Activation of the plant mitochondrial potassium channel by free fatty acids and acyl-CoA esters: a possible defence mechanism in the response to hyperosmotic stress

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Abstract

The effect of free fatty acids (FFAs) and acyl-CoA esters on K⁺ uptake was studied in mitochondria isolated from durum wheat (Triticum durum Desf.), a species that has adapted well to the semi-arid Mediterranean area and possessing a highly active mitochondrial ATP-sensitive K⁺ channel (PmitoK ATP), that may confer resistance to environmental stresses. This was made by swelling experiments in KCl solution under experimental conditions in which PmitoK ATP activity was monitored. Linoleate and other FFAs (laurate, palmitate, stearate, palmitoleate, oleate, arachidonate, and the non-physiological 1-undecanesulphonate and 5-phenylvalerate), used at a concentration (10 μM) unable to damage membranes of isolated mitochondria, stimulated K⁺ uptake by about 2–4-fold. Acyl-CoAs also promoted K⁺ transport to a much larger extent with respect to FFAs (about 5–12-fold). In a different experimental system based on safranin O fluorescence measurements, the dissipation of electrical membrane potential induced by K⁺ uptake via PmitoK ATP was found to increase in the presence of 5-phenylvalerate and palmitoyl-CoA, both unable to elicit the activity of the Plant Uncoupling Protein. This result suggests a direct activation of PmitoK ATP. Stimulation of K⁺ transport by FFAs/acyl-CoAs resulted in a widespread phenomenon in plant mitochondria from different mono/dicotyledonous species (bread wheat, barley, triticale, maize, lentil, pea, and topinambur) and from different organs (root, tuber, leaf, and shoot). Finally, an increase in mitochondrial FFAs up to a content of 50 nmol mg⁻¹ protein, which was able to activate PmitoK ATP strongly, was observed under hyperosmotic stress conditions. Since PmitoK ATP may act against environmental/oxidative stress, its activation by FFAs/acyl-CoAs is proposed to represent a physiological defence mechanism.

Key words: Acyl-CoA esters, durum wheat mitochondria, electrical membrane potential, free fatty acids, mitochondrial swelling, osmotic stress, plant mitochondria, potassium channel, salt stress.

Introduction

Plant mitochondria possess protein-mediated K⁺ uptake systems, the first of which, an ATP-sensitive K⁺ channel, was discovered about ten years ago in durum wheat mitochondria (DWM) (Pastore et al., 1999a). This channel has been named PmitoK ATP in analogy with the animal counterpart, mitoK ATP (Garlid, 1996). Other ATP-sensitive
K⁺ import pathways have been described in mitochondria from etiolated pea stems (Petrussa et al., 2001, 2004; Chiandussi et al., 2002; Casolo et al., 2003), from soybean cell cultures (Casolo et al., 2005), from embryogenic cultures of Picea abies (L.) Karst., Abies cephalonica Loud (Petrussa et al., 2008a), Abies alba Mill. (Petrussa et al., 2009), and from Arum spadix and tubers (Petrussa et al., 2008b). The existence of an ATP-insensitive quinone-inhibited K⁺ transporter has been reported in mitochondria from potato tubers, etiolated seedlings of maize, and tomato fruits (Ruy et al., 2004). A large-conductance calcium-activated K⁺ channel has also been described recently in potato tuber mitochondria (Koszela-Piotrowska et al., 2009). K⁺ uptake has also been shown in mitochondria from etiolated seedlings of bread wheat, barley, spelt, rye, from green leaves of spinach, from potato tubers (Pastore et al., 1999a) and from potato cell cultures (Fratianne et al., 2001).

Among mitochondrial K⁺ channels, PmitoKATP shows intriguing effects on the bioenergetics of isolated mitochondria suspended in media containing K⁺. This channel catalyses the electrophoretic uniport of K⁺ across the inner mitochondrial membrane towards the matrix. The cooperation between PmitoKATP and the K⁺/H⁺ exchanger, very active in plant mitochondria (Diolez and Moreau, 1985), allows the operation of a K⁺ cycle that may induce H⁺ re-entry in the mitochondrial matrix. Whereas, in mammals, the K⁺ cycle cannot uncouple mitochondria, the maximal rate of this cycle being only about 20% of that of the proton-ejection of the respiratory chain (Pastore et al., 1999a), in plant mitochondria the activity of this K⁺ pathway is expected to be approximately equal to the proton-ejecting capacity. In fact, the K⁺ cycle flux is sufficient to collapse the proton motive force (Δp) of isolated mitochondria completely (Pastore et al., 1999a, 2007), by dissipating, in particular, the electrical membrane potential (ΔΨ), the main component of Δp in plant mitochondria (Douce, 1985, and references therein). By lowering ΔΨ and Δp, PmitoKATP may dampen the generation of reactive oxygen species (ROS) in mitochondria purified in vitro (Pastore et al., 1999a, 2007). This is explained according to the ‘mild uncoupling’ theory proposed by Skulachev (Skulachev, 1998). Briefly, the occurrence of a deep positive relationship between ΔΨ and ROS production by the respiratory chain has been well established (in particular, the semiquinone CoQH₂ is involved in the non-enzymatic one-electron reduction of oxygen to the superoxide anion, that may generate other ROS): high ΔΨ values (over 140 mV) may induce a dramatic stimulation of superoxide production; accordingly a small ΔΨ decrease, below the critical level, may also markedly decrease superoxide generation. This means that some increase in the proton leak may prevent a burst of superoxide production. In light of this, regulatory mechanisms able to control ΔΨ levels, such as H⁺ re-entry generated by the K⁺ cycle due to the PmitoKATP-K⁺/H⁺ antiporter combined function, as well as the free fatty acid (FFA)-induced H⁺ transport by the Uncoupling Proteins (UCPs), may decrease ROS formation in mitochondria isolated in vitro. The capability of PmitoKATP to control mitochondrial ROS generation has been demonstrated in DWM from both control (Pastore et al., 1999a) and salt- and osmotically stressed seedlings (Trono et al., 2004). Therefore, PmitoKATP has been suggested to co-operate with the cellular antioxidant systems and with the other plant mitochondrial energy-dissipating systems, i.e. the UCP (Pastore et al., 2000) and the Alternative Oxidase (AOX) (Pastore et al., 2001), to defend the cell from oxidative stress occurring when plants suffer environmental stresses (Pastore et al., 2007).

In plant cells, the K⁺ cytosol concentration is homeostatically maintained at very high levels (80–100 mM) (Leigh and Wyn Jones, 1984) by the activity of K⁺ influx and efflux channels (for the regulation mechanisms see Lebudy et al., 2007; Szczerba et al., 2009). Since, in DWM, a K⁺ content ranging from 50–100 mM, similar to that of cytosol, was measured by means of atomic absorption technique (D Pastore et al., unpublished data), the electrophoretic K⁺ uptake via a fully opened PmitoKATP is mainly driven by high ΔΨ (ranging from 170–200 mV in different DWM preparations; Trono et al., 2004). This transport is active enough to dissipate Δp completely, with the possible impairment of mitochondrial functions. Therefore, a careful and fine modulation of PmitoKATP activity is expected to occur in vivo. In light of this, studies of PmitoKATP regulation to characterize physiological modulators appear to be necessary to understand the in vivo function of this channel fully, in particular, under environmental/oxidative stress conditions. PmitoKATP is known to be modulated by physiological compounds, including the inhibitors ATP and NADH and the activators GTP, palmitoyl-CoA, and CoA; PmitoKATP is also activated as a consequence of mitochondrial treatment with the superoxide anion (Pastore et al., 1999a, 2007). In particular, with respect to ROS and ATP modulation, a feedback mechanism has been depicted under hyperosmotic/oxidative stress conditions: the increased ROS generation (Trono et al., 2004) and the inhibited ATP synthesis (Flagella et al., 2006) may elicit PmitoKATP activity, which, in turn, may control large-scale ROS production (Trono et al., 2004; Pastore et al., 2007). For a recent review about ROS homeostasis and signalling during drought and salinity stresses see Miller et al. (2010). In accordance with the involvement of the ROS-activated PmitoKATP in the environmental stress response, it should also be underlined that ROS can activate plasma membrane non-selective cation influx channels in plant cells, as well as K⁺ efflux channels, both playing a critical role in ROS-mediated plant regulatory reactions, including the plant cell response to stress (Demidchik and Maathuis, 2007; Demidchik et al., 2010).

In this study, the possibility of a PmitoKATP modulation by FFAs and their acyl-CoA esters derivatives has been evaluated. This is in light of the ability of palmitoyl-CoA to activate PmitoKATP strongly (Pastore et al., 1999a) and of FFAs to activate or inhibit other mitochondrial dissipative systems, such as (P)UCP (Vercesi et al., 2006; Echtay, 2007).
and AOX (Sluse et al., 1998), respectively, as well as to modulate the Plant Inner Membrane Anion Channel (PIMAC) (Laus et al., 2008) and the mammalian mitochondrial K+ channel (Schönfeld et al., 2003).

Our results show that, in DWM, FFAs and acyl-CoAs activate K+ transport; by enlarging the study to mitochondria from different species and organs, this effect has been found to be widespread in plants. Under hyperosmotic (NaCl or mannitol) stress conditions, endogenous FFAs have been found to increase up to a content able to activate PmitoKATP strongly. This suggests that activation may have a role to play in environmental/oxidative stress because of the crucial role of plant mitochondria in orchestrating drought tolerance (Atkin and Macherel, 2009).

Materials and methods

Chemicals and plant material

All chemicals (at the highest commercially available purity) were purchased from Sigma Chemical Co. (St Louis, MO, USA). Substrates were used as Tris-(hydroxymethyl)-aminomethane (TRIS) salts at pH 7.20. Carboxylic anhydride p-(trifluoromethoxy)-phenylhydrazone (FCCP), valinomycin, and FFAs were dissolved in ethanol.

Certified seeds of durum wheat (Triticum durum Desf., cv. Ofanto), bread wheat (Triticum aestivum L.), barley (Hordeum vulgare L.), triticale (Triticum×secalae), maize (Zea mays L.), and tubers of topinambur (Jerusalem artichoke: Helianthus tuberosus L.) were kindly supplied from the Cereal Research Centre (Foggia, Italy). Certified seeds of pea (Pisum sativum L.) and lentil (Lens esculenta M.) were purchased from an agricultural pharmacy.

Seedling growth

Durum wheat seeds were germinated in deionized water for 3 d in darkness, at 25 °C and 80–85% RH, as reported in Pastore et al. (1999b). Severe salt- and osmotic-stressed seedlings of durum wheat were obtained as described in Trono et al. (2004) and Soccio et al. (2010), by substituting water with 0.21 M NaCl solution (conductivity 20 dS·m−1; Ψw = −1.04 MPa) and 0.42 M mannitol solution (having the same osmolality as the NaCl solution), respectively.

Bread wheat, barley, triticale, pea, and lentil seeds were grown essentially as described for durum wheat seedlings in darkness, at 25 °C and 80–85% RH, for 4 d and 6 d, respectively (comprising 24 h and 60 h of light exposure, respectively).

Mitochondria isolation

DWM were purified from 3-d-old etiolated shoots, as previously reported in Pastore et al. (1999b) with minor modifications. The grinding and washing buffers were: (i) 0.5 M sucrose, 4 mM cysteine, 1 mM EDTA, 30 mM TRIS-HCl (pH 7.5), 0.1% (w/v) defatted BSA, 0.6% (w/v) polyvinylpyrrolidone (PVP)-360; and (ii) 0.5 M sucrose, 1 mM EDTA, 10 mM TRIS-HCl (pH 7.4), 0.1% (w/v) defatted BSA, respectively. Washed mitochondria were purified by isopycnic centrifugation in a self-generating density gradient containing 0.5 M sucrose, 10 mM TRIS-HCl (pH 7.2) and 28% (v/v) Percoll (colloidal PVP-coated silica) in combination with a linear gradient of 0% (top) to 10% (bottom) PVP-40 (Moore and Proudlive, 1987). The final mitochondrial suspension was diluted with an appropriate volume of a sucrose-free washing buffer in order to obtain a 0.3 M sucrose concentration. This protocol gives mitochondria with high intactness of inner and outer membranes and well-coupled (Pastore et al., 1999b).

Mitochondria from salt- and osmotic-stressed seedlings of durum wheat were purified as reported in Trono et al. (2004) and Soccio et al. (2010); they also showed high membrane intactness and a sufficient functionality to study mitochondrial bioenergetics (Trono et al., 2004; Soccio et al., 2010).

Mitochondria from etiolated shoots of bread wheat, barley, triticale, and lentil and from pea roots were obtained essentially as above reported for durum wheat. With respect to mitochondria from green leaves of durum wheat and maize, homogenization was carried out by using a Waring blender homogenizer (speed setting 3×3 s, three times); purification was performed as described for DWM, with the only exception that, in order to prevent chlorophyll contamination, washed mitochondria were passed two consecutive times through the Percoll gradient. Mitochondria from Jerusalem artichoke (topinambur) tubers were isolated essentially as in Liden and Møller (1988) from fresh cut slices of fully mature tubers in the quiescent state, and stored at 4 °C for no more than three months. The grinding and washing buffers were: (i) 0.4 M sucrose, 1 mM EDTA, 30 mM TRIS-HCl (pH 8.2), 0.1% (w/v) defatted BSA, 0.05% (w/v) cysteine; and (ii) 0.3 M sucrose, 1 mM EDTA, 10 mM TRIS-HCl (pH 7.4), 0.1% (w/v) defatted BSA, 0.05% (w/v) cysteine, respectively. Mitochondria obtained from different plant species and organs showed good membrane intactness and functionality, as indicated by the capability to generate high ΔΨ due to succinate oxidation and by the failure of swelling in sucrose medium. Mitochondrial preparations lacking these requisites were discarded.

Mitochondrial protein content was determined by the method of Lowry modified according to Harris (1987), using BSA as a standard.

Swelling experiments

Swelling experiments were performed as described by Pastore et al. (1999a). Absorbance changes at 546 nm of a mitochondrial suspension (0.05 mg ml−1 protein) in 0.36 M sucrose, 0.18 M KCl or 0.18 M tetraethylammonium chloride (TEACl) iso-osmotic solutions (2 ml), buffered with 20 mM TRIS-HCl (pH 7.2), were monitored at 25 °C as a function of time, by a PerkinElmer Lambda 18 UV/Vis spectrophotometer (PerkinElmer, Wellesley, MA, USA). Mitochondrial swelling in iso-osmotic KCl solution depends on both K+ and Cl− influxes, that occur at different rates; in the height of this, the rate of the global process (swelling) reflects the rate of the slowest one. The rate-limiting step of the swelling in KCl is K+ rather Cl− uptake, as demonstrated by the increase in the swelling rate induced by the addition of the K+ ionophore valinomycin, which strongly increases K+ permeability across the inner mitochondrial membrane (see, for example, Fig. 1A). In the light of this, swelling in KCl solution represents a useful tool to study K+ uptake, while swelling in KCl medium in the presence of valinomycin may allow Cl− transport to be measured.

Swelling rate was expressed as ΔΔA546nm min−1 mg−1 protein and calculated as the slope relative to the initial part of the experimental trace. Swelling rate in KCl medium in the presence of acyl-CoA esters was calculated as the highest slope of the experimental curve, occurring about 15 s after the beginning of the swelling.

Fluorimetric measurements of ΔΨ changes

The fluorescent probe safranin O was used to estimate ΔΨ changes, as reported by Moore and Bonner (1982). The fluorescence intensity changes of safranin O were recorded at 25 °C, using excitation and emission wavelengths of 520 nm and 570 nm, respectively, by means of a PerkinElmer LS-50B spectrofluorimeter. The incubation medium (2 ml) contained 0.3 M mannitol,
5 mM MgCl₂, 20 mM TRIS-HCl (pH 7.2), 2.5 μM safranin O, and 0.1 mg ml⁻¹ DWM protein [(safranin O)/(DWM protein) ratio value of 25]. The reaction was started by the addition of 5 mM succinate, as a respiratory substrate.

Calibration of safranin O fluorescence changes as a function of K⁺ diffusion potentials was performed using rat liver mitochondria, as reported by Zotti et al. (1993). Rat liver mitochondria were isolated according to Pastore et al. (1994); the K⁺ diffusion potential in rat liver mitochondria was induced by the addition of 0.05 μg ml⁻¹ valinomycin (Åkerman and Wikström, 1976).

**Determination of FFA content in DWM from control and stressed seedlings**

**Extraction of lipids and separation of FFAs:** Total lipids were extracted from mitochondrial preparations obtained from both control and severe salt- and osmotic-stressed seedlings. In brief, 0.8 ml of mitochondrial suspension (1 mg ml⁻¹ protein) were added together with 0.6 ml of diethyl ether-hexane 1:1 (v/v), to which a known amount of heptadecanoic acid (17:0) had previously been added as the internal standard; the resulting mixture was vigorously shaken and subsequently centrifuged at 1700 g for 10 min. The organic phase was collected and the extraction was repeated another three times. The combined organic phases were diluted with hexane to a final volume of 5 ml. The separation of FFAs from bound FAs present in lipidic mitochondrial extract was achieved by the solid-phase extraction (SPE) procedure. The extraction was performed according to Gambacorta et al. (2009), with minor modifications. The sample obtained was evaporated to dryness under a nitrogen stream.

**Quantification of FFAs:** Quantitative analysis of palmitate, linoleate, and linolenate in the fraction obtained from SPE was carried out by means of gas chromatography coupled with flame ionization detection. The FFA dry residue was subjected to acid-catalyzed methylation; the methylated FAs were extracted with 100 l of hexane, centrifuged at 1000 g for 1 min, and the supernatant was collected. The sample (2 μl) was analysed by Carlo Erba HRGC 5300 mega series chromatograph equipped with a SUPELCOWAX10 capillary column (30 m×0.25 mm i.d. ×0.25 μm film thickness, Supelco Inc, Bellerofonte, PA). Methyl esters were identified and quantified by a comparison of their retention times and areas with that of known standards.

Linoleate content in the SPE fraction was also confirmed by means of gas chromatography coupled to mass spectrometry. In this case, the FFA dry residues were reconstituted in 10 μl of diethyl ether and 2 μl of the sample was injected into the gas chromatographic system. A 6890N series gas chromatograph (Agilent Technologies) with an Agilent 5973 mass selective detector and equipped with a ZB-FFAP capillary column (30 m×0.25 mm i.d. ×0.25 μm film thickness, Restek, Bellerofonte, PA) was used.

**Results**

In order to gain a first insight into the effect of FFAs on K⁺ permeability in purified DWM, K⁺ uptake was studied by means of swelling experiments in iso-osmotic KCl solutions.

As shown in Fig. 1A, DWM absorbance in 0.36 M sucrose solution was found to remain constant during the time, thus demonstrating the intactness of mitochondrial membranes. No swelling in the sucrose solution was observed after the addition of either 10 μM linoleate or linoleoyl-CoA. The ineffectiveness of linoleate and linoleoyl-CoA excludes damage to the inner membrane under the adopted experimental conditions as a consequence of detergent action. Moreover, a control was made that linoleate did not promote aspecific permeabilization processes attributable to the cyclosporin A (CsA)-sensitive mitochondrial permeability transition (MPT) (for a review see Halestrap, 2009) or to the non-classical CsA-insensitive permeability transition (NCPT) (Sultan and Sokolove, 2001), evaluated

**Fig. 1.** Effect of linoleate and linoleoyl-CoA on DWM swelling in iso-osmotic solution of KCl, of KCl plus valinomycin, and of TEACl. (A) DWM (0.1 mg protein) were suspended in 2.0 ml of a medium consisting of 20 mM TRIS-HCl pH 7.2 and 0.36 M sucrose or 0.18 M KCl or in KCl medium supplemented with: 10 μM linoleate (Lin); 10 μM linoleoyl-CoA (Lin-CoA); 10 μM Lin plus 0.1% (w/v) BSA; 10 μM Lin-CoA plus 0.1% (w/v) BSA; 0.5 μg valinomycin (Val). At the time indicated by the arrows 10 μM Lin or Lin-CoA and 0.5 μg Val were added. Swelling was continuously monitored by measuring the absorbance decrease at 546 nm and 25 °C, as reported in the Materials and methods. (B) Swelling rates in 0.36 M sucrose, 0.18 M TEACl, 0.18 M KCl, and 0.18 M KCl plus 0.5 μg Val either in the absence or presence of Lin or Lin-CoA are reported as a function of Lin (full symbol) or Lin-CoA (empty symbol) concentration. Data are expressed as mean value ± standard deviation (n=3).
essentially as reported in Schönfeld and Bohnensack (1997) and Sultan and Sokolove (2001), respectively (data not shown).

On the other hand, DWM exhibited a fast and clearly evident swelling in iso-osmotic KCl solution, depending on K* and Cl* uptake mediated by PmitoK\textsubscript{ATP} (Pastore et al., 1999\textit{a}) and PIMAC (Laus et al., 2008), respectively. Swelling rate in KCl was strongly increased by the K* ionophore valinomycin, either added in the course of the swelling or already present in the reaction medium, thus indicating that the swelling rate in KCl solution is a measure of the rate of K* uptake, while the swelling rate in KCl plus valinomycin reflects the rate of Cl* uptake (see also the Materials and methods). When 10 \( \mu \text{M} \) linoleate was added to KCl medium, a large increase in the rate of K* uptake of about 160\% with respect to the control was observed, further increased by valinomycin, so indicating K* uptake as the rate-limiting step under these conditions as well. About 55\% inhibition of this linoleate-stimulated swelling rate was induced by the addition of 0.1\% BSA, that binds FFAs. Complete recovery was not obtained in the presence of higher BSA concentrations [up to 0.5\% (w/v)] (data not shown). Since acyl-CoAs are obligate intermediates of FFA metabolism, the effect of linoleoyl-CoA on K* transport was also evaluated. In the presence of 10 \( \mu \text{M} \) linoleoyl-CoA, a much more evident increase of about 55\% of swelling rate in KCl solution was found, slightly inhibited by BSA, in accordance with the BSA ability to bind acyl-CoAs (Richards et al., 1990).

The effect of increasing linoleate and linoleoyl-CoA concentrations on DWM swelling in sucrose, KCl, and KCl plus valinomycin was also evaluated (Fig. 1B). Both linoleate and linoleoyl-CoA, up to 30 \( \mu \text{M} \), did not significantly affect DWM permeability to sucrose. Consistently, under these experimental conditions, no significant release into the extramitochondrial phase of the matrix malate dehydrogenase was observed (data not shown). By contrast, an increasing stimulation of K* uptake (30–285\%) was observed with increasing linoleate concentrations from 2 \( \mu \text{M} \) to 20 \( \mu \text{M} \). As expected, in the light of the recently demonstrated FFA-dependent inhibition of anion uptake via PIMAC (Laus et al., 2008), linoleate also induced a progressive decrease of the swelling rate in KCl plus valinomycin (representing the rate of Cl* uptake). As a result of the combined linoleate capability to activate K* uptake and inhibit anion transport, when FFA concentration was higher than 20 \( \mu \text{M} \), the swelling rates in KCl were the same in both the absence and presence of valinomycin and represented a measure of Cl* uptake. Increasing concentrations of linoleoyl-CoA from 1 \( \mu \text{M} \) to 20 \( \mu \text{M} \) caused an increasing activation of K* transport, which resulted at any concentration higher than the linoleate-dependent one. Since linoleoyl-CoA does not affect PIMAC activity (Laus et al., 2008), under these experimental conditions, at 20 \( \mu \text{M} \) linoleoyl-CoA it is possible to observe maximal measurable activation of K* transport.

To unravel the role of PmitoK\textsubscript{ATP} in the activation of K* transport by linoleate and linoleoyl-CoA, swelling in the TEACl medium was studied. This depends on the uptake of TEA*, a cation not transported by either mitoK\textsubscript{ATP} (Beavis et al., 1993) or PmitoK\textsubscript{ATP} (Pastore et al., 1999\textit{a}) (Fig. 1B). Although no significant increase in the swelling rate was observed up to about 15 \( \mu \text{M} \) linoleate, an evident stimulation of TEA* transport was observed at higher linoleate concentrations. This stimulation was prevented by 0.1\% BSA (data not shown). A comparison between swellings in KCl and TEACl suggests that, at linoleate concentrations higher than 15 \( \mu \text{M} \), the involvement of a ‘valinomycin-like effect’ due to the formation of cation–FFA complexes (Sharpe et al., 1994; Zeng et al., 1998) should also be considered to explain the activation fully. By contrast, the activation of K* transport observed up to about 15 \( \mu \text{M} \) linoleate appears only to be PmitoK\textsubscript{ATP}-dependent, the contribution of the ‘valinomycin-like effect’ to K* permeabilization being negligible. Consistently, linoleoyl-CoA, which is unable to form the cation complex, was found not to affect TEA* uptake in the 2.5–15 \( \mu \text{M} \) concentration range (Fig. 1B).

On the whole, the results of Fig. 1 show that FFA concentrations lower than 15 \( \mu \text{M} \) have to be used to evaluate the effect of FFAs on K* transport via PmitoK\textsubscript{ATP}. This is in order to avoid confusion consequent on both a possible rate-limiting step due to Cl* uptake (see above) and a ‘valinomycin-like effect’.

Other natural unsaturated and saturated FFAs with different chain length and their CoA ester derivatives were evaluated for their capability to activate K* uptake in DWM (Fig. 2A, B). To begin with it was determined that all the FFAs/acyl-CoAs tested did not affect DWM permeability to sucrose in a 1–20 \( \mu \text{M} \) concentration range. All the FFAs and acyl-CoAs (10 \( \mu \text{M} \)) tested were found to promote K* uptake; in all cases the activation due to the acyl-CoA was much higher, from 3–6 times, than that of the respective FFA. Two non-natural FFAs, 1-undecanesulphonate and 5-phenylvalerate, were also found to activate the swelling rate in KCl strongly (Fig. 2B). Since these latter two FFAs are not transported by PUCP in DWM (Pastore et al., 2000), as well as in other plant mitochondria (Jezek et al., 1997), our results rule out a possible mechanism of K* transport activation involving a combined action of PUCP with a K*/H* antiporter (see Ruy et al., 2004, and the Discussion).

In order to confirm the involvement of PmitoK\textsubscript{ATP} in the FFA-induced stimulation of K* transport, another method based on \( \Delta \psi \) measurement using the fluorimetric probe safranin O was used. This method allows PmitoK\textsubscript{ATP} activity to be evaluated by measuring the decrease in \( \Delta \psi \) due to the addition of KCl to respiring mitochondria. This can be done at much lower KCl concentrations with respect to the swelling experiments (Pastore et al., 1999\textit{a}), so allowing the effect of FFAs and acyl-CoAs to be checked with a completely different experimental condition. To exclude PUCP activity (Fig. 3B, C), use was made of the non-natural FFA phenylvalerate in \( \Delta \psi \) experiments. Moreover, the use of this FFA has another advantage: the
Fig. 2. Effect of several FFAs and acyl-CoA esters on the swelling rate in KCl solution. Swelling experiments were carried out as reported in Fig. 1A, in 2.0 ml of 0.18 M KCl solution in the absence and presence of either 10 μM FFAs or 10 μM acyl-CoAs. Swelling rates are expressed as a percentage of the control. In (A) the effect of unsaturated FFAs and their acyl-CoA ester derivatives and in (B) the effect of saturated FFAs and their acyl-CoAs as well as of 5-phenylvalerate and 1-undecanesulphonate are shown. The statistical analysis was carried out according to the Tukey’s test (α=0.01).

The effect of phenylvalerate on ΔΨ changes is reported in Fig. 3B. The reaction was started by adding succinate to DWM in order to induce a rapid ΔΨ increase until about 180 mV. In the absence of phenylvalerate, sequential additions of KCl were found progressively to depolarize 180 mV. In the absence of phenylvalerate, sequential DWM in order to induce a rapid

ΔΨ decrease was observed in the presence of phenylvalerate, palmitoyl-CoA (Fig. 5B ), while, as expected, DWM were depolarized at a significant rate by the addition of 15 mM KCl; no Na+-dependent ΔΨ decrease was observed in the presence of palmitoyl-CoA, respectively. Consistently, ATP was able to prevent a K+-dependent ΔΨ decrease more efficiently in control mitochondria than in DWM treated with palmitoyl-CoA (Fig. 4B). Similar results were obtained in the presence of linoleoyl-CoA and arachidonoyl-CoA (data not shown). Therefore, acyl-CoAs may also cause some activation of the ATP-inhibited channel.

ΔΨ experiments were also carried out in order to check PmitoKATP activation by acyl-CoAs. In Fig. 4 the effect of palmitoyl-CoA on DWM ΔΨ changes is shown; when palmitoyl-CoA was added to succinate-respiring DWM, only a negligible ΔΨ decrease was observed, thus showing that it cannot activate PUCP (Fig. 4A); by contrast, addition of KCl was found to decrease ΔΨ to a much higher rate and extent compared with the control. ΔΨ was restored by externally added ATP up to values of about 155 mV and 125 mV in the absence and presence of palmitoyl-CoA, respectively. Consistently, ATP was able to prevent a K+-dependent ΔΨ decrease more efficiently in control mitochondria than in DWM treated with palmitoyl-CoA (Fig. 4B). Similar results were obtained in the presence of linoleoyl-CoA and arachidonoyl-CoA (data not shown). Therefore, acyl-CoAs may also cause some activation of the ATP-inhibited channel.

Some experiments were also performed to evaluate the K+/Na+ selectivity with respect to the FFA/acyl-CoA effect. To do this, ΔΨ experiments were carried out, since they allow ion concentrations resembling the physiological ones to be adopted. In particular, the effect on Na+ uptake of a FFA (phenylvalerate) and an acyl-CoA (palmitoyl-CoA) was tested by measuring the Na+-induced ΔΨ decrease in succinate-respiring DWM (Fig. 5). As shown in Fig. 5A, sequential additions of 5 mM NaCl did not affect the ΔΨ of succinate-respiring DWM, while, as expected, DWM were depolarized at a significant rate by the addition of 15 mM KCl; no Na+-dependent ΔΨ decrease was observed in the presence of phenylvalerate, that induced, on the contrary, a K+-dependent depolarization at a higher rate and extent with respect to the control. Similarly, in the presence of palmitoyl-CoA (Fig. 5B), a slight ΔΨ decrease was found due to NaCl additions, while a very strong K+-dependent ΔΨ decrease was observed with respect to the control. These observations confirm the selectivity of the transport of K+ with respect to Na+ by FFAs/acyl-CoAs.

In order to ascertain whether activation of K+ uptake by FFAs and acyl-CoAs is a property common to other plant mitochondria, K+ transport was studied by means of swelling experiments in mitochondria isolated from different mono and dicotyledonous, C₃ and C₄ plant species,
including durum wheat, bread wheat, barley, triticale, maize, pea, topinambur, and lentil and from different organs, such as the shoot, leaf, root, and tuber (see the Materials and methods). All the species/organs investigated exhibited no swelling in the sucrose solution (data not shown) and evident swelling in the KCl medium occurring at different rates (Table 1), but it was always increased by valinomycin, as well as being promoted by linoleate (data not shown). In this case, analysis of the effect of FFAs/acyl-CoAs on plant mitochondrial K⁺ uptake was extended to the palmitate/palmitoilCoA and oleate/oleoyl-CoA pairs (Table 1). In all the species/organs tested, the swelling rate in KCl was activated by FFAs up to about 4 times with respect to the control. In all cases, acyl-CoAs induced an activation of K⁺ uptake much higher than FFAs, ranging from 3–11 times compared with the control. In the light of these results, activation of K⁺ transport by FFAs/acyl-CoAs may be considered a widespread phenomenon in the plant kingdom, thus suggesting some basic physiological significance.

In the light of the role of PmitoKATP against environmental/oxidative stress, the hypothesis of the occurrence under stress conditions of a PmitoKATP regulation pathway involving FFAs and their CoA ester derivatives was checked. As shown in Fig. 6A, mitochondria purified from severely salt-stressed seedlings, that showed a good intactness as demonstrated by the constancy of the absorbance in sucrose solution, exhibited a large amplitude swelling in KCl solution, which was found to be much higher than that obtained with DWM from control seedlings (dotted line), and to be largely prevented by BSA. This suggests an activation by FFAs. Consistently, a small amount of commercial phospholipase A₂ (PLA₂) purified from bee venom, an enzyme able specifically to hydrolyse membrane glycerophospholipids at the sn-2 position to yield FFAs and lysophospholipids, caused an evident BSA-sensitive increase of the swelling rate in KCl solution of DWM from control seedlings; under our experimental conditions, PLA₂ did not affect mitochondrial integrity, as shown by the lack of any effect on sucrose trace (Fig. 6B).

To confirm whether and to what extent the mitochondrial content of FFAs may increase under hyperosmotic stress conditions, it was studied in NaCl- and mannitol-stressed DWM (Table 2). The content of all the tested FFAs was found to rise remarkably, with higher increases under salt stress with respect to the osmotic one. In particular, carried out as reported in the Materials and methods. DWM (0.2 mg protein) were suspended in 2.0 ml of a medium consisting of 20 mM TRIS-HCl pH 7.2, 0.3 M mannitol, 5 mM MgO₂, 2.5 µM safranin O, with the safranin O fluorescence continuously monitored as a function of time. At the time indicated by the arrows succinate, phenylvalerate, KCl, ATP (together with 2 µg oligomycin and 10 µM atractyloside), Val and FCCP were added at the reported concentrations. In (C) 0.5 mM ATP plus 10 µM atractyloside and 2 µg oligomycin were already present in the reaction medium.
linoleate and palmitate, the most abundant and the most active FFA, respectively, were found to undergo about 30-fold and 26-fold increases with respect to the control under salt stress and about 27-fold and 18-fold increases under osmotic stress, respectively. The total content of FFAs was found to rise, as a result of stress imposition, from about 2 nmol mg\(^{-1}\) protein to about 50 nmol mg\(^{-1}\) protein. The possible occurrence of a significant increase of PmitoK\(_{\text{ATP}}\) activity within this concentration range was evaluated. This was done by using palmitate and palmitoyl-CoA as the FFA/acyl-CoA pair. As expected, an increase in swelling rate with increasing either palmitate or palmitoyl-CoA concentrations was observed, with a much higher activation in the case of the acyl-CoA (Fig. 7). Interestingly, when low increasing concentrations (20–50 nmol mg\(^{-1}\) protein or 1–2.5 \(\mu\)M) of palmitate were added to KCl medium already containing palmitoyl-CoA at a concentration (2.5 \(\mu\)M) resembling the physiological one (Larson and Graham, 2001), a synergistic action was observed with a very strong activation of K\(^{+}\) transport.

In the whole, these results are in accordance with the hypothesis of PmitoK\(_{\text{ATP}}\) activation as a result of FFA release from mitochondrial membrane phospholipids under stress.
Table 1. Swelling rate in iso-osmotic KCl solution of mitochondria from different plant species and organs and effect of palmitate, oleate, palmitoyl-CoA, and oleoyl-CoA

Swelling experiments were carried out as described in Fig. 1A. Mitochondrial proteins (0.1 mg) were suspended in 2 ml of 0.18 M KCl solution either in the absence or presence of the listed compounds at the reported concentrations. For each plant species the organs used to obtain mitochondria are indicated.

<table>
<thead>
<tr>
<th>Monocotyledonous</th>
<th>Dicotyledonous</th>
</tr>
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<tbody>
<tr>
<td>Shoot</td>
<td>Shoot</td>
</tr>
<tr>
<td>Durum wheat</td>
<td>Pea</td>
</tr>
<tr>
<td>Bread wheat</td>
<td>Topinambur</td>
</tr>
<tr>
<td>Barley</td>
<td>Lentil</td>
</tr>
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</table>

| Swelling rate in 0.18 M KCl (ΔΛΨ460nm min⁻¹ mg⁻¹ protein) | 0.33±0.01b | 0.28±0.01 | 1.54±0.09 | 0.60±0.04 | 0.54±0.02 | 0.60±0.05 | 0.63±0.04 | 0.69±0.05 | 0.52±0.01 |
| Swelling rate (% of the control) | +10 μM Palmitate | 277±26 | 326±4 | 175±27 | 210±14 | 276±18 | 230±21 | 295±20 | 287±26 | 202±8 |
| | +10 μM Oleate | 287±25 | 430±30 | 138±17 | 194±8 | 296±23 | 185±15 | 179±17 | 217±5 | 173±16 |
| | +5 μM Palmitoyl-CoA | 550±54 | 964±36 | 417±11 | 873±63 | 705±27 | 538±52 | 914±19 | 402±17 | 708±11 |
| | +5 μM Oleoyl-CoA | 667±65 | 1160±25 | 411±45 | 916±83 | 726±17 | 685±61 | 503±33 | 326±51 | 885±7 |

a C₃ species.
b Mean value ± standard deviation (n=3).

Discussion

In this study, the property of FFAs/acyl-CoAs to enhance K⁺ permeability of DWM and plant mitochondria from different species/organisms has been shown to involve the PmitoK_ATP activation. Under our experimental conditions (1–10 μM FFAs/acyl-CoAs), the occurrence of an impairment to the inner membrane due to FFA/acyl-CoA detergent action was ruled out, and the contribution of FFA ionophoric activity was shown to be negligible. The involvement of MPT- or NCPT-like processes was also excluded in the light of (i) the absence of DWM swelling in succrose medium in the presence of linoleate and the classical MPT/NCPT inducers, Pi and Ca²⁺ (data not shown) and (ii) the FFA-stimulated K⁺ uptake in the absence of Pi and Ca²⁺, as well as in the presence of CsA and the chelating agent EGTA (data not shown). Moreover, the hypothesis of a PUCP involvement was rejected considering the effectiveness in promoting K⁺ transport of phenylvalerate and undecanesulphonate, FFAs not transported by PUCP in DWM (Pastore et al., 2000). These observations also exclude the operation in DWM of the mechanism proposed by Ruy et al. (2004) to explain the sensitivity to BSA and ATP of the swelling of potato mitochondria in KCl. This is based on the occurrence of an ATP-insensitive K⁺ transporter and an ATP-sensitive and FFA-promoted K⁺ pathway involving (i) K⁺ uptake through the K⁺/H⁺ antiporter in co-operation with (ii) PUCP-mediated H⁺ cycling.

The direct involvement of PmitoK_ATP in the activation of K⁺ transport by FFAs was suggested by swelling experiments with phenylvalerate, unable to activate PUCP and to exhibit a flip-flop movement across membranes and, as a consequence, an ionophoric ‘valinomycin-like’ activity. Phenylvalerate, in fact, remarkably enhanced K⁺ transport, but not the PmitoK_ATP-independent TEA⁺ uptake. The activation of PmitoK_ATP by FFAs/acyl-CoAs was clearly demonstrated in energized DWM by means of fluorimetric measurements of ΔΨ changes using safranin O as the probe. Phenylvalerate and palmitoyl-CoA were found to stimulate depolarization, induced by the addition of low KCl (but not NaCl) concentrations to succinate-respiring DWM. This stimulation was partially recovered/prevented by the PmitoK_ATP inhibitor ATP, so indicating that FFAs/acyl-CoAs are activators of PmitoK_ATP able to modulate ATP sensitivity.

The capability of FFAs/acyl-CoAs to promote K⁺ uptake appears to be a widespread property in the plant kingdom, but this is not surprising. In many different mammalian tissues a role of FFAs in the modulation of different plasma membrane K⁺ channels, including also the ATP-regulated channels, has been reported (Kim and Pleumsamran, 2000; Liu et al., 2001; Zhao et al., 2008). Moreover, it should also be considered that the interactions of voltage-sensing domains of voltage-activated plasma membrane K⁺ channels with the surrounding lipid membrane have recently been demonstrated to play a critical role in pore gating (Swartz, 2008; Kreplky et al., 2009; Milescu et al., 2009). With respect to plant cells, the FFA capability both to activate inwardly rectifying K⁺ channels and to inhibit outward K⁺ channels has been demonstrated in guard cell plasma membranes (Lee et al., 1994). By contrast, very few literature data are available about an FFA-dependent modulation of mitochondrial K⁺ channels. Long-chain FFAs have been demonstrated to permeabilize the inner membrane of rat liver mitochondria to K⁺ (and Cl⁻), by promoting a rapid depolarization of mitochondrial Mg₂⁺, that activates latent Mg₂⁺-sensitive ion-conducting pathways, including the K⁺ transporter, the K⁺/H⁺ exchanger, and the IMAC, probably by a direct Mg₂⁺ withdrawal from protein-binding sites (Schönfeld et al., 2003, 2004, and references therein). To the best of our knowledge, this is the first demonstration of an FFA-induced activation of K⁺ uniport in plant mitochondria. Unlike rat liver
mitochondria, in DWM this activation should not involve an endogenous Mg^{2+} release, PmitoKATP activity being Mg^{2+}-insensitive (Pastore et al., 1999a).

As for acyl-CoAs, they represent one of the most important classes of activators of plasma membrane ATP-regulated K^{+} channels in pancreatic β-cells (Schulze et al., 2003; Brännström et al., 2007, and references therein; Webster et al., 2008, and references therein) and in cardiac muscle cells (Liu et al., 2001; Schulze et al., 2003). On the other hand, in rat liver mitochondria palmitoyl-CoA and oleoyl-CoA are known to inhibit mitoKATP strongly (Paucek et al., 1996). Unlike rat liver mitochondria, acyl-CoAs act as powerful activators of PmitoKATP in DWM, as observed for the plasma membrane K_{ATP} channels of cardiac and pancreatic cells.

Since PmitoKATP was reported to act against environmental/oxidative stress, a possible physiological role of

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**Table 2.** FFA content in DWM from control and severe salt- and osmotic-stressed seedlings

FFA content was measured as described in the Materials and methods.

<table>
<thead>
<tr>
<th>FFA</th>
<th>FFA content (nmol mg^-1 protein)</th>
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<tr>
<td></td>
<td>Control</td>
</tr>
<tr>
<td>16:0 Palmitate</td>
<td>0.4±0.1^a</td>
</tr>
<tr>
<td>18:2 D_{9,12} Linoleate</td>
<td>1.1±0.2</td>
</tr>
<tr>
<td>18:3 D_{9,12,15} or D_{6,9,12} Linolenate</td>
<td>0.4±0.1</td>
</tr>
<tr>
<td>Total</td>
<td>1.9±0.4</td>
</tr>
</tbody>
</table>

^a Mean value ± standard deviation (n=3).
PmitoK\(_{\text{ATP}}\) modulation by FFAs/acyl-CoAs under salt stress was evaluated. Salinity, in fact, heavily affects intracellular Na\(^+\)/K\(^+\) homeostasis and K\(^+\) transporters are known to play a crucial role in the plant response to salt stress (Chen et al., 2007, and references therein). In particular, it was evaluated whether and how PmitoK\(_{\text{ATP}}\) activity may be related to FFA content in DWM from salt- and osmotically stressed seedlings. Under NaCl and mannitol stress applied at germination, an increase in FFA-dependent PUCP activity was measured in DWM, that was suggested to depend on an increased availability of endogenous FFAs (Trono et al., 2006). At the same time, an increase of PmitoK\(_{\text{ATP}}\) activity was observed (Trono et al., 2004; Pastore et al., 2007); here, it appears that this may be dependent, in part, on an increased content of endogenous FFAs. Consistently, several studies have related changes in the structural and functional properties of mitochondrial membranes with the plant’s adaptation to stresses. Modifications of linolenic acid content of mitochondrial membranes have been observed in soybean seedlings in response to salicylic acid treatment (Matosa et al., 2009). A higher ratio between unsaturated and saturated 18-carbon FAs of mitochondrial membranes and a higher membrane fluidity were suggested to be involved in chilling tolerance of maize seedlings (De Santis et al., 1999). A loss of membrane phospholipids was measured in mitochondria from castor bean endosperm exposed to hydrogen peroxide treatment (Kwon et al., 1998). Mitochondria isolated from mild osmotically stressed wheat shoots showed altered FA and phospholipid composition and transport processes (Klein et al., 1986).

The increase of FFAs measured in DWM under severe salt and osmotic stress conditions was remarkable (up to about 50 nmol mg\(^{-1}\) protein). The same amount of an externally added FFA (palmitate) caused a significant 2-fold activation of PmitoK\(_{\text{ATP}}\). Interestingly, when the FFA was added in the presence of a basal concentration of an acyl-CoA, the activation was very strong: the palmitate/palmitoyl-CoA pair generated a 12-fold activation of the PmitoK\(_{\text{ATP}}\) when palmitate and palmitoyl-CoA were both 2.5 \(\mu\)M (i.e. 50 nmol mg\(^{-1}\) protein). This PmitoK\(_{\text{ATP}}\) activation is comparable with that of PUCP (from 7–15-fold) measured under severe salt and osmotic stress (Trono et al., 2004). It should be noted that the acyl-CoA basal concentration (2.5 \(\mu\)M) used in our experiment may be considered physiological, since it was found to range between about 2.5 \(\mu\)M and 6 \(\mu\)M in different species and developmental stages (Larson and Graham, 2001).

Now the question arises about how the FFA content may increase under stress. Interestingly, mammalian mitochondria possess some PLA\(_2\) isoforms able to hydrolyse membrane glycerophospholipids and to release FFAs (Nakamura et al., 1991; Ghosh et al., 2006; Kinsey et al., 2007, and references therein); these enzymes may be activated by ROS (Madesh and Balasubramanian, 1997; Goto et al., 1999; Guidarelli and Cantoni, 2002) or under stress conditions, for example, under cerebral (Bonventre and Koroshetz, 1993; Adibhatla and Hatcher, 2006) and renal (Nakamura et al., 1991) ischemia-reperfusion, and are implicated in the pathogenesis of some neurodegenerative diseases (Sun et al., 2007). To date, no information is available concerning the occurrence of a PLA\(_2\) activity in...
plant mitochondria, but the ability of a commercial PLA
catalytic action of DWM swelling in KCl suggests that a similar
method might operate in durum wheat under hyper-
osmotic stress conditions to activate PmiKATP. This point
will merit further investigation.

In conclusion, the PmiKATP activation by FFAs/acyl-
CoAs demonstrated in this study allows a regulation
pathway operating under hyperosmotic/oxidative stress to
be suggested: under these conditions an increase in mito-
chondrial FFA content occurs that may activate Pmito-
KATP and also generate acyl-CoAs (Fig. 8). The combined
FFA/acyl-CoA activation of PmitoKATP may be about as
strong as that of PUCP under the same stress conditions.
This pathway is to be added to the other, recently described,
pathway involving ATP and ROS (see Introduction).
PmitoKATP activation under stress conditions due to the
functioning of both the FFA/acyl-CoA and ROS/ATP
pathways may collapse ΔΨm, so controlling the harmful
large-scale ROS production according to a feedback mecha-
nism. To date, it is not clear whether this FFA/acyl-CoA
modulation of PmitoKATP may play a role in the manifes-
tation of programmed cell death (PCD). However, the
involvement of plasma membrane ROS-activated K+ chan-
nels in stress-induced PCD (Demidchik et al., 2010, and
references therein), as well as some mitochondrial K+ATP
channels (Casolo et al., 2005; Petrussa et al., 2009) is well
established.

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expressed form of human cytosolic phospholipase A2β (cPLA2β):
Plant mitochondrial potassium channel and FFAs/acyl-CoAs


