The Existence of the K⁺ Channel in Plant Mitochondria

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In this study, evidence is given that a number of isolated coupled plant mitochondria (from durum wheat, bread wheat, spelt, rye, barley, potato, and spinach) can take up externally added K⁺ ions. This was observed by following mitochondrial swelling in isotonic KCl solutions and was confirmed by a novel method in which the membrane potential decrease due to externally added K⁺ is measured fluorimetrically by using safranine. A detailed investigation of K⁺ uptake by durum wheat mitochondria shows hyperbolic dependence on the ion concentration and specificity. K⁺ uptake electrogenicity and the non-competitive inhibition due to either ATP or NADH are also shown. In the whole, the experimental findings reported in this paper demonstrate the existence of the mitochondrial K⁺ ATP channel in plants (PmKATP). Interestingly, Mg²⁺ and glyburide, which can inhibit mammalian K⁺ channel, have no effect on PmKATP. In the presence of the superoxide anion producing system (xanthine plus xanthine oxidase), PmKATP activation was found. Moreover, an inverse relationship was found between channel activity and mitochondrial superoxide anion formation, as measured via epinephrine photometric assay. These findings strongly suggest that mitochondrial K⁺ uptake could be involved in plant defense mechanism against oxidative stress due to reactive oxygen species generation.

One of most outstanding problems in mitochondria bioenergetics concerns the mitochondrial permeability to metabolites, organic compounds, including vitamins, their derived cofactors, and metal ions. The mitochondrial inner membrane contains metabolite carriers (for review, see Refs. 1 and 2), responsible for shuttling substrates between matrix and cytosol and for catabolism dependent on matrix enzymes, as well as vitamin and cofactor translocators (for review, see Refs. 3 and 4). Moreover, the inner membrane also contains the cation carriers and channels that regulate cell and mitochondrial physiology. In particular, as regards K⁺ ion, in mammalian mitochondria, the transport properties are such that net potassium flux across the mitochondrial membrane determines mitochondrial volume (Refs. 5 and 6 and references therein). It has been shown that K⁺ uptake is mediated by diffusion leak, driven by the high electric membrane potential maintained by redox-driven electrophoretic proton ejection, and that regulated K⁺ efflux is mediated by the inner membrane K⁺/H⁺ antiporter (see Ref. 7). There is also evidence for the existence of an inner membrane protein designed to catalyze electrogenic K⁺ uptake into mammalian (5–12) and yeast (13, 14) mitochondria. As far as plant mitochondria are concerned, even though mitochondrial structure and function are expected to be strictly dependent on K⁺ transport across the mitochondrial membrane, the knowledge of K⁺ permeability is not established at present. Indeed, the presence of a powerful K⁺/H⁺ antiporter, which partially collapses ΔpH, thereby increasing ΔΨ, has been shown (Refs. 15 and 16 and references therein). On the other hand, among a variety of compounds (including reducing sugars, proline, and Cl⁻), K⁺ could play a significant role in the leaf osmotic adjustment in response to water stress (17). Thus, the purpose of this investigation was an attempt to determine whether and how K⁺ can enter per se plant mitochondria. They were isolated from a number of different plant species, and a detailed investigation was carried out using durum wheat mitochondria. The latter were chosen on the grounds of their high K⁺ permeability. The existence of a K⁺ channel, which is probably involved in plant defense against oxidative stress, is shown.

EXPERIMENTAL PROCEDURES

Chemicals and Plant Material—All reagents were purchased from Sigma. They were of purest available grade and they were used without further purification. Substrates were used as Tris salts at pH 7.20. Solution pH was adjusted with either Tris or HCl. Valinomycin, oligomycin, nigericin, and diazoxide were dissolved in ethanol.

Certified seeds of durum wheat (Triticum durum Desf.), bread wheat (Triticum aestivum L.) and spelt (Triticum dicoccum Schühh.), rye (Secale cereale L.), and barley (Hordeum vulgare L.) were from the Italian Cereal Crop Institute. Potato (Solanum tuberosum L.) tuber and spinach (Spinacea oleracea L.) leaves were from the local market.

Isolation of DWM—Durum wheat seeds, cv. Ofanto (250 g) were sowed on a distilled water-saturated polyurethane foam sheet, and they were covered with a Whatman filter paper. They were then grown in the dark at 25 °C and 90% relative humidity for 72 h in an Heraeus HPS 1500 incubator.

About 200 g of etiolated shoots (1–2 cm long) were removed from seedlings, and mitochondria were isolated essentially as in Ref. 18 with minor modifications; the grinding and washing buffers were 0.5 mM sucrose, 4 mM cysteine, 1 mM EDTA, 30 mM Tris-HCl, pH 7.50, 0.1% (w/v) defatted BSA, 0.6% (w/v) PVP, and 0.5 mM sucrose, 1 mM EDTA, 10 mM Tris-HCl, pH 7.40, 0.1% (w/v) defatted BSA, respectively. Purification of washed mitochondria was performed by isopycnic centrifugation in a self-generating density gradient containing 0.5 mM sucrose, 10 mM Tris-HCl, pH 7.20, and 28% (w/v) Percoll (colloidal PVP-coated silica, Amersham Pharmacia Biotech) in combination with a linear gradient of 0% (top) to 10% (bottom) (w/v) PVP-40 (19). The final mitochondrial
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**FIG. 1. DWM swellings.** DWM (0.1 mg of protein) were added to 2 ml of 0.36 M sucrose (A and B), 0.18 M KCl (A and B), potassium acetate (KAc) (A) or TEACl (B) or 0.14 M ammonium phosphate (NH₄P) (A), added with 2 mM Tris-HCl, pH 7.00, in the absence of externally added respiratory substrates. Swellings were continuously monitored at 546 nm and 25 °C. Where indicated, the medium contained 0.1 μg of valinomycin (Val), 1 mM ATP, 2 μg of oligomycin (Oligo), or 10 μM atracyloside (Atr); at the arrows either 0.1 μg of valinomycin (Val) or 10 nM nigericin (Nig) were added.

The purified mitochondria showed 95% and 90% intactness of inner and outer membrane, respectively, determined as in Ref. 18. They were tightly coupled: oxidation of 2-oxoglutarate occurs with a respiratory control ratio equal to 6.7 ± 1.4 (S.E., four experiments) and with an ADP/O ratio, i.e. the ratio between the phosphorylated ADP and reduced oxygen, equal to 3.6 ± 0.15 (S.E., four experiments). Oxygen uptake was measured at 25 °C by means of a Gilson Oxygraph model 5/6-servo channel, pH 5, equipped with a Clark-type electrode (5331; YSI, Yellow Spring, OH) in a medium consisting of 0.3 M mannitol, 5 mM MgCl₂, 10 mM KCl, 0.1% (w/v) defatted BSA, 10 mM potassium phosphate buffer, pH 7.20.

Swelling Experiments—Swelling experiments were performed at 25 °C by monitoring photometrically at 546 nm the absorbance of a suspension of mitochondria (0.1 mg of protein) in different isotonic media as a function of time. In the swelling experiments devoted to investigate the MgCl₂ effect, this was not present or included in the medium at 0.1 and 5 mM concentrations.

Fluorimetric Measurements of ΔΨ Changes—Mitochondrial ΔΨ changes were monitored at 25 °C essentially as in Ref. 21, by measuring safranine fluorescence changes (λ/em = 520 nm, 480 nm) by means of the Perkin-Elmer LS50B spectrophotometer. The reaction medium (2 ml) contained DWM (0.2 mg of protein), 0.3 mM mannitol, 5 mM MgCl₂, 20 mM Tris-HCl, pH 7.20, 2.5 μM safranine. In the ΔΨ experiments aimed at investigating the MgCl₂ effect, this was either omitted from the medium or used at 0.1 and 5 mM concentrations.

Calibration of the safranine fluorescence decrease as a function of K⁺ diffusion potential was performed according to Ref. 22 by using rat liver mitochondria isolated as in Ref. 23; the K⁺ diffusion potential in rat liver mitochondria was induced by adding 0.05 μg/ml valinomycin (24).

Superoxide Anion Assay—Production of superoxide anion was determined essentially as in Ref. 25 by monitoring photometrically (480 nm, ε₅₅°₄ = 4.00 mM⁻¹ cm⁻¹) the rate of epinephrine oxidation to adrenochrome. The assay was carried out in the presence of 5 mM succinate in 2 ml of a medium consisting of 100 mM KCl, 110 mM mannitol, 5 mM MgCl₂, 2 EU of catalase, 10 mM Tris-HCl, pH 7.20; 10 μM cytochrome c was also present to account for the swelling-dependent endogenous cytochrome c release (26). Mitochondria (0.1 mg of protein) were incubated for 10 min during which any mitochondrial swelling was completed, then the reaction was started by adding 1 mM epinephrine. In certain experiments the osmoticum was 0.5 mM KCl plus 309 mM mannitol instead of 100 mM KCl plus 110 mM mannitol.

In order to establish whether superoxide anion formation can affect PmitoK ATP activity, superoxide anion was generated by using the properly developed superoxide anion-producing system consisting of 0.1 mM xanthine plus 0.038 EU of xanthine oxidase. This system was added to 2 ml of 0.18 M KCl, 2 mM Tris-HCl, pH 7.00. 30 s later mitochondria were added, and the swelling was continuously monitored. In ΔΨ experiments, the superoxide anion producing system was added to 2 ml of the safranine medium containing mitochondria; after 30 s of incubation, 5 mM succinate was added, then the ΔΨ decrease was induced by KCl (25 mM) addition. Swelling and ΔΨ experiments were carried out as described in the respective sections.

**RESULTS**

DWM Swelling—In order to gain a first insight into the K⁺ permeability of purified DWM, swelling experiments were carried out, using isolated mitochondria in the absence of externally added respiratory substrates. In control experiments, the absorbance of DWM, suspended in 0.36 M sucrose solution, was found to remain constant during the time, since sucrose cannot enter mitochondria (Fig. 1A, trace a); conversely, DWM were found to swell in 0.14 M NH₄P solution (Fig. 1A, trace b). The mitochondrial K⁺ permeability was checked by suspending DWM in potassium acetate and in KCl isotonic solutions. In the
light of the existence of K⁺/H⁺ antiport, previously reported in plant mitochondria (Refs. 15 and 16 and references therein), spontaneous swelling was found in potassium acetate with initial swelling rate equal to 0.08 ΔA/min-mg protein (Fig. 1A, trace c). A further addition of nigericin, which allows K⁺/H⁺ exchange, has been proven to increase swelling rate up to 0.16 ΔA/min-mg protein.

Surprisingly, DWM were found to swell rapidly in KCl (initial rate equal to 0.12 ΔA/min-mg protein) (Fig. 1A, trace d); since Cl⁻ can enter plant mitochondria (27), such a swelling indicates that K⁺ can penetrate per se mitochondria in the absence of valinomycin. When valinomycin, which allows K⁺ permeability, was added at the end of the spontaneous swelling, little additional absorbance decrease was observed. No significant changes in both rate and extent of swelling was found in the presence of the respiratory substrate succinate or salicylhydroxamic acid, which can inhibit cytochrome c oxidase and plant alternative oxidase, respectively (data not shown).

When DWM were suspended in KCl in the presence of valinomycin, a very high rate swelling was found to occur (initial rate was 1.9 ΔA/min-mg protein) (Fig. 1A, trace e), indicating that K⁺ rather than Cl⁻ uptake via FIMAC (27) represents the rate-limiting step of DWM swelling in KCl.

In the light of the already reported ATP inhibition of K⁺ channel in mammalian mitochondria (6, 7, 9, 10, 12), we investigated whether externally added ATP can prevent DWM swelling in KCl (Fig. 1B). First, in a control experiment it was observed that ATP (1 mM) did not affect DWM absorbance in sucrose medium (Fig. 1B, traces f and g); however, ATP inhibited both the initial rate (about 25%) and the extent of the KCl swelling (Fig. 1B, traces k and l). Inhibition was insensitive to oligomycin and atracyloside, powerful inhibitors of ATP synthase and of ADP/ATP translocator, respectively (Fig. 1B, trace j), which have no effect on the KCl swelling (data not shown).

Valinomycin, added at the end of the swelling, induced an additional significant swelling (Fig. 1B, traces j and k). This finding indicates that K⁺ uptake rather than Cl⁻ uptake was inhibited by ATP under these experimental conditions (1 mM ATP, absence of valinomycin). Swelling experiments in KCl plus valinomycin occurred at very fast rates, and these rates were inhibited by ATP (Fig. 1B, traces m and n), raising the possibility of ATP inhibition of Cl⁻ transport. In the same experiment, swelling in TEA⁺ medium was also carried out either in the absence or in the presence of ATP (see Ref. 10); ATP was found to cause no significant change in TEACl swelling, which occurs with a low rate (Fig. 1B, traces h and i).

These qualitative findings are consistent with the existence of a mitochondrial K⁺ATP channel; however, two findings render them insufficient to establish such a process with certainty. ATP inhibition of Cl⁻ transport could explain the data; moreover, swelling in KCl plus succinate is unexpected if mitochondria maintain their membrane potential during respiration.

The Effect of Externally Added K⁺ on Mitochondrial ΔΨ—The observed K⁺ uptake as well as the possible potassium cycle in Garlid's terms (6) are expected to cause membrane potential decrease, which could occur as a result of K⁺ and/or H⁺ transport by DWM (see Scheme 1). We monitored ΔΨ using a safranine probe, and observed that succinate addition (5 mM) to DWM in the absence of K⁺ caused a rapid increase of ΔΨ to about 185 mV (Fig. 2A). Phosphate caused a slight increase of ΔΨ, as expected if it enters via proton-compensated symport (21, 24), whereas ADP caused a decrease of ΔΨ, as expected from the increased proton current secondary to ATP synthesis. The ATP synthase inhibitor, oligomycin, partially restored ΔΨ, and the uncoupler, FCCP, abolished ΔΨ (Fig. 2A). Taken together, these findings indicate that DWM exhibit normal functionality with respect to ΔΨ in K⁺-free medium.

Entirely different results were obtained when KCl was added to the medium (Fig. 2, B–D). When KCl was added after ener-
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The ΔΨ decrease was monitored as reported in Fig. 2B (Control trace), and it was expressed as the percentage of the rate of ΔΨ decrease, caused by externally added FCCP (1 μM). The rates of passive swelling in both KCl and potassium acetate (Kac), carried out as reported in Fig. 1A (traces c and d), are expressed as the percentage of the rate of mitochondrial swelling in ammonium phosphate. The swelling in Kac was performed in presence of 1% (w/v) BSA except for Triticum durum.

| Plant species | Depolarization | KCl swelling | Kac swelling |
|---------------|----------------|--------------|--------------|
| Triticum durum| Complete       | 55 ± 6.1%    | 155 ± 17.7%  |
| Desf.         | Complete       | 44 ± 5.3%    | 138 ± 14.6%  |
| Triticum aestivum | L.     | Complete | 55 ± 4.8%    | 88 ± 9.5%    | 105 ± 12.2% |
| Schuler        | Complete       | 47 ± 4.9%    | 290 ± 30.3%  | 200 ± 19.5%  |
| Hordeum vulgare| L.            | ND           | ND           | 260 ± 29.6%  | 235 ± 25.7% |
| Solanum       | up to 90 mV    | 17 ± 5.1%    | 64 ± 7.3%    | 70 ± 8.9%    |
| Spinacea oleracea | L.      | up to 70 mV  | 19 ± 4.8%    | 140 ± 13.2%  | 103 ± 13.4% |

* Mean value ± S.E. (three experiments).

** Certain Features of K⁺ Uptake by DWM—** In order to gain some insight into the mechanism by which K⁺ enters DWM, the dependence of the K⁺ uptake rate was investigated as a function of 0.005–25 mM KCl concentration (Fig. 4). Hyperbolic dependence on the concentration was found, suggesting that K⁺ uptake takes place in a protein-dependent manner, probably via PmtoK	extsubscript{ATP}. In three different experiments, Kₘ and V	extsubscript{max} were 2.2 ± 0.78 mM (S.E.) and 12.5 ± 1.96 mV/s (S.E.), respectively. The rate of ΔΨ decrease due to FCCP addition was found to be greater than that observed with all KCl additions (Fig. 4, inset), confirming that this rate is not limited by the rate of safranine response.

To gain an insight into the specificity of PmtoK	extsubscript{ATP}, we compared the swelling response to various monovalent chloride salts (Fig. 5A). The rate and amplitude of mitochondrial swelling varied in the order Cs⁺ > K⁺ > Rb⁺ > Na⁺ > Li⁺. We also examined their ability to collapse ΔΨ in respiring mitochondria (Fig. 5B). Na⁺ and Li⁺ had no significant effect, whereas K⁺, Cs⁺, and Rb⁺ caused ΔΨ decrease at a significant rate.

In order to ascertain whether and how the K⁺ channel is ΔΨ-dependent, DWM, previously energized by succinate, were treated with FCCP in the nanomolar concentration range, designed to partially collapse membrane potential. Then the dependence of the rate of ΔΨ decrease on the actual mitochondrial ΔΨ was investigated, using 25 mM KCl (Fig. 6). A fast decrease of K⁺-dependent depolarization, *i.e.*, of the K⁺ channel

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**Fig. 3. Inhibition by ATP and NADH of the rate of K⁺-induced ΔΨ decrease in DWM.** The Dixon plots relative to ATP (A) and NADH (B) inhibition were obtained by means of ΔΨ measurements carried out as in Fig. 2B (Control trace). KCl concentrations were: 25 mM (○) and 0.5 mM (●). Bars represent the S.E. relative to three different experiments.

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**Table I**

| Plant species | Depolarization | KCl swelling % rate | Kac swelling % rate |
|---------------|----------------|---------------------|---------------------|
| Triticum durum| Complete       | 55 ± 6.1%           | 155 ± 17.7%         |
| Desf.         | Complete       | 44 ± 5.3%           | 138 ± 14.6%         |
| Triticum aestivum | L.     | Complete | 55 ± 4.8%           | 88 ± 9.5%           | 105 ± 12.2% |
| Schuler        | Complete       | 47 ± 4.9%           | 290 ± 30.3%         | 200 ± 19.5% |
| Hordeum vulgare| L.            | ND                  | ND                  | 260 ± 29.6%         | 235 ± 25.7% |
| Solanum       | up to 90 mV    | 17 ± 5.1%           | 64 ± 7.3%           | 70 ± 8.9%          |
| Spinacea oleracea | L.      | up to 70 mV         | 19 ± 4.8%           | 140 ± 13.2%        | 103 ± 13.4% |

* Mean value ± S.E. (three experiments).

† Not determined.
activity, was found in ΔΨ range varying from about 175 to 140 mV. The rate was found to remain rather constant in the range 140–95 mV.

**Effector Sensitivity of PmitoK ATP**—We examined inhibitors and activators of mammalian mitoK ATP to determine whether PmitoK ATP shared any or all of the ligands previously reported for mitoK ATP (10, 12, 30, 31). Results are reported in Table II. Like mammalian mitoK ATP, PmitoK ATP is inhibited by ATP (Figs. 2A and 3A) and ADP, and ATP inhibition is prevented or reversed by GTP and diazoxide. PmitoK ATP is also stimulated by the sulfhydryl group reagents mersalyl and N-ethylmaleimide. In contrast to mammalian mitoK ATP, PmitoK ATP is activated, rather than inhibited, by palmitoyl-CoA, and it is not inhibited by glyburide. The glyburide effect was examined in both the absence or presence of Mg$^{2+}$ (0.1 mM) plus ATP (1 mM) plus either diazoxide (10 μM) or GTP (1 mM), which proved to be essential for inhibition due to glyburide of mitochondrial K$^+$ channel in mammalian (31); however, PmitoK ATP was not inhibited by glyburide under any condition. A difference was also observed in the effect of Mg$^{2+}$ ion. Mg$^{2+}$ ion is required for inhibition of mammalian mitoK ATP by ATP and CoA esters; however, it has no effect on its own (6). In contrast, Mg$^{2+}$ had no effect on PmitoK ATP, and ATP inhibition did not require Mg$^{2+}$.

PmitoK ATP also differs from the plant inward rectifying K$^+$ of nonmitochondrial membranes because it is not inhibited by Al$^{3+}$, Ba$^{2+}$, and TEA$^-$. Inhibition by NADH (Figs. 2A and 3B) and Zn$^{2+}$ and stimulation by coenzyme A are also distinctive of the plant mitochondrial channel.

**PmitoK ATP Activity and Superoxide Anion Generation**—Since electron flow via the respiratory chain can produce oxygen radical species, which are assumed to damage severely both animal and plant cells (32, 33), and given that a number of energy dissipating processes have been proposed to decrease the mitochondrial reactive oxygen species generation (34–41), investigation was made in order to establish whether K$^+$ uptake and superoxide anion generation are somehow related. Superoxide anion formation was monitored as in Ref. 25 by measuring the absorbance increase due to the epinephrine to adrenochrome conversion. Measurements were done in medium containing low (0.5 mM) and high (100 mM) K$^+$, in which PmitoK ATP is less and more active, respectively (Fig. 7). DWM respiring in 0.5 mM KCl produced superoxide anion at a rate equal to 40 nmol/min/mg protein. This rate was decreased to 19 nmol/min/mg protein in 100 mM KCl. Control was made in...
A series of compounds that have known effects on mammalian mitoK<sub>ATP</sub> channel and plant plasma membrane K<sup>+</sup> channel were investigated with respect to their effect on DWM K<sup>+</sup> channel activity, evaluated by ∆ψ and/or passive swelling experiments. ∆ψ experiments were carried out as reported in Fig. 2B. The investigated compounds were added to mitochondria; after 90 s of incubation, succinate (5 mM) was added to the sample; then, after 120 s, depolarization was induced by adding KCl (5 mM). Swelling experiments were carried out as in Fig. 1A (trace d) in a medium containing the listed compounds. Activation and inhibition are reported as the percentage of the control rate of ∆ψ as reported in Fig. 2 with the arrow.

Moreover, ATP and mersalyl, an inhibitor and an activator, respectively, of PmitoK<sub>ATP</sub> proved to enhance and to prevent, respectively, the superoxide anion generation by DWM in 100 mM KCl medium (data not shown).

We examined the possibility that superoxide anion might modulate PmitoK<sub>ATP</sub>. DWM were incubated with xanthine plus xanthine oxidase, which generate superoxide anion, then the swelling in KCl (Fig. 8A) and ∆ψ response to KCl (Fig. 8B) were monitored. Xanthine oxidase per se had no effect on both swelling and ∆ψ decrease rate; on the contrary, xanthine was found to cause 35% increase in the rates. When added together, xanthine and xanthine oxidase were found to cause approximately 100% increase in PmitoK<sub>ATP</sub> activity. Such an increase was found to be partially prevented by SOD. Under our experimental conditions, mitochondria treated with superoxide anion show the same polarization rate and ∆ψ of the control (data not shown), thus indicating that the increase of K<sup>+</sup> uptake is not due to gross membrane damage.

### TABLE II

| Compound                      | Effect on DWM K<sup>+</sup> channel | Effect on other K<sup>+</sup> channels | Ref.     |
|-------------------------------|-------------------------------------|---------------------------------------|---------|
| Mg<sup>2+</sup> (0, 0.1, and 5 mM)<sup>a,b,c</sup> | No inhibition                       | Inhibition<sup>f</sup>                | (5–7, 8, 12)<sup>j</sup> |
| Zn<sup>2+</sup> (400 μM)<sup>d</sup>       | Inhibition (∆ψ ± 12.2%)              | Activation<sup>j</sup>                | (51)     |
| Al<sup>3+</sup> (50 μM)<sup>d</sup>      | No inhibition                       | Inhibition<sup>k</sup>                | (42, 43) |
| Ba<sup>2+</sup> (1 mM)<sup>a</sup>       | No inhibition                       | Inhibition<sup>d,e</sup>              | (42, 51) |
| Na<sup>+</sup> (10 mM)<sup>d</sup>       | No inhibition                       | Inhibition<sup<k</sup>                 | (42, 43) |
| Ca<sup>2+</sup> (1–5 μM)<sup>d</sup>     | No inhibition                       | Inhibition<sup>j</sup>                | (6, 7, 51) |
| EGTA (10 mM)<sup>d</sup>               | No effect                           | Inhibition<sup>j</sup>                | (6, 9, 3, 52)<sup>j</sup> |
| Glyburide (5–10 μM)<sup>m,n,b,a</sup>   | No inhibition                       | Prevention of ATP inhibition         | (9)<sup>j</sup> |
| 4-Aminopyridine (5 mM)<sup>m,n</sup>    | Prevention of swelling experiments   | Reversion of ATP inhibition          |         |
| Dazoxine (10 μM)<sup>m</sup>           | Prevention of ATP inhibition        | Reversion of ATP inhibition          |         |
| GTP (1 mM)<sup>m</sup>                | Prevention of ATP inhibition        | Prevention of ATP inhibition         |         |
| ADP (1 mM)<sup>m</sup>                | Prevention of ATP inhibition        | Prevention of ATP inhibition         |         |
| ADP plus F<sub>4</sub> (1 + 1 mM)<sup>m</sup> | Prevention of ATP inhibition       | Prevention of ATP inhibition         |         |
| AMP (1 mM)<sup>m</sup>                | Prevention of swelling experiments   | Prevention of swelling experiments   |         |
| NAD<sup>+</sup> (1 mM)<sup>m</sup>      | Prevention of swelling experiments   | Prevention of swelling experiments   |         |
| Phosphate (1 mM)<sup>m</sup>           | Prevention of swelling experiments   | Prevention of swelling experiments   |         |
| Palmitoyl-CoA (1 mM)<sup>b</sup>       | Prevention of swelling experiments   | Prevention of swelling experiments   |         |
| Coenzyme A (100 μM)<sup>d</sup>        | Prevention of swelling experiments   | Prevention of swelling experiments   |         |
| Mersalyl (1 mM)<sup>b</sup>            | Activation (+102 ± 21.8%)            | Activation (+102 ± 21.8%)            | (6, 12)  |
| N-Ethylmaleimide (30 nmol/mg)<sup>b</sup> | Activation (+102 ± 21.8%)            | Activation (+102 ± 21.8%)            | (6, 12)  |

<sup>a</sup> Evaluated by ∆ψ experiments.
<sup>b</sup> Evaluated by swelling experiments.
<sup>c</sup> Mg<sup>2+</sup> at the reported concentrations was used in both the absence and presence of 1 mM ATP. The osmolarity of the ∆ψ medium and of the swelling medium was kept constant by acting on mannitol and KCl concentrations, respectively.
<sup>d</sup> The osmolarity of the medium was kept constant by acting on mannitol concentration.
<sup>e</sup> Glyburide was tested in both the absence and presence of Mg<sup>2+</sup> (0.1 and 5 mM) plus ATP (1 mM) plus either diazoxide (10 μM) or GTP (1 mM).
<sup>f</sup> Mean value ± S.E. (three experiments).
<sup>g</sup> Effect on mammalian mitoK<sub>ATP</sub> channel.
<sup>h</sup> Effect on the plant plasma membrane K<sup>+</sup> channel.
<sup>i</sup> Garlid’s group found Mg<sup>2+</sup> inhibition only in the presence of ATP.
<sup>j</sup> In mammalian mitochondria, glyburide inhibition occurs only in the presence of Mg<sup>2+</sup> plus ATP plus either diazoxide or GTP (31).
<sup>k</sup> In Ref. 53, no inhibition due to 4-aminopyridine was reported in rat liver mitochondria.

**DISCUSSION**

It is central in the understanding of ion membrane permeability and transport to recognize that there is a vectorial component whereby ions are translocated through a protein embedded in a lipid bilayer membrane. Plant cells contain K<sup>+</sup> channels in the plasma membrane, in the tonoplast (Refs. 42 and 43 and references therein), in the chloroplast envelope (44, 45) and probably in the Golgi (45). To the best of our knowledge, K<sup>+</sup> channels have not previously been reported in plant mitochondria. Our first observation was that DWM swell spontaneously in isotonic KCl (Fig. 1). The inner membranes of plant mitochondria transport Cl<sup>–</sup> ions via the pH-regulated PIMAC (27). Since mitochondria swell rapidly in KCl, it follows that

| ![Fig. 7](image7.png) | **Fig. 7.** Superoxide anion (O<sub>2</sub>) generation by DWM in media containing low and high KCl concentration. The superoxide anion generation by mitochondria was assayed as reported under “Experimental Procedures” in the absence or the presence of SOD (10 μg/ml). The medium was maintained either 100 mM KCl plus 110 mM mannitol; 5 mM EGTA was always present. Reaction was started by adding 1 mM epinephrine at the time indicated by the arrow. |
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Plant mitochondria also contain a K⁺ conductance pathway.

KCl swelling was inhibited by external ATP, and ATP inhibition was insensitive to oligomycin and atracyloside. Moreover, 1 mM ATP inhibited swelling in KCl plus valinomycin only slightly and had no effect on the rate of swelling in TEACl (see Ref. 10). These findings strongly indicate that ATP specifically inhibits swelling by inhibiting K⁺ uniport; however, the question whether ATP inhibits K⁺ or Cl⁻ influx is not entirely clear in these protocols. Thus, we observed that 5 mM ATP inhibited swelling in KCl plus valinomycin (data not shown).

A clearer picture emerged from the measurements in respiring mitochondria, in which we monitored ∆Ψ as a measure of K⁺ cycling across the membrane. In particular, these experiments (Fig. 2) demonstrate that endogenous K⁺ influx uncouples mitochondria to a remarkable degree. DWM and mammalian mitochondria differ strongly in this respect. The K⁺ cycle in mammalian mitochondria cannot uncouple completely, because the maximal rate of the cycle, given by the V₉₉ of the K⁺/H⁺ antiporter (15, 16) in plant mitochondria implies that a matching K⁺ channel activity should also exist in these membranes, as has been found. Evidently the activity of each pathway is approximately equal to the proton-ejecting capacity of the electron transport chain. This quantitative difference between mammalian and plant mitochondria is also reflected in the energy dependence of swelling in K⁺ salts; swelling of respiring rat liver mitochondria is due to electroneutral uptake of acetate and/or phosphate in combination with electrophoretic uptake of K⁺, driven by ∆Ψ. Cl⁻ does not penetrate significantly, because the endogenous K⁺ cycle flux pathways are insufficient to collapse ∆Ψ. In contrast, respiring plant mitochondria swell in both respiring and non-respiring states, because the active K⁺ cycle completely collapses ∆Ψ, permitting electrophoretic Cl⁻ uptake.

We were able to show that uncoupling is specific for K⁺ (Cs⁺, Rb⁺) ion and does not occur with Na⁺ or Li⁺. Moreover, the K⁺-specific uncoupling is inhibited by ATP. Taking the rate of ∆Ψ change to be proportional to the rate of electrophoretic K⁺ influx, we observed hyperbolic dependence on K⁺ concentration. The apparent Kₘ for K⁺ uptake was about 2 mM, which is lower than the value of 32 mM for the purified mitoKATP from rat liver mitochondria (7). ATP and NADH inhibition is non-competitive with K⁺ values equal to 290 and 390 μM, respectively; moreover, ATP shows a Kᵢ higher than the one observed in rat liver and beef heart mitochondria (7). ATP inhibition of PmitoKATP is independent of the presence of Mg²⁺ ions, whereas ATP inhibition of mammalian mitoKATP exhibits an absolute requirement for Mg²⁺ (6). Moreover, PmitoKATP was activated, rather than inhibited, by palmitoyl-CoA, which is a potent inhibitor of mammalian mitoKATP. In both of these respects, PmitoKATP more closely resembles mammalian plasma membrane K⁺ATP, channels (47). The K⁺ channel opener, diazoxide, reversed inhibition by ATP; however, PmitoKATP was insensitive to glyburide under all conditions tested. This property raises the possibility that PmitoKATP, unlike mammalian K⁺ATP channels, may not be regulated by a sulfonylurea receptor.

Since electrophoretic K⁺ uptake dissipates energy and uncouples oxidative phosphorylation, the mitochondrial K⁺ channel must be regulated in vivo. The observed inhibition by ATP and NADH meets this requirement, but it remains to be determined how the channel is opened under physiological conditions. The possible role of ATP and other ligands in regulating mammalian mitoKATP has been reviewed (6, 12). The physiological role of PmitoKATP is also unknown. In vitro results in a KCl medium cannot be extrapolated to behavior in vivo; however, it seems clear from the agreement between rates of K⁺/H⁺ antiport and K⁺ uniport via PmitoKATP that plant mitochondria are capable of complete uncoupling. The demonstration that operation of PmitoKATP reduces superoxide anion formation is not surprising, because most modes of uncoupling have this effect (34, 35, 40, 41, 48–50). Of potentially greater interest is the finding that superoxide anion formation stimulated PmitoKATP, suggesting a possible feedback mechanism to protect against reactive oxygen species.

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