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A Highly Active ATP-Insensitive K^+ Import Pathway in Plant Mitochondria

Fernando Ruy,[1] Anibal E. Vercesi,[1] Paula B. M. Andrade,[2] M. Lucia Bianconi,[3] Hernan Chaimovich,[2] and Alicia J. Kowaltowski[2,4]

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We describe here a regulated and highly active K^+ uptake pathway in potato (*Solanum tuberosum*), tomato (*Lycopersicon esculentum*), and maize (*Zea mays*) mitochondria. K^+ transport was not inhibited by ATP, NADH, or thiol reagents, which regulate ATP-sensitive K^+ channels previously described in plant and mammalian mitochondria. However, K^+ uptake was completely prevented by quinine, a broad spectrum K^+ channel inhibitor. Increased K^+ uptake in plants leads to mitochondrial swelling, respiratory stimulation, heat release, and the prevention of reactive oxygen species formation. This newly described ATP-insensitive K^+ import pathway is potentially involved in metabolism regulation and prevention of oxidative stress.

KEY WORDS: Potassium channel; *Solanum tuberosum*; reactive oxygen species; uncoupling; heat.

INTRODUCTION

The impermeability of the mitochondrial inner membrane to ions plays an important role in bioenergetics and is essential for adequate mitochondrial function. It ensures that redox energy released through substrate oxidation can be maintained as a proton gradient, which fuels ATP synthesis (Mitchell, 1961). Moreover, the proton gradient can also be used in other processes such as Ca^{2+} transport (Gunter *et al.*, 1994) or heat production, mediated by the mammalian brown adipose tissue uncoupling protein (UCP1; Nicholls *et al.*, 1978).

Surprisingly, plant mitochondria present the ability to dissipate or prevent the formation of the proton gradient. Two energy-dissipating systems that can significantly decrease the efficiency of oxidative phosphorylation have been described in plant mitochondria: the plant uncoupling protein (PUMP; Vercesi *et al.*, 1995) and the alternative oxidase (AOX; Huq and Palmer, 1978; Rich, 1978).

Similarly to UCP1, PUMP stimulates proton gradient dissipation by promoting H^+ reentry into the mitochondrial matrix through a fatty acid cycle. In this cycle, PUMP catalyzes the transport of anionic fatty acids from the matrix to cytosol. The return of these fatty acids in the protonated form, via a "flip-flop" mechanism (Skulachev, 1991, 1999), results in a futile H^+ cycle, dissipating protonmotive force (Jezek *et al.*, 1996). PUMP-stimulated proton cycling is sensitive to allosteric inhibition by purine nucleotides such as ATP and GTP and is strongly inhibited by BSA, which binds free fatty acids (Jezek *et al.*, 1996; Sluse *et al.*, 1998).

AOX shunts electrons from the cytochrome pathway at the level of ubiquinone, reducing oxygen to water and avoiding H^+ transport from the matrix into the intermembrane space. Thus, electron flux through AOX is not coupled to ATP synthesis, dissipating energy as heat. The activity of AOX is insensitive to cytochrome pathway

[1] Departamento de Patologia Clínica, Faculdade de Ciências Médicas, Universidade Estadual de Campinas, Campinas, SP, Brazil.
[2] Departamento de Bioquímica, Instituto de Química, Universidade de São Paulo, São Paulo, SP, Brazil.
[3] Departamento de Bioquímica Médica, Instituto de Ciências Biomédicas, Universidade Federal do Rio de Janeiro, Rio de Janeiro, RJ, Brazil.
[4] To whom correspondence should be addressed; e-mail: alicia@iq.usp.br.

Key to abbreviations: AOX, alternative oxidase; BSA, bovine serum albumin; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; mitoK_{ATP}, mitochondrial ATP-sensitive K^+ channels; PIMAC, plant inner-membrane anion channel; PUMP, plant uncoupling protein; UCP, uncoupling protein.

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inhibitors such as antimycin or cyanide, but can be inhibited by benzohydroxamate or linoleic acid, an abundant free fatty acid in plants (Sluse *et al.*, 1998).

Although both energy dissipating systems in plant mitochondria lead to the same effect (uncoupling of respiration from oxidative phosphorylation), there is a basic difference between these pathways: while PUMP decreases the proton gradient, AOX prevents its formation by shunting electrons from the respiratory chain. Another difference is the opposite effect of free fatty acid regulation. While PUMP is activated, AOX is inhibited by free fatty acids. Thus, both systems seem not to work at their maximal activity simultaneously. Most probably, these energy-dissipating systems work sequentially during plant development (Almeida *et al.*, 1999).

Recently a new dissipative mechanism was described in plant mitochondria involving K^+ import into the mitochondrial matrix and K^+/H^+ exchange (Chiandussi *et al.*, 2002; Pastore *et al.*, 1999; Paucek *et al.*, 2002; Petrussa *et al.*, 2001). In this manner K^+, the most abundant cation in the cytosol, regulates coupling between respiration and ATP synthesis in plant mitochondria. The K^+ import pathway was studied in detail in durum wheat and pea stem mitochondria, but also evidenced in spinach, potato, and barley. In these plants, the pathway appears to be inhibited by ATP, suggesting the channel is similar to mammalian ATP-sensitive K^+ channels (mitoK_{ATP}). The discovery of a mitoK_{ATP} in plant mitochondria brought new implications to the physiology of this organelle, because the existence of a K^+ import channel acting together with a potent K^+/H^+ exchanger in these mitochondria (Diolez and Moreau, 1985) would allow proton potential regulation through a K^+ cycle (Garlid, 1996).

We decided to study K^+ transport in plant mitochondria in more detail, using potato (*Solanum tuberosum*), tomato (*Lycopersicon esculentum*), and maize (*Zea mays*), plants in which the roles of PUMP and AOX have been previously characterized. Surprisingly, we did not find an ATP-sensitive K^+ channel. Instead, we report the existence of a highly active ATP-insensitive K^+ import pathway in plant mitochondria.

EXPERIMENTAL PROCEDURES

Mitochondrial isolation. Potato mitochondria were isolated by differential centrifugation as described by Beavis and Vercesi (1992). Green stage tomato mitochondria were isolated as described by Almeida *et al.* (1999) and purified on a Percoll gradient (Van den Bergen *et al.*, 1994). Maize mitochondria were prepared following Martins and Vercesi (1985). Protein content in the mitochondrial suspension was determined using the Biuret method (Gornall *et al.*, 1949).

Swelling measurements. Increases in matrix volume of de-energized plant mitochondria were measured by following the decrease in light scattering of the mitochondrial suspension at 520 nm, using an SLM Aminco DW 2000 or a Multispec Shimadzu 1501 spectrophotometer.

O_2 consumption measurements. Oxygen consumption was measured using a Clark-type electrode (Yellow Springs Instruments) in a temperature-controlled chamber (25°C).

Mitochondrial H_2O_2 release. H_2O_2 production was assessed at 25°C by the oxidation of Amplex Red in the presence of extramitochondrial horseradish peroxidase bound to H_2O_2. Amplex Red fluorescence was monitored at excitation wavelengths of 365 and 450 nm, respectively, on a Hitachi F4500 fluorimeter.

Isothermal titration calorimetry. Isothermal titration calorimetry experiments were conducted in a VP-ITC microcalorimeter from Microcal Llc (Northampton, MA, U.S.A.). The implementation principles of isothermal titration calorimetry were described by Wiseman *et al.* (1989). The experiments were started by injecting mitochondria after the reaction medium reached equilibrium at 30°C in the 1.45-mL sample cell. Total heat (QT) released in the reactions was calculated by integrating the total area under the peak of the calorimetric thermograms for the first 6 min after injection.

Data analysis. Traces shown are representative data of at least three similar experiments. Linear regression fits were calculated using Origin® software. Averages and standard errors (SEM) were determined using SigmaStat® ($n \geq 3$).

RESULTS

Potato Mitochondria Are Permeable to K^+ Ions

To assess inner-membrane permeability to K^+ in plant mitochondria, we suspended de-energized potato mitochondria in hyposmotic media. Under these conditions, mitochondria suspended in Cl^- salts swell if their inner membrane is permeable to the cation, since Cl^- is transported into the plant mitochondrial matrix by a highly active channel (PIMAC; Beavis and Vercesi, 1992). We observed that the inner-membrane conductance for ions followed the order: $K^+ > Li^+ \gg Na^+$ (Fig. 1, see swelling rates in the figure legend), indicating the presence of a selective K^+ ion transport pathway in the inner-membrane of potato mitochondria. The addition of valinomycin, a K^+ ionophore, enhanced ion uptake (line e), suggesting that mitochondrial swelling is limited by K^+ transport rates

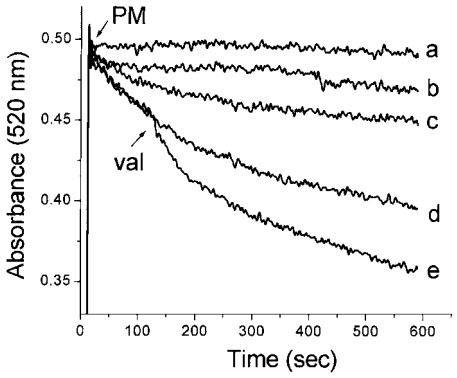

Fig. 1. Potato mitochondria swell in K^+ ions. Isolated potato mitochondria (PM, 0.2 mg/mL) were added to a buffer containing 10 mM HEPES, 2 μM antimycin, 1 μg/mL oligomycin, and 100 mM sucrose (line a); 50 mM NaCl (line b); 50 mM LiCl (line c); or 50 mM KCl (lines d and e), in which the buffer pH was adjusted to 7.2 using NaOH (lines a and b), LiOH (line c), or KOH (lines d and e). Valinomycin (val -1 μM) was added where indicated by the arrow (line e). Absorbance changes/min during the first 2 min were 5.4×10^{-4} (line a), 4.7×10^{-3} (line b), 1.2×10^{-2} (line c), and 2.1×10^{-2} (line d).

and not by Cl^- transport through PIMAC (Beavis and Vercesi, 1992).

To investigate if mitochondrial K^+ permeability was due to an ATP-sensitive K^+ channel (mitoK_{ATP}), we tested if K^+-induced swelling was inhibited by ATP or ATP plus Mg^{+2}. Under conditions similar to those used by Pastore *et al.* (1999), we observed an inhibitory effect of ATP (Fig. 2(A), line a) or a partial inhibitory effect of ATP + Mg^{2+} (line b) on control K^+ uptake (line c). However, in plant mitochondria incubated under these de-energized conditions, K^+ uptake may occur through both a

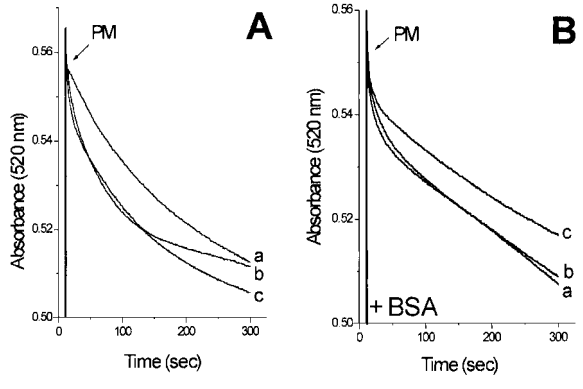

Fig. 2. ATP-insensitive K^+ transport in potato mitochondria. PM (0.2 mg/mL) were added to a buffer containing 10 mM K^+-HEPES (pH 7.2), 50 mM KCl, 2 μM antimycin, and 1 μg/mL oligomycin, in the absence (Panel A) or presence (Panel B) of 1% BSA. Swelling is shown under control conditions (lines c) and in the presence of 1 mM ATP (lines a) or 1 mM ATP plus 2 mM Mg^{2+} (lines b).

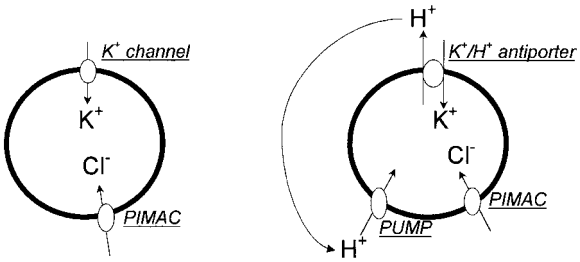

Fig. 3. Pathways through which K^+ can enter plant mitochondria. In plant mitochondria incubated in hyposmotic KCl media, Cl^- ions enter the mitochondrial matrix through PIMAC. K^+ uptake may occur through both a K^+ uniporter (left) or the K^+/H^+ antiporter (right), with H^+ cycling maintained by PUMP.

K^+ uniporter or the K^+/H^+ antiporter, very active in plants (Diolez and Moreau, 1985; Fig. 3). In the latter uptake mechanism, H^+ cycling is maintained by PUMP (Vercesi *et al.*, 1995). To avoid K^+ transport through the second mechanism, we added BSA to the reaction medium, removing fatty acids essential for H^+ transport by PUMP. As seen in Fig. 2(B), swelling occurs in the presence of BSA (line c) and is not prevented by ATP (line b) or ATP + Mg^{2+} (line a). Therefore, potato mitochondria are permeable to K^+ ions, but this permeability is not inhibited by ATP. In the absence of BSA, the inhibitory effect of ATP is probably due to the prevention of PUMP-mediated H^+ transport.

To confirm that the partial inhibitory effect of ATP on mitochondrial swelling is related to PUMP-mediated H^+ transport across the inner membrane, we also studied mitochondria in which H^+ transport was maximized by the protonophore FCCP, eliminating any possible PUMP effect. As observed in Fig. 4, swelling in KCl media under

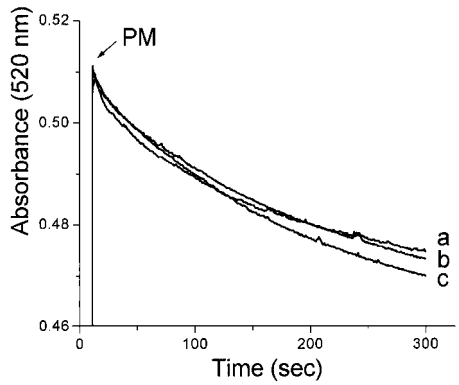

Fig. 4. ATP insensitive K^+ transport in uncoupled potato mitochondria. PM (0.2 mg/mL) were added to a buffer containing 10 mM K^+-HEPES (pH 7.2), 50 mM KCl, 2 μM antimycin, 1 μg/mL oligomycin, and 0.5 μM FCCP in the presence of 1 mM ATP plus 2 mM Mg^{2+} (line a), no further additions (line b), or 1 mM ATP (line c).

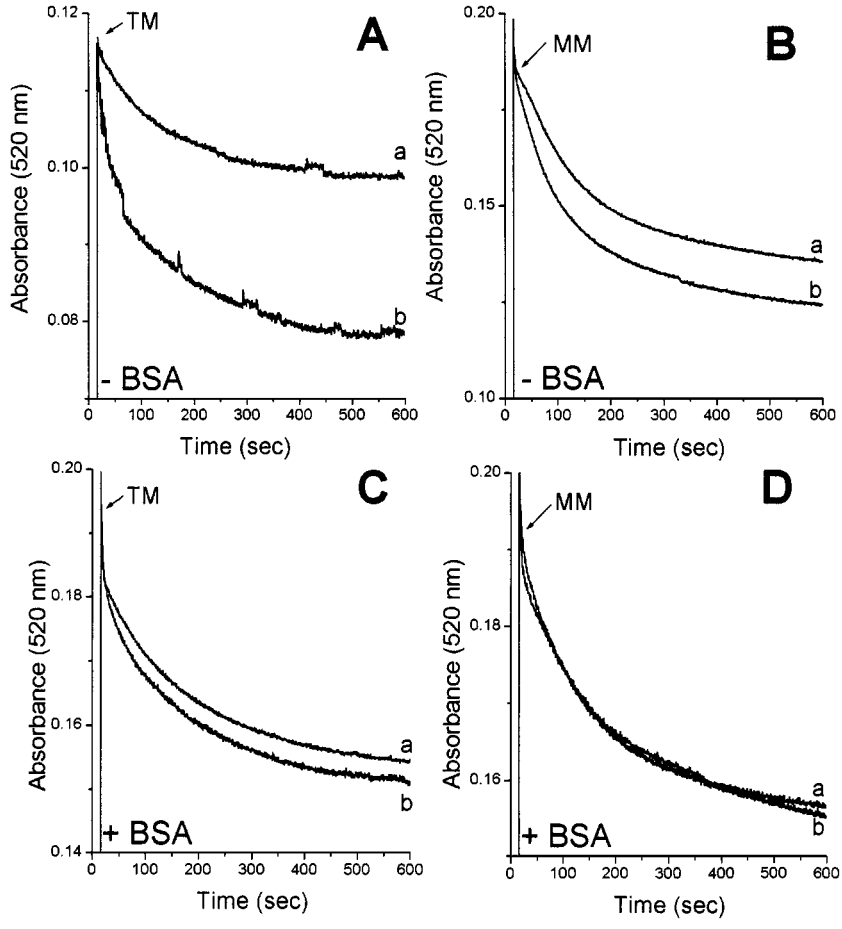

Fig. 5. K^+ transport in tomato and maize mitochondria. Isolated tomato or maize mitochondria (TM or MM, 0.2 mg/mL) were added to a buffer containing 10 mM K^+-HEPES (pH 7.2), 50 mM KCl, 2 μM antimycin, and 1 μg/mL oligomycin in the absence (Panels A and B) or presence (Panels C and D) of 1% BSA, as indicated. ATP (1 mM) was present in lines a.

these conditions (line b) is also not affected by the presence of ATP (line c) or ATP + Mg^{2+} (line a).

We reproduced the potato mitochondrial swelling experiments shown in Fig. 2, using tomato and maize (Fig. 5) and found that tomato and maize mitochondria also swell in KCl (lines b), in a manner uninhibited by ATP (lines a, Panels C and D) unless BSA was absent from the incubation medium (lines a, Panels A and B). Thus, ATP-insensitive K^+ uptake seems to be present in a variety of plant mitochondria.

Next, we measured mitochondrial oxygen consumption in the presence of BSA and under conditions that allow the buildup of a membrane potential, further preventing the uptake of K^+ ions by the K^+/H^+ antiporter. Figure 6(A) shows that uptake of externally added K^+ by the mitochondrial suspension respiring on succinate (plus rotenone) promotes an increase in O_2 consumption due to K^+ cycling (K^+ entry mediated by the ATP-insensitive K^+ pathway and exchange for H^+ by the K^+/H^+ antiporter). Respiratory increases were not observed after the addition of equal amounts of NaCl, indicating that they are not due to osmotic changes in the media (not shown). The increase in respiratory rates promoted by K^+ salts exhibits a hyperbolic dependence with ion concentration, as expected for channel-mediated K^+ transport (Fig. 6(B)), with $K_{1/2} = 27.9 \pm 1.6$ mM and maximal respiratory stimulation of 70.8 ± 1.3 nmoles $O_2 \cdot \text{min}^{-1} \cdot \text{mg} \cdot \text{protein}^{-1}$ ($n = 5$). This is a significant increment in respiratory rates, capable of diminishing oxidative phosphorylation and reflecting K^+ transport rates much higher than those found for mammalian mitoK_{ATP}s (Bajgar *et al.*, 2001; Kowaltowski *et al.*, 2001).

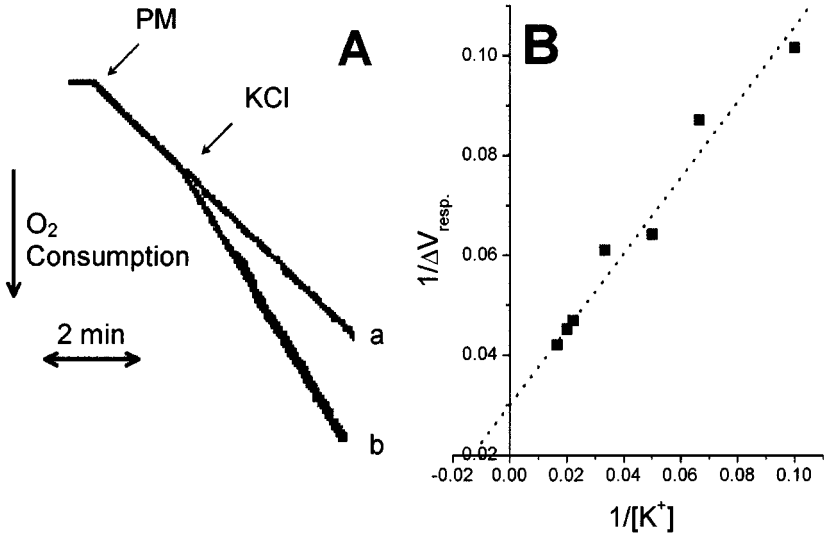

Fig. 6. K^+ transport increases potato mitochondrial respiration. PM (1 mg/mL) respiration was measured in a buffer containing 250 mM sucrose, 10 mM Na^+-HEPES, 1% BSA, 2 mM Na^+-succinate, 1 μg/mL oligomycin, and 2 μM rotenone (pH 7.2). In Panel A, KCl (60 mM, line b) was added where indicated. In Panel B, changes in respiratory rates are plotted against [K^+] added to the reaction media, using experimental conditions identical to Panel A.

Regulation of K^+ Uptake

The K^+ effect on respiratory rates was not altered by the addition of 1 mM ATP (Fig. 7, line b) or ATP + Mg^{2+} (not shown), confirming that the K^+ channel in potato mitochondria is not a mitoK_{ATP}. A variety of compounds that regulate other K^+ channels were then tested to further characterize this transport. We observed that the potato channel is not regulated by NADH, 5-hydroxydecanoate, or glybenclamide (not shown), but is sensitive to quinine, a broad-spectrum K^+ channel inhibitor (Fig. 7, line a), in a dose-dependent manner ($K_{1/2} = 254 \pm 10$ μM, $n = 3$).

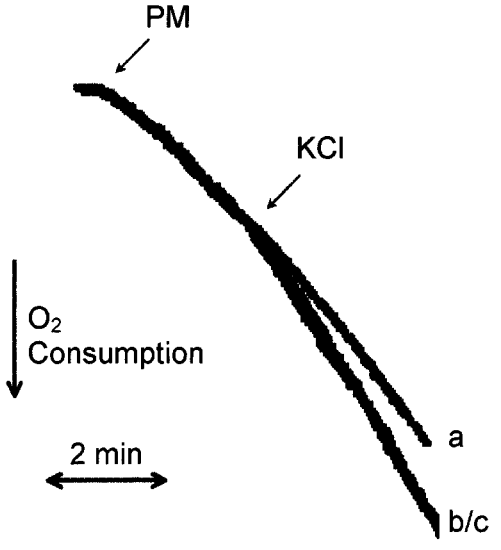

Fig. 7. Regulation of K^+-stimulated respiration. PM (1 mg/mL) respiration was measured under the conditions described in Fig. 6, in the presence of 150 μM quinine (line a), 1 mM ATP (line b), or no further additions (line c). KCl (30 mM) was added where indicated by the arrow.

Unfortunately, quinine doses above 150 μM decrease maximal respiratory rates (not shown). To exclude the possibility that quinine acts inhibiting respiration and not K^+ transport, we tested the compound using nonrespiring mitochondrial swelling experiments, and found that quinine inhibits K^+ transport in the absence of respiration (Fig. 8(A)). Since under these conditions the inhibitory effect of quinine could be due to PIMAC inhibition (Beavis and Vercesi, 1992), we also monitored mitochondrial swelling in KSCN buffer, in which swelling depends only on K^+ uptake. Indeed, under these conditions, experiments in which K^+ transport was maximized by nigericin and FCCP indicated that the mitochondrial inner membrane is freely permeable to SCN^- even in the presence of quinine (not shown). In the absence of nigericin and FCCP (Fig. 7(B)), the inhibitory effect of quinine on mitochondrial swelling in KSCN confirms that the drug directly prevents K^+ uptake, strongly suggesting this uptake is mediated by a quinine-sensitive channel.

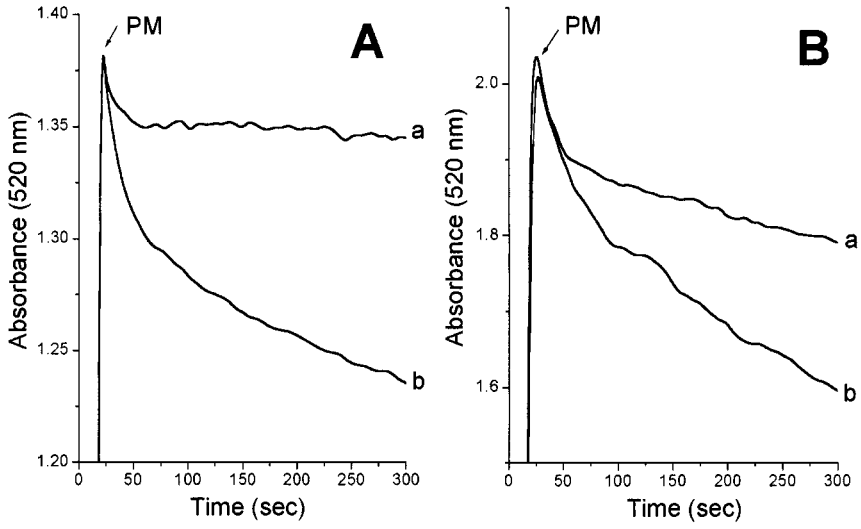

Fig. 8. K^+-induced swelling is inhibited by quinine. PM (0.2 mg/mL) were added to a buffer containing 10 mM K^+-HEPES (pH 7.2), 50 mM KCl (Panel A) or 50 mM KSCN (Panel B), 2 μM antimycin, and 1 μg/mL oligomycin. K^+ uptake was measured by following light scattering in the absence (lines b) or presence (lines a) of 1 mM quinine.

K^+ Transport Increases Heat and Decreases Reactive Oxygen Release

Since plant mitochondrial K^+ transport significantly increased respiration in the absence of oxidative phosphorylation, we hypothesized that it acts as a proton potential dissipative pathway in conjunction with the K^+/H^+ exchanger. To confirm this hypothesis experimentally, we measured heat release promoted by K^+ transport under conditions in which other plant mitochondrial dissipative pathways (PUMP and AOX) were inhibited through the use of BSA and benzohydroxamate (Schonbaum *et al.*, 1971). Figure 9(A) shows typical traces of heat release from potato mitochondria measured using isothermal titration calorimetry. In the absence of K^+, total heat exchange after 6 min was -7.8 mcal (line a). Adding K^+ to the reaction media promoted a marked stimulation of mitochondrial heat release (line b, QT $= -9.5$ mcal), suggesting that K^+ transport in potato mitochondria promotes futile H^+ cycling and generates heat.

In addition to regulating metabolism and generating heat, H^+ cycling has the beneficial effect of preventing mitochondrial reactive oxygen formation due to increased respiratory rates (Korshunov *et al.*, 1997; Kowaltowski *et al.*, 1998). Indeed, we found that potato mitochondria incubated in K^+ salts generated significantly less H_2O_2 (Fig. 9(B), line b) when compared to mitochondria incubated in the absence of K^+ (line a). Thus, the prevention of mitochondrial oxidative stress may be an additional function of plant mitochondrial K^+ import pathways.

DISCUSSION

We describe here a highly active K^+ import pathway in plant mitochondria which promotes mitochondrial swelling, increases respiratory rates, stimulates heat release, and decreases mitochondrial reactive oxygen species generation. All these characteristics are typical of a pathway that dissipates the mitochondrial proton potential and decreases the efficiency of oxidative phosphorylation.

K^+ uniporters in the inner mitochondrial membrane have been previously described in mammalian tissue and are strongly inhibited by Mg^{2+}–ATP (Bajgar *et al.*, 2001; Garlid, 1996; Kowaltowski *et al.*, 2001). A striking characteristic of these mammalian channels is their low conductivity—although they increase inner-membrane permeability to K^+, the transport rate of the ion is so low that the overall effect on the membrane potential is often undetectable (Bajgar *et al.*, 2001; Kowaltowski *et al.*, 2001) and changes in the efficiency of oxidative phosphorylation are minimal.

In contrast to K^+ transport characteristics in mammalian mitochondria, Pastore *et al.* (1999) and Chiandussi *et al.* (2002) found that K^+ was imported at high rates into plant mitochondria, uncoupling respiration from oxidative phosphorylation. These authors also reported that ATP inhibited the import pathway, in the absence of Mg^{2+}. However, we found here that the inhibition reported by these authors is only observed in the absence of the fatty acid quencher BSA (Figs. 2 and 4, see scheme in Fig. 3). This suggested two possibilities: (i) plant mitoK_{ATP} depends

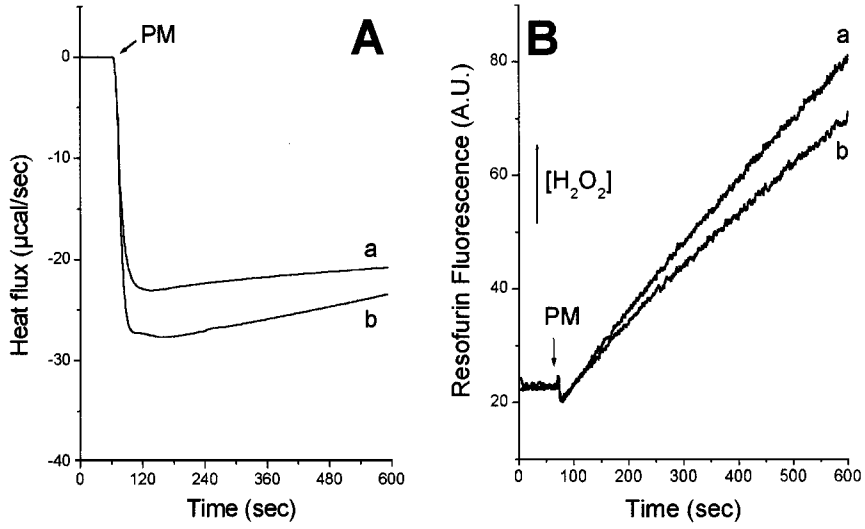

Fig. 9. Heat and reactive oxygen release effects of K^+ transport. PM (0.2 mg/mL) were added where indicated to a buffer containing 10 mM Na^+-HEPES (pH 7.2), 250 mM sucrose, 2 mM Na^+-succinate, 2 μM rotenone, and 1% BSA, in the presence (lines b) or absence (lines a) of 20 mM KCl. In Panel A, 2 mM benzohydroxamate was present in both traces, and heat changes were monitored as described in Experimental Procedures. In Panel B, 30 μM Amplex Red and 1 U/mL horseradish peroxidase were present to monitor H_2O_2 release.

on fatty acids or (ii) ATP does not inhibit plant K^+ transport and the ATP effect observed previously is attributable to PUMP. The first possibility was excluded by the finding that mitochondria incubated in the presence of BSA increase in volume (Fig. 2) and present larger oxygen uptake rates (Fig. 5) when treated with K^+. ATP and other known mitoK_{ATP} inhibitors did not prevent these respiratory changes (Fig. 6), further supporting the notion that plant mitochondria present a highly active ATP-insensitive K^+ import pathway. However, although our data show that high K^+ transport rates in plant mitochondria occur through an ATP-insensitive pathway, they do not exclude the concomitant existence of mitoK_{ATP}s in these mitochondria (Paucek *et al.*, 2002), as long as these channels present limited K^+ transport rates, as observed in mammalian tissue (Bajgar *et al.*, 2001; Kowaltowski *et al.*, 2001).

The ATP-insensitive K^+ import pathway described here exhibits saturation kinetics (Fig. 6), and can be inhibited by quinine (Figs. 7 and 8), supporting the idea that this transport is not a consequence of the inner-membrane K^+ "leak," but a K^+ channel. The $K_{1/2}$ of this putative channel (~30 mM) and the fact that it is not inhibited by physiological quantities of ATP suggests it can be fully active within intact cells, where K^+ concentrations are approximately 140 mM (Hanson, 1985).

K^+ transport promoted by the plant pathway is quite large compared to mammalian mitoK_{ATP} (Bajgar *et al.*, 2001; Kowaltowski *et al.*, 2001), indicating that extensive quantities of K^+ can be imported into the plant mitochondrial matrix through this putative channel. This could promote mitochondrial swelling and even rupture if plant mitochondria did not present a highly efficient K^+/H^+ exchanger (Diolez and Moreau, 1985), which removes matrix K^+ in a manner stimulated by mitochondrial swelling. In fact, maximal respiratory rates are maintained for long periods after the addition of K^+ to potato mitochondria (Fig. 6(A)), suggesting no organelle rupture and cytochrome c loss occurs. As a result, the most evident effect of this K^+ transport in vivo is probably a decrease in mitochondrial coupling, dissipating energy and avoiding ATP synthesis. Supporting this idea, K^+ transport in these mitochondria increases heat release (Fig. 9).

The presence of a channel with a primarily dissipative function in the mitochondrial inner membrane is surprising, since two distinct dissipative pathways have previously been described in plant mitochondria: AOX and PUMP (Huq and Palmer, 1978; Jezek *et al.*, 1996; Rich, 1978). It is thus interesting to investigate when each of these pathways acts. Since the activation of plant K^+ transport generates heat, we tested the effect of cold exposure on this transport. We found that refrigerating potatoes for 2–3 days prior to mitochondrial isolation did not increase K^+ transport and change the affinity for the ion or regulatory characteristics (not shown). Thus, heat generation may not be the primary role of plant K^+ transport.

Similarly to both PUMP and AOX (Kowaltowski *et al.*, 1998; Popov *et al.*, 1997), K^+ transport decreased mitochondrial reactive oxygen release (Fig. 9). This may be an important function for dissipative pathways, which prevent reactive oxygen release by decreasing the accumulation of electrons at initial steps of the respiratory chain, where they can generate superoxide radicals, and by decreasing the local oxygen tensions (Korshunov *et al.*, 1997). Supporting this hypothesis, PUMP knockout plants were found to be more susceptible to oxidative stress. Furthermore, exogenous additions of oxidants promote increased PUMP expression (Brandalise *et al.*, 2003a,b).

Finally, an interesting hypothesis for the existence of many dissipative pathways in plant mitochondria is that these pathways are necessary for plant metabolic regulation (Sluse and Jarmuszkiewicz, 2002). Mitochondria contribute to anabolic function when photosynthetic activity is high, synthesizing most precursors for macromolecules in the tricarboxylic acid cycle. Therefore, the tricarboxylic acid cycle must function independently of the bioenergetic status and ATP levels. In plant mitochondria, dissipative pathways ensure the oxidation of excess reducing equivalents even when ATP levels are high, allowing the tricarboxylic acid cycle to function. In this sense, further studies determining endogenous K^+ transport regulation and its interaction with other dissipative pathways should help establish the exact role of each pathway.

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