ATP-Sensitive Cation-channel in Wheat (Triticum durum Desf.): Identification and Characterization of a Plant Mitochondrial Channel by Patch-clamp

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Abstract
Indirect evidence points to the presence of K+ channels in plant mitochondria. In the present study, we report the results of the first patch clamp experiments on plant mitochondria. Single-channel recordings in 150 mM potassium gluconate have allowed the biophysical characterization of a channel with a conductance of 150 pS in the inner mitochondrial membrane of mitoplasts obtained from wheat (Triticum durum Desf.). The channel displayed sharp voltage sensitivity, permeability to potassium and cation selectivity. ATP in the mM concentration range completely abolished the activity. We discuss the possible molecular identity of the channel and its possible role in the defence mechanisms against oxidative stress in plants.

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Introduction
Potassium is the most abundant cation in plant cells, where it plays major roles in biochemical and biophysical processes [1]. A large number of K+ channels and transporters have now been identified at the molecular level in this kingdom or are currently under investigation, demonstrating the complex nature of K+ transport in plants. K+ transport in plants is not restricted to the plasmamembrane, but like in animal systems, it takes place also in intracellular compartments and in particular in the bioenergetic organelles mitochondria [2-4] and chloroplasts [5].

In animal mitochondria five different K+-selective channels have been identified and characterized, namely an ATP-sensitive potassium channel [6], two calcium-activated ones [7, 8], a voltage-gated channel [9] and a two-pore potassium channel [10]. These channels mediate the influx of potassium into the matrix according to the electrochemical gradient for this ion. The suggested physiological roles of these channels include regulation of mitochondrial matrix volume, respiration and membrane potential [11]. In addition, mitochondrial potassium channels seem to play an important role in protection against ischemic damage [12] and in regulation of apoptosis [13].

While much attention has been devoted to mitochondrial K+ channels in animal systems, very few publications deal with plant mitochondrial K+ channels.
We have previously described, using classical bioenergetics techniques, an ATP-inhibited mitochondrial K+ uptake pathway in wheat and other species [2] and suggested that it could be involved in plant defence mechanisms against oxidative stress due to reactive oxygen species generation [14]. A similar, ATP-sensitive K+ uptake system, which was however induced by oxygen species generation [14].

A fourth mitochondrial plant K+ channel is the recently described calcium-activated mitoBKCa from potato tuber mitochondria [15]. Activity of this channel was detected in a reconstituted system, using planar lipid bilayer techniques.

To directly identify plant K+ channels in the native mitochondrial membrane, we took advantage of the patch clamp technique, applied for the first time to isolated plant mitochondria. We report here the identification and characterization by single channel recording of an ATP-sensitive cation channel in mitochondria isolated from wheat (Triticum durum Desf.).

**Materials and Methods**

*Triticum durum* Desf. seeds were germinated in the dark at 95% humidity and 25 °C for two days and only the etiolated seedlings (200 g) were used for further processes. Wheat mitochondria were then isolated by differential centrifugation, either by slight modification of a previously described procedure [2] or by the method described in Virolainen et al. [16]. Total extract was obtained by homogenizing the seedlings. The isolated mitochondria were assayed for membrane potential by using safranine or TMRE (not shown). Mitochondria thus obtained were washed with the experimental medium and sealed were established under symmetrical ionic conditions. Seal configuration was mitochondrion-attached. Voltage was controlled manually via an Axopatch 200 unit. All data were filtered at 10 kHz and recorded on tape using a VR-10B (Instrutech) adaptor, and recovered later for off-line analysis. Axon pClamp 6.0 software was used for voltage control and data analysis. The voltages reported in this paper are those applied to the patch-clamp pipette interior. Current (cations) flowing from the pipette to the ground electrode was considered as positive and plotted upwards. For determination of the selectivity, the experimental medium was modified by employing symmetrical 150 mM KCl in place of K-glucanate. After initially recording with symmetrical salt conditions, bath [KCl] was increased by withdrawing an aliquot of the medium and adding back the same volume of a solution having identical composition except for a higher (2 M) [KCl]. For Na+ versus K+ selectivity, the standard experimental medium was replaced with an identical one containing Na-glucanate instead of K-glucanate. In all cases connection to the Ag/AgCl ground electrode was via a 1M KCl agar bridge. In pharmacological experiments small volumes of concentrated drug solution were added, and the bath contents were thoroughly mixed.

**Results**

*Characterization of plant mitochondria preparation*

Patch-clamp of plant mitochondria turned out to be technically much more difficult to perform than patch-clamp of mammalian mitochondria, possibly due to differences in lipid composition and to the small size of the swollen mitoplasts [16]. We tested mitochondria isolated from five different plant systems (carrot, Arabidopsis, cauliflower, rice and wheat) but the percentage of high-resistance seals between the patch-clamp pipette and mitochondrial inner membrane was extremely low in all cases. We modified pH, ionic strength, concentration of calcium and swelling protocol (osmotic...
shock or Ca\textsuperscript{2+}/Pi, see Materials and Methods) to increase the percentage of successful seals, but to no avail. Despite the low percentage of successful trials (< 5% vs. ~ 50% in mammalian mitochondria) we were able to obtain single channel recordings. Wheat (Triticum durum Desf.) mitoplasts proved to be the best system.

To characterize the mitochondria from a biochemical point of view and for possible contaminations, we performed biochemical assays. Western blotting of wheat mitochondrial fractions confirmed the enrichment of typical mitochondrial marker cytochrome c and of the organellar ATP-ase β-subunit in purified mitochondria versus total extract, at equal protein quantities loaded on the SDS-PAGE. A very slight contamination by the plasmamembrane (PM) marker H\textsuperscript+-ATP-ase and the ER marker BiP can be observed in the isolated mitochondria (Fig. 1A). The PM contamination was completely abolished and the ER contamination greatly reduced when the mitochondria obtained by differential centrifugation were further purified on Percoll gradient (Fig. 1A). However, these Percoll-purified mitochondria did not form high-resistance seals in patch clamp experiments and this step greatly reduced the yield of the purification. We also verified whether the isolated mitochondria (not Percoll-purified) contained plastid contaminations: our preparation was free of Rubisco and of P45, marker proteins of the plastid stroma and inner membrane, respectively (Fig. 1B).

These biochemical data indicated thus that the mitochondria used in our previous studies [2] and in the patch clamp experiments contain minor contaminations by ER and plasmamembrane but not by chloroplasts/plastids. A slight contamination by these membrane fractions is known to occur also in the case of mammalian mitochondria (due also to the presence of the so-called MAMs (mitochondria-associated membranes)), successfully used for patch clamp experiments.

For patch clamping, wheat germ mitoplasts (swollen mitochondria without outer membrane) were obtained either by inducing swelling in KCl medium or by presumably inducing the permeability transition as described in the experimental section. The resulting mitoplasts were morphologically undistinguishable from those of mammals under the microscope (see [7, 8, 19]), except for the fact that they were smaller, with an average diameter of 1-2 μm, in accordance with the observations of Virolainen et al [16]. Mitoplasts were easily recognizable due to their size, round shape and the presence of a “cap region”. (Fig.1C).

**Electrophysiological characterization of the wheat germ mitochondrial ATP-sensitive cation channel activity**

To focus on potassium channels, patch-clamp recordings were obtained in K-gluconate medium.

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Fig. 1. Biochemical characterization of isolated mitochondria. A) Upper (top) three-lane panels: Western blots on isolated rat liver mitochondria, total wheat germ extract (ET) and wheat germ mitochondria (Mito) isolated according to the protocol described in [2]. 50 μg/lane of total proteins were loaded. Anti-ATP-ase of organelles recognizes an approx. 50 kDa band in rat mitochondria, and a 55 kDa band with the predicted MW in wheat germ. Anti-cytochrome c antibody reacts with 12 kDa and 14 kDa cytochrome c in rat and wheat, respectively. Please note the increase in the signal for both proteins in the mitochondrial fraction with respect to the total extract in wheat, indicating that our preparation was enriched in mitochondria. Lower left panels: Isolated mitochondria (Mito) are slightly contaminated by PM and ER as revealed by the presence of PM-H\textsuperscript{+}-ATP-ase and BiP. Isolated PM fraction and ET were loaded as controls. Lower right panels: The same antibodies were used for Percoll-purified mitochondria (MitoP). 50 and 75 μg/lane of total proteins were loaded for left and right panels, respectively. B) Isolated chloroplasts (Chl), mitochondria (Mito) and ET were loaded and developed with anti-P45, an inner chloroplast membrane marker and anti-Rubisco, a stromal enzyme responsible for carbon fixation. 50 μg/lane of total proteins were loaded. C) Phase contrast image of a typical wheat mitoplast with a schematic representation of the cell-attached configuration.
Gluconate is a large anion which does not permeate through most chloride channels. In a part of the experiments the mitoplast membrane patches did not exhibit channel activity, indicating a relatively low density of potassium channels in this system. However, a K+-permeable channel with characteristic behavior could be clearly identified in 21 seals out of 69 seals established on wheat mitoplasts. Fig. 2A shows a typical recording of this activity. Only one or at most a few channels were active in any given membrane patch. The characteristics and kinetic behavior of the channel seemed to be independent of the calcium concentration used in the experimental medium (not shown). Fig. 2B shows a plot of the current flowing through the wheat mitoplast K+-permeable channel versus voltage. The current reported was determined by averaging individual measurements obtained at the various voltages. The channel displayed a conductance of 150-155 pS in the negative pipette voltage range, a strong voltage sensitivity and an open probability (Po) value approaching 0 at positive pipette potentials (i.e. negative, physiological matrix potential) (Fig. 2C), suggesting that the channel tends to be closed under physiological conditions.

In symmetrical 150 mM KCl this channel showed basically identical biophysical properties to those observed in K-gluconate (the same kinetics, ohmic behavior, V-dependence) (not shown). To determine its selectivity we first measured the reversal potential (E\text{rev}) in a KCl gradient (Fig. 3A). Given the conventions and experimental conditions, negative currents at 0 mV applied voltage correspond to a net flow of K+ ions into the pipette, i.e., to cation-selectivity of the channel, as expected. In the presence of a 4.07 fold [KCl] gradient (bath versus pipette) the reversal potential was about +23mV (see Fig. 3A), corresponding to a ratio of permeability coefficients P_K/P_{Cl}=5.5. Under these ionic conditions a reversal potential of +36 mV is predicted for a perfectly potassium selective channel. To check for selectivity between K+ and sodium, we measured the conductance of the channel in standard experimental medium (containing 150 mM K-glucolate) and then after exchanging the bath medium with one containing Na-glucolate instead of K-glucolate
The conductance did not change significantly, indicating a low selectivity for potassium toward sodium.

To check whether the channel described above might correspond to the ATP-sensitive potassium uptake system we have previously identified [2], we tested the effect of ATP. 1 mM ATP caused a significant reduction of the open probability of the channel (Fig. 4B) as observable also from the current traces before and after the addition of ATP (Fig. 4A). The activity was nearly completely abolished at higher concentrations of ATP (Fig. 4B).

**Discussion**

In the present paper, we describe for the first time the application of the patch clamp technique to mitochondria isolated from plants and present the characterization of the channel activity we observed most often (in 30% of the seals, indicating a relatively low density of the channel). The channel displays a conductance of 150 pS in 150 mM potassium, a strong voltage dependence and rectification, a relatively low selectivity for potassium over chloride and basically no selectivity for $K^{+}$ vs. $Na^{+}$. Importantly, channel activity is completely blocked by mM concentrations of ATP. This latter characteristic suggests that the channel activity we recorded by patch clamp might correspond to the potassium uptake system previously observed in the same system, i.e. in wheat germ mitochondria by classical bioenergetic approaches [2]. In that paper we reported that in addition to a potassium/proton antiporter, energized mitochondria accumulate potassium in an ATP and NADH-sensitive manner. Furthermore, we studied the effect of various classical potassium channel modulators and inhibitors of mammalian $K_{ATP}$ and observed that magnesium (0.1 and 5 mM), TEA$^-$ (10 mM), calcium (1-3 μM), barium (1 mM) and aminopyridine (5 mM) did not have an inhibitory effect on the potassium uptake. In the absence of the determination of the channel’s molecular identity, it is unclear whether the lack of effect of these inhibitors on the $K_{ATP}$ activity have structural reasons. Unfortunately, the limited success rate of the patch clamp experiments did not allow us to perform a detailed pharmacological characterization of the observed activity preventing us to definitively identify the channel observed by patch-clamp with the activity we previously studied [2]. The technical difficulty to perform patch clamp experiments on plant mitoplasts is mainly due to their small size, but possibly also to the lipid composition of the inner membrane. In any case, the sensitivity to ATP points to the identification of the $K_{ATP}$ channel by patch clamp.

The group of Vianello and colleagues have also reported the existence of a Cyclosporine A-induced, ATP-inhibited potassium channel in de-energized as well as in energized pea mitochondria [3]. They concluded that the channel is voltage-dependent, with a tendency to close at increasing $\Delta \Psi_m$ and that its inhibition by ATP is partially reversible by addition of GTP and diazoxide, openers of the $K_{ATP}$ of mammalian mitochondria [20]. The channel we observe is clearly voltage-dependent and is open at positive matrix potential. However, in contrast to our findings described in this paper, both papers reported a selectivity for potassium over sodium in swelling and membrane potential measurement experiments. On the other hand, while swelling did not occur in sucrose medium, in sodium chloride swelling could be observed, although to a lower extent than in potassium chloride, suggesting that the plant $K_{ATP}$ is not perfectly selective for potassium [20].
In addition to the mitoK\textsubscript{ATP}, an ATP- and NADH-insensitive, highly active potassium uptake pathway has been identified in plant mitochondria [4]. Besides the ATP-sensitive channel, in patch-clamp experiments in a few cases we have observed channels with higher conductances, one resembling the mitochondrial megachannel [21] (not shown). It might \textit{a priori} be possible that the same channel protein gives rise to ATP-dependent and ATP-insensitive activities, depending on the association or lack of regulatory subunits, which might dissociate depending on the isolation protocol used. Alternatively, the channel described by Ruy and colleagues [4] might correspond to the large-conductance calcium-activated channel that was recently observed by the group of Szewczyk [15]. Further work is required to test these hypotheses.

The question arises concerning the possible molecular identity of the channel we observed. The mammalian K\textsubscript{ATP} channel of the plasmamembrane is composed of the Kir6.1 or Kir6.2 inward rectifying potassium channel subunits and of a sulfonylurea receptor subunit (SUR 1, 2A, 2B). Whether the mammalian mitoK\textsubscript{ATP} has the same composition, is still under debate (for a recent discussion see, e.g., [11, 22]). In alternative to a Kir/SUR complex, mitoK\textsubscript{ATP} activity has been proposed to be due to a complex comprising a mitochondrial ABC transporter, the adenine nucleotide translocator, phosphate carrier, the ATP-ase and succinate dehydrogenase [23]. Interestingly, plant mitoK\textsubscript{ATP} appears to play a major role in the \textit{in vitro} regulation of succinate dehydrogenase (SDH). Affourtit et al. [24] showed that SDH is inactivated by K\textsuperscript{+} and reactivated by low concentrations of nucleotides acting from the intermembrane space, and hypothesized that these effects are mediated via plant mitoK\textsubscript{ATP}.

In plants, only one Kir-like channel exists, the single pore potassium channel AtKCO3 (in \textit{Arabidopsis}), belonging to the TPK (two-pore potassium channel) family. AtKCO3 however does not show any sequence homology with Kir6.1 and Kir6.2. Furthermore, Kir6.1 and Kir6.2 do not display any homology to any channel protein in \textit{Arabidopsis}. For AtKCO3 a mitochondrial targeting is predicted, although with low probability; and according to some localization prediction algorithms. It must be mentioned however, that numerous KCO two-pore K\textsuperscript{+} channels (TPK) have a predicted localization in chloroplasts but have been shown nonetheless to be located in the vacuolar membrane [25]. Thus, such predictions must be considered with caution, and at the moment a localization of KCO3 in mitochondria \textit{in vivo} cannot be excluded with certainty. The voltage dependence of the observed channel activity might be compatible with this channel being a member of the TPK family (TPK1 has been shown to behave as a voltage dependent channel). A variant of TPK1 in potato for example has a strong predicted localization in mitochondria. However, the high conductance, contrasting with that observed for TPK1 (22-45 pS) [26], and the high permeability to sodium argue against the proposal that the channel we observe might correspond to a TPK1-like channel. The channel activity of AtKCO3 is not known, preventing a comparison with the mitoplast channel.

The strong voltage-dependence of the channel activity we observe points to the possibility that it might be a \textit{shaker}-like channel. In \textit{Arabidopsis} there are nine \textit{shaker}-like channels identified and none of them (except AtKC1, a regulatory silent subunit) shows a strong prediction for mitochondrial localization. On the other hand, AKT1 or an AKT1-like channel is predicted to be located in mitochondria in several other plant species, including barley, grape, rice and wheat (\textit{Triticum aestivum}). In wheat root an AKT1-like channel, which has a strong prediction for mitochondrial targeting, has been cloned (Q9M671) [27]. The conductances (25 pS for OsAKT1 in 100/150 mM KCl [28]) and permeabilities to sodium of AKT1 from rice (strong mitochondrial targeting predicted) and of the wheat mitoplast channel are very different, and argue against an identification. It should be mentioned however that \textit{shaker}-like channels may have a different conductance in the presence or absence of their regulatory β-subunits (e.g. [29]). A regulatory β-subunit has been identified by proteomics in rice mitochondria, although the channel associated with it is not known [30]. Thus, it cannot be excluded that the activity we observe is due to a \textit{shaker}-like channel with altered characteristics due to the lack of β-subunit, or, alternatively, to the presence of another regulatory protein. Indeed, in the mammalian system the participation of SUR in mitoK\textsubscript{ATP} formation has been proposed. Interestingly, SUR1 (AAC36724) and SUR2A (NP_005682.2) subunits both show a high degree of sequence homology with several members of the multidrug-resistance protein family in \textit{Arabidopsis}, including the ABC transporters of mitochondrion (AtATM1-3) (32% identity and 53% positive over 1381 aminoacids (BLASTP algorithm) between SUR1 and AtMRP1). While AtATM1-3 proteins are implicated in iron homeostasis [31], AtMRP5 has been proposed to regulate a potassium channel activity [32]. Thus, one possibility is that an ABC-transporter protein associates with e.g. AtKCO3 or AKT1-like subunits to give rise to the ATP-dependent channel activity we observe.

Both the high conductance and the poor selectivity
for potassium over sodium are characteristic instead of the very heterogeneous non-selective cation channels (NSCC). Furthermore, some members of this family do not respond to classical inhibitors like TEA+, a characteristic also of the K+ uptake system we studied in wheat germ mitochondria [2]. Some members have also been reported to function as voltage-sensitive and ATP-inhibited channels [33]. In plants there are numerous genes encoding for members of the cyclic-nucleotide-gated (CNG) and ionotropic glutamate receptor (iGLR) family which may a priori give rise to NSCC activity [33]. AtCNGC13, 16 and 18, as well as GLR3.3 and 3.5 have strong targeting sequence for mitochondria, but their activity at single channel level has not been observed to our knowledge, preventing thus a possible identification of these gene products with the channel we observe.

The activation of a $K_{ATP}$ in mammalian system is known to regulate matrix volume, decrease mitochondrial membrane potential and prevent mitochondrial ROS formation. In plants it is well known that cellular reactive oxygen species (ROS) production can be increased as a result of plant exposure to various environmental factors inducing oxidative stress; mitochondria, in particular, were reported to show increased ROS generation under drought and salt stress [14 and refs therein]. In durum wheat, mito$K_{ATP}$ is activated by ROS [2]; the activated mito$K_{ATP}$ may cooperate with a very active K+/H+ antiporter, thus generating a K+ cycle able to completely uncouple mitochondria and dampen ROS generation [2, 34]. Therefore we suggested that the channel may act against the oxidative stress occurring when plants are exposed to environmental stresses [2]. Consistently, we observed a 400% increase in mito$K_{ATP}$ activity in mitochondria from water stress-adapted potato cell and in mitochondria from osmotic and salt stressed durum wheat seedlings with respect to control condition. At the same time, a decrease of about 60% of mitochondrial ROS generation occurred [14, 34 and refs therein]. Under severe salt stress a strong decrease (about -70%) of ATP synthesis is also observable [35], that might result in a reduced inhibition of the channel (since mito$K_{ATP}$ shows low affinity towards ATP). In fact, we measured a $K_i$ equal to about 0.3 mM ATP by using an indirect method [2] and, similarly, an $IC_{50}$ of 0.5 mM ATP by using patch-clamp experiments (this paper). In contrast, the mammalian counterpart is strongly inhibited by very low ATP concentration ($K_{0.5}$ = 22-40 μM), suggesting that in mammals ATP can hardly modulate the degree of channel opening in vivo [36].

Recently, we have reported that activation of mito$K_{ATP}$ may also depend on the increase of mitochondrial free fatty acids and acyl-CoA esters occurring under hyperosmotic stress [37] and that a plant inner membrane anion channel (PIMAC) may work in coordination with mito$K_{ATP}$ under de-energized conditions [38]. Interestingly, activation of plant mito$K_{ATP}$ was generally observed alongside with uncoupling protein, but not with alternative oxidase [34, 37 and refs therein]. For detailed reviews on the physiological roles of different plant mito$K_{ATP}$ see also [39] and [40].

In summary, the present work identifies for the first time an ATP-sensitive cation channel activity in native mitochondrial inner membrane in plants, namely in wheat. Further work is needed to understand the molecular identity of the channel in order to fully prove in the future its physiological role in intact plants by using genetic tools.

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