+31 mV and +34 mV, respectively) (Fig. 3 and [13]). Possibility that both channels might be Ca^{2+}-sensitive, one responding to much lower (contaminating) Ca^{2+} concentrations than the other, is unlikely. During mitochondria preparation, 3.0–1.5 mM EGTA and 3 mM EDTA were present in the isolation media [13]. Moreover, addition of 0.1 mM EDTA during a PLM recording of the mitoBK Ca^{2+}-insensitive channel did not change its electrophysiological properties and subsequent addition of 0.4 mM Ca^{2+} did not lead to sensitivity to the cation (data not shown). The discrepancies in the response of these channels to calcium may be due to inherent differences in protein structure. It has been reported that differential expression of the regulatory β1 subunit can modify the calcium-sensitivity of mammalian plasma-membrane BK$_{Ca}$ channels [35]. In mammalian cells, the β1 subunit of the BK$_{Ca}$ channels appears to be a key controller of single-channel activity [36]. Considering the possibility that regulatory subunits might sometimes be lost during the purification/incorporation steps, it is difficult to unequivocally exclude this possibility. However, in the case of purification, it is rather impossible as for the same SMP fraction (in all studied SMP preparations), activities of both channels, the mitoBK Ca-sensitive channel and the mitoBK Ca-insensitive channel, were detected after reconstitution.

The conductance of the mitoBK channel (approx. 312 pS) described in this study is about twice that of the mitoK$_{ATP}$ channel (approx. 164 pS) and both displayed similar reversal potentials. However, the activity of the mitoBK Ca^{2+}-insensitive channel was insensitive to 1 mM ATP (data not shown). This excludes the possibility that the measured conductance represents a doublet of cooperating mitoK$_{ATP}$ channels.

As in the case of all described BK$_{Ca}$ channels and mitoBK$_{Ca}$ channels (including the potato tuber mitoBK$_{Ca}$ channel, [13]), the activity of the reconstituted mitoBK Ca^{2+}-insensitive channel of potato tuber mitochondria described in this study was blocked by IbTx with sub-micromolar affinity. IbTx has been reported to bind to the external mouth of BK$_{Ca}$ channels through a bimolecular reaction that physically blocks the conduction pathway [37]. Thus, our results suggest that the different large-conductance potassium channel types share some common structural features at the toxin-binding site.

4.4. The mitoCl channel

In almost 14% of successful reconstitutions in the PLM system, the mitoCl channel with conductance of approx. 120 pS and voltage-dependent activity was observed under asymmetrical (50/450 mM KCl) conditions. The potato tuber mitoCl channel displayed electrophysiological properties similar to certain single-channel behaviours reported for mitochondrial inner membrane anion channels of mammals. It displayed channel conductance and voltage-dependent activity (almost no channel openings at negative potentials) similar to a 108 pS mitochondrial anion channel detected in certain mammalian cells [17,38–40]. However, in contrast to the potato tuber mitoCl channel, the mammalian 108 pS anion channel is not inhibited by DIDS [17]. This study is the first electrophysiological study (to the best of our knowledge) that confirms the presence of anion channels in the inner membrane of plant mitochondria. To date, anion uniport pathway named PIMAC (plant inner membrane anion channel) have been characterised in plant mitochondria only by swelling assays with isolated mitochondria [41,42].

5. Conclusions

Reconstitution of potato tuber mitochondrial inner membrane into PLMs revealed four different ion conductance pathways: (i) a mitoK_{ATP} channel activity with conductance of approx. 164 pS, (ii) a mitoBK Ca^{2+}-insensitive iberiotoxin-sensitive channel activity with conductance of approx. 312 pS, (iii) a mitoCl DIDS-inhibited channel activity with conductance of approx. 117 pS, and (iv) a previously described mitoBK_{Ca} channel activity with conductance of 502–615 pS [13]. These results indicate the existence of numerous ion pathways in the plant inner mitochondrial membrane that require further elucidation. The physiological role of these ion pathways in plant cells especially awaits exploration. K^+ entry into the mitochondrial matrix of potato tuber mitochondria through K^+ channels, i.e., the mitoK_{ATP} channel, the mitoBK Ca^{2+}-insensitive channel and the mitoBK_{Ca} channel, caused significant (up to 14 mV) but not collapsing $\Delta\Psi$ depolarisation. However, plant mitochondrial K^+ channels function as energy-dissipating systems that are unlikely to be able to divert energy from oxidative phosphorylation. Similarly to the mammalian and protozoan mitoK_{ATP} channels, the mitoK_{ATP} channel of potato tuber mitochondria mediates low flux of K^+ and may contain the pore-forming subunit of the Kir-like family.

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