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Modulation of a Plant Mitochondrial K^+_{ATP} Channel and Its Involvement in Cytochrome *c* Release

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Pea stem mitochondria, resuspended in a KCl medium (de-energized mitochondria), underwent a swelling, as a consequence of K^+ entry, that was inhibited by ATP. This inhibition was partially restored by GTP and diazoxide (K^+_{ATP} channel openers). In addition, glyburide and 5-hydroxydecanate (K^+_{ATP} channel blockers) induced an inhibition of the GTP-stimulated swelling. Mitochondrial swelling was inhibited by H_2O_2, but stimulated by NO. The same type of responses was also obtained in succinate-energized mitochondria. When the succinate-dependent transmembrane electrical potential ($\Delta\Psi$) had reached a steady state, the addition of KCl induced a dissipation that was inhibited by H_2O_2 and stimulated by NO. The latter stimulation was prevented by carboxy-PTIO, a NO scavenger. Phenylarsine oxide (a thiol oxidant) and NEM (a thiol blocker) stimulated the KCl-induced dissipation of $\Delta\Psi$, while DTE prevented this effect in both cases. In addition, DTE transiently inhibited the NO-induced dissipation of $\Delta\Psi$, but then it caused a more rapid collapse. These results, therefore, show that the plant mitochondrial K^+_{ATP} channel resembles that present in mammalian mitochondria and that it appears to be modulated by dithiol–disulfide interconversion, NO and H_2O_2. The aperture of this channel was linked to the partial rupture of the outer membrane. The latter effect led to a release of cytochrome *c*, thus suggesting that this release may be involved in the manifestation of programmed cell death.

KEY WORDS: Cytochrome *c*; mitochondria; K^+_{ATP} channel; *Pisum sativum*.

INTRODUCTION

Potassium is an essential element for plant cells that can accumulate it in different compartments. The cytoplasmic concentration of K^+, expressed as activity (a_K), is in the order of 80 mM, while that of the vacuole is approximately 100 mM (Tester and Leigh, 2001). The mitochondrial matrix concentration is also very high (120–140 mM, corresponding to a calculated a_K of 80–90 mM) (Hanson, 1985). The matrix level is regulated by a K^+_{ATP} channel (Pastore *et al*., 1999), localized in inner membrane, whose opening can be induced by cyclosporin A (Petrussa *et al*., 2001). On the same membrane there are, in addition, an electroneutral K^+/H^+ exchanger (Hanson, 1985) and a putative chloride channel, recently identified (Lurin *et al*., 2000). These systems can act in concert to accomplish a K^+ cycling, which, as already demonstrated for mammalian mitochondria (Bernardi, 1999; Garlid *et al*., 1995), may be related to energy dissipation and volume regulation.

The biochemical and molecular characteristics of the plant mitochondrial K^+_{ATP} channel are still unknown and its functions can only be hypothesized. The K^+ cycling, linked also to this K^+_{ATP} channel, seems to be responsible for both the prevention of reactive oxygen species generation (Pastore *et al*., 1999) and the release of cytochrome *c* (Petrussa *et al*., 2001), which, at least

Key to abbreviations: BSA, bovine serum albumin; carboxy-PTIO, 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide, potassium salt; DTE, dithioerythritol; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenyl-hydrazone; NEM, *N*-ethyl maleimide; PCD, programmed cell death; PhArOx, phenylarsine oxide; PTP, permeability transition pore; SNP, sodium nitroprusside; $\Delta\Psi$, transmembrane electrical potential.

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in mammalian mitochondria, constitutes a crucial step in the chain of events leading to the manifestation of programmed cell death (PCD), also named apoptosis (Bernardi *et al*., 2001; Gottlieb, 2000; Green and Reed, 1998; Pedersen, 1999; Susin *et al*., 1998). In this paper, we present evidence on some characteristics of the K^+_{ATP} channel of pea stem mitochondria, including the modulation by NO, H_2O_2, and redox agents, whose opening can lead to cytochrome *c* release.

MATERIALS AND METHODS

Plant Material

Pea (*Pisum sativum* L., var. Alaska) seedlings were grown in sand for 5 days in darkness at 25°C. Stems were cut in small pieces and then used for isolation of mitochondria.

Mitochondria Isolation

Pea stem mitochondria were isolated as previously described (Petrussa *et al*., 2001) with minor modifications. The pellet (mitochondrial fraction) was suspended in 2 mL of resuspending buffer (0.4 M sucrose, 20 mM HEPES-Tris, pH 7.5 and 0.1%, w/v, fatty acid-free BSA), washed with 20 mL of the same medium, without BSA, and finally centrifuged at $28{,}000 \times g$ for 5 min. The pellet, resuspended in 1 mL of the same buffer, contained about 4 mg protein/mL and was stored on ice.

Membrane Electrical Potential ($\Delta\Psi$) Measurements

Safranin O was used to estimate $\Delta\Psi$ changes, as previously described (Petrussa *et al*., 2001), evaluating variations of fluorescence (ΔF) expressed in arbitrary units (a.u.). The incubation medium contained 0.4 M sucrose, 20 mM HEPES-Tris (pH 7.5), 0.1% (w/v) fatty acid-free BSA, 5 μM safranin O, and 0.1 mg/mL of mitochondrial protein in a final volume of 2 mL.

Swelling Experiments

Swelling experiments were performed as described by Pastore *et al*. (1999). Absorbance changes at 540 nm of the mitochondrial suspension (0.2 mg protein/mL) in 0.2 M KCl and 2 mM Tris-HCl (pH 7.2) were monitored at 25°C by a Perkin-Elmer λ 15 spectrophotometer.

Outer Membrane Integrity Assay

The intactness of the outer mitochondrial membrane was monitored by KCN-sensitive, succinate-cytochrome *c* oxidoreductase activity as described by Douce *et al*. (1973). The incubation medium was the same used for swelling experiments, with the addition of 1 mM KCN and 50 μM oxidized cytochrome *c*. After 3 min, when the swelling was completed, 5 mM succinate was added, and the reaction was followed as absorbance increase at 550 nm, at 25°C, by a Perkin-Elmer λ 15 spectrophotometer. The initial rate, after the succinate addition, was converted to percentage values. The complete rupture of the outer membrane was performed when the incubation medium was made of bidistilled water.

Cytochrome *c* Release Measurements

After swelling experiments, samples of mitochondria were collected and centrifuged at $100{,}000 \times g$ for 40 min by a Beckman L7-55 centrifuge (Ty 70ti rotor) to obtain supernatants. Soluble proteins were concentrated about 13-fold by 5,000 MWCO concentrators VIVASPIN 500 (Sartorius, Göettingen, Germany) at $10{,}000 \times g$ for 10 min in an Eppendorf 5415C centrifuge and separated by SDS-PAGE (15% acrylamide/4% acrylamide stacker) to detect the presence of cytochrome *c*, according to the method described by Mather and Rottenberg (2001) with minor changes. The resolved polypeptides were electroblotted to nitrocellulose membrane (BioRad, Hercules, CA) in 25 mM Tris-0.2 M glycine-20% (v/v) methanol transfer buffer for 90 min at 15 V. The blots were saturated for 1 h in a blocking buffer (5% skim milk in Tris-saline buffer) and then incubated overnight at 4°C with anticytochrome *c* monoclonal antibody (7H8.2C12), as primary antibody (1/500). After washing, the membrane was incubated with antimouse peroxidase-conjugated IgG (1/80,000) for 1 h at room temperature. Labelling was detected using the "SuperSignal West Dura" substrate, chemiluminescence reagent, according to the supplier's manual (Pierce). The membrane was then exposed to an X-ray film (X-OMAT, East Man Kodack, Rochester, NY) for 5 min.

Protein Determination

The mitochondrial protein was determined by the Bradford method (Bradford, 1976), using the BioRad protein assay.

Chemicals

Na-ATP, Na-GTP, BSA, oligomycin, sodium nitroprusside, KCl, phenylarsine oxide, glucose oxidase, β-D-glucose, hydrogen peroxide, *N*-ethyl maleimide, diamide, dithioerythritol, 5-hydroxydecanoic acid (sodium salt), diazoxide, the antimouse peroxidase IgG were purchased from Sigma, St. Louis, MO. Horse heart cytochrome *c* was purchased from Boehringer Mannheim, West Germany. Cyclosporin A was a generous gift of Novartis Pharma AG, Switzerland. Uric acid was a generous gift of the Department of Chemistry, University of Udine. 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (potassium salt) (carboxy-PTIO) and glyburide were from Molecular Probes, The Netherlands. Anticytochrome *c* monoclonal antibody was purchased from Pharmingen, San Diego, CA. West Dura SuperSignal was purchased from Pierce, Rockford, IL. Sodium nitroprusside solution was freshly prepared in 50 mM Tris-HCl (pH 7.2). Phenylarsine oxide was dissolved in dimethyl sulfoxide. Diazoxide was solubilized in 0.1 N NaOH. Carboxy-PTIO was dissolved in water.

Data Presentation

All the experiments were repeated at least three times, and the figures shown represent a typical experiment.

RESULTS

Effect of Openers and Blockers on K^+_{ATP} Channel of Pea Stem Mitochondria

It is well known that pharmacological (diazoxide) and physiological (GTP) agents can induce the opening of the K^+_{ATP} channel of mammalian mitochondria and that this opening can be inhibited by some blockers (glyburide and 5-hydroxydecanoic acid) (Jabůrek *et al*., 1998). As previously shown (Petrussa *et al*., 2001), the addition of pea stem mitochondria to a sucrose-based medium did not induce changes of absorbance (Fig. 1, trace a). However, when mitochondria were resuspended in iso-osmotic NaCl- (trace b) or KCl- (trace c) based media, a slight or a strong decrease of absorbance, respectively, took place, indicating that the organelles underwent a swelling. The KCl-dependent swelling was strongly inhibited by ATP (trace d), but stimulated by valinomycin (Petrussa *et al*., 2001). Therefore, these traces refer to the previously identified K^+_{ATP} channel, which exhibits a good, but not complete, selectivity for K^+ (Petrussa *et al*., 2001). Both diazoxide (trace e) and GTP (trace f) partially relieved the ATP-induced inhibition of the channel. The GTP-induced restoration (Fig. 2, compare traces a, b, and c) was inhibited by both 5-hydroxydecanoic acid and glyburide (traces d and e). Therefore, these results show that plant mitochondria respond to K^+ channel openers and blockers in a way similar to that already demonstrated for mammalian mitochondria (Jabůrek *et al*., 1998).

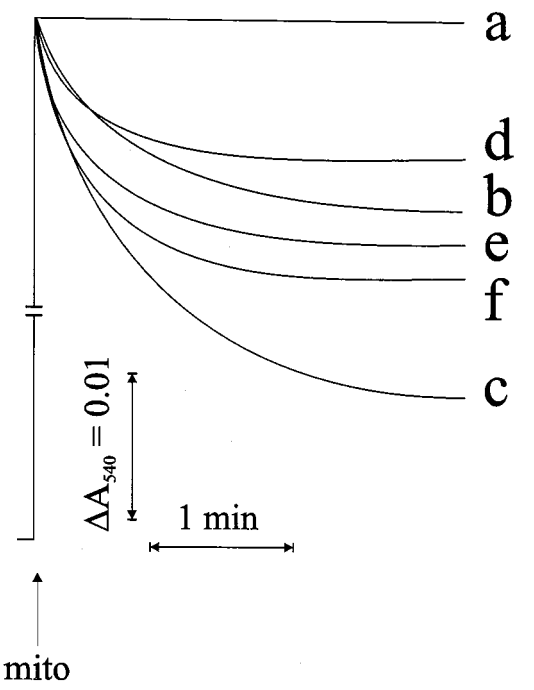

Fig. 1. Effect of K^+ channel openers (diazoxide and GTP) on K^+-dependent swelling of pea stem mitochondria: a, control (0.4 M sucrose); b, control (0.2 M NaCl); c, control (0.2 M KCl); d, 1 mM ATP (1 μg/mL oligomycin, 0.2 mM $MgCl_2$); e, 1 mM ATP (1 μg/mL oligomycin, 0.2 mM $MgCl_2$) plus 20 μM diazoxide; f, 1 mM ATP (1 μg/mL oligomycin, 0.2 mM $MgCl_2$) plus 100 μM GTP.

Effect of H_2O_2 and NO on K^+_{ATP} Channel in De-Energized and Energized Pea Stem Mitochondria

Figure 3 (panel A) shows that the mitochondrial swelling in de-energized organelles (trace a) was inhibited by both the addition of exogenous H_2O_2 (trace b) and the presence in the incubation mixture of a H_2O_2-generating system (trace c). Conversely, panel B shows that the swelling (trace a) was stimulated by NO liberated by sodium nitroprusside (trace b) present in the incubation mixture. This stimulation was abolished by carboxy-PTIO

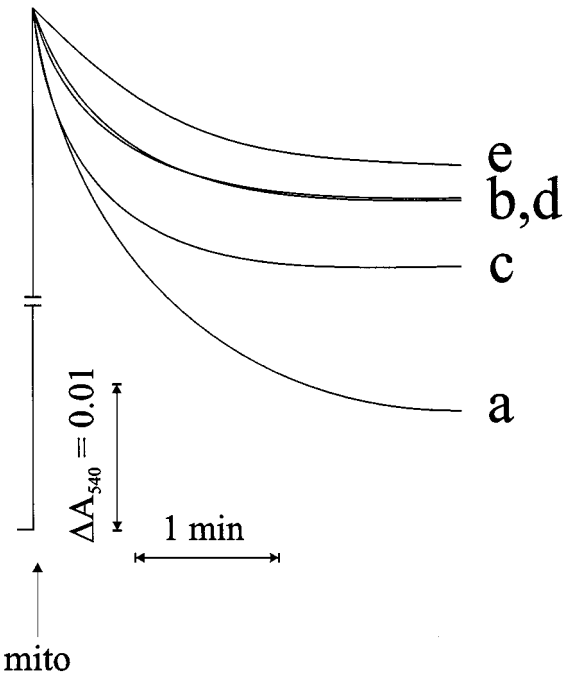

Fig. 2. Effect of K^+ channel blockers (glyburide and 5-hydroxydecanoic acid) on K^+-dependent swelling stimulated by GTP in pea stem mitochondria: a, control (0.2 M KCl); b, 1 mM ATP (1 μg/mL oligomycin, 0.2 mM $MgCl_2$); c, 1 mM ATP (1 μg/mL oligomycin, 0.2 mM $MgCl_2$) plus 100 μM GTP; d, 1 mM ATP (1 μg/mL oligomycin, 0.2 mM $MgCl_2$) plus 100 μM GTP and 50 μM 5-hydroxydecanoic acid; e, 1 mM ATP (1 μg/mL oligomycin, 0.2 mM $MgCl_2$) plus 100 μM GTP and 20 μM glyburide.

(trace c), a well-known NO scavenger (Amano and Noda, 1995), and again inhibited by ATP (trace d).

A pattern of responses very similar was also obtained in mitochondria energized by succinate (Fig. 4). The addition of KCl to mitochondria that had reached a stable value of $\Delta\Psi$, induced a slight and in part transient dissipation of the potential (panel A, trace a). This dissipation was inhibited by either exogenously supplied H_2O_2 (trace b) or the H_2O_2-generating system (trace c). Conversely, the KCl-induced dissipation of $\Delta\Psi$ (panel B, trace a) was strongly enhanced by NO (nitroprusside in the incubation mixture) (trace b). This stimulation was completely prevented by carboxy-PTIO (trace c) and ATP (trace d), but not by uric acid, a peroxynitrite scavenger (result not shown).

Effect of Sulfhydryl-Modifying Agents on K^+_{ATP} Channel Opening Induced by KCl in Energized Pea Stem Mitochondria

Figure 5 shows that phenylarsine oxide (PhArOx) and diamide (not shown), two sulfhydryl group oxidants, strongly stimulated the KCl-induced dissipation in energized mitochondria (compare traces a and b). This effect was dependent on the concentration of phenylarsine oxide (see inset) and was almost completely abolished by DTE (trace c). Panel B shows that also a sulfhydryl group blocker, *N*-ethyl maleimide (NEM), induced a similar stimulation of the KCl-induced dissipation (compare traces a and b) which was concentration-dependent (see inset) and inhibited by DTE (trace c).

These results show, hence, that the pea stem mitochondrial K^+_{ATP} channel can be modulated by the redox state or modification of some sulfhydryl groups. To verify if NO also interacted with the same target, the effect of DTE on NO-stimulated, KCl-induced dissipation of $\Delta\Psi$ was assayed. Figure 6 shows that the NO-stimulated dissipation of $\Delta\Psi$ (compare traces a and b) was initially blocked for few minutes by DTE, but then a more rapid dissipation occurred (trace c).

Mitochondrial Outer Membrane Rupture and Cytochrome *c* Release Induced by Modulation of the K^+_{ATP} Channel

Pea stem mitochondria, resuspended in a sucrose-based medium, had a high degree of integrity of the outer membrane (Table I). This integrity was partially lost when the same organelles were resuspended in KCl. This effect, although to a little extent, was increased by both valinomycin, which allows K^+ entry, and cyclosporin A, which stimulates the K^+_{ATP} channel opening (Petrussa *et al.*, 2001). These results, therefore, show that the mitochondrial swelling dependent on K^+_{ATP} channel opening was accompanied by the partial rupture of the outer mitochondrial membrane.

To verify whether this swelling was linked to the release of proteins from the intermembrane space, the presence of cytochrome *c* in supernatants of pea stem mitochondria, treated with different modulators of the K^+_{ATP} channel, was detected by western blot analysis (Fig. 7). When mitochondria were resuspended in a sucrose-based medium, no release of cytochrome *c* was observed (result

Table I. Effect of Valinomycin and Cyclosporin A on the Outer Membrane Integrity in Pea Stem Mitochondria

Additions	% of integrity
0.4 M sucrose	94
0.2 M KCl	80
+50 nM valinomycin	75
+300 nM cyclosporin A	76

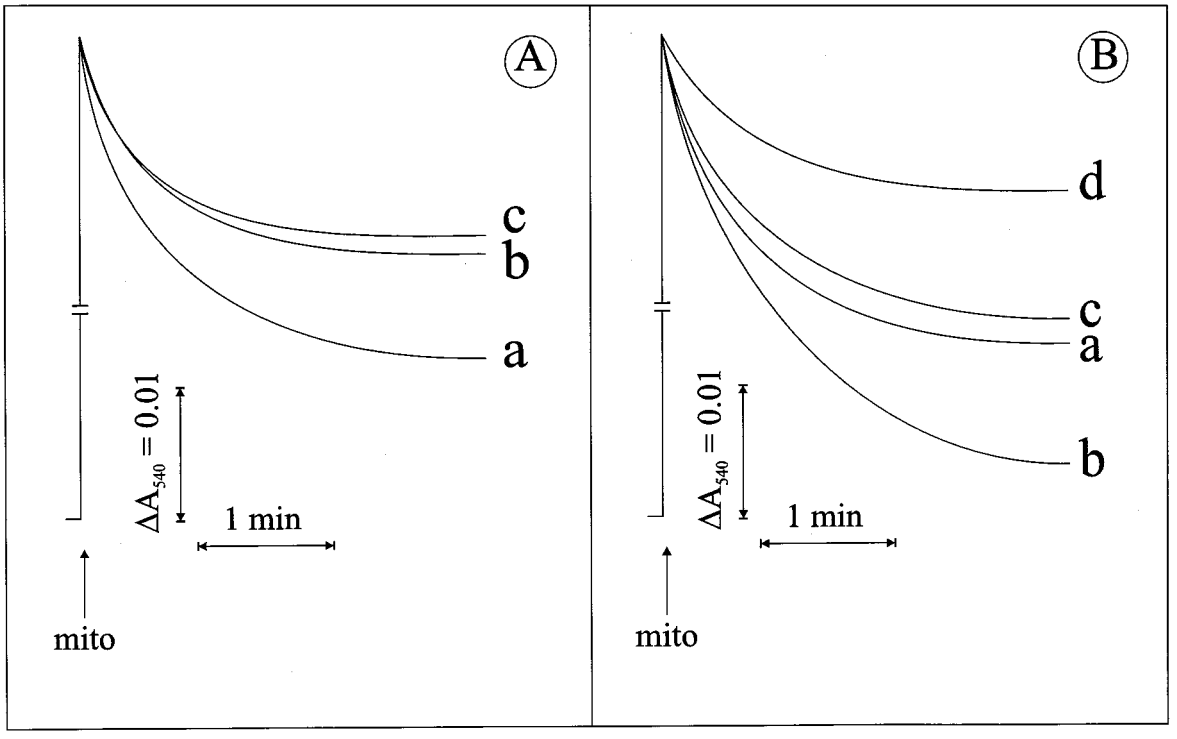

Fig. 3. Effect of H_2O_2 (A) or NO (B) on K^+-dependent swelling of pea stem mitochondria. (A): a, control (0.2 M KCl); b, 40 μM H_2O_2; c, 0.5 mM glucose/0.5 IU/mL glucose oxidase. (B): a, control (0.2 M KCl); b, 250 μM SNP; c, 250 μM SNP plus 50 μM carboxy-PTIO; d, 250 μM SNP plus 1 mM ATP (1 μg/mL oligomycin, 0.2 mM $MgCl_2$).

not shown), but if they were resuspended in KCl, a little amount of cytochrome *c* was detectable (lane 1). This release was inhibited by ATP (lane 2) and increased by NO (lane 3). Both diamide (lane 4), phenylarsine oxide (lane 5) and NEM (lane 6), which induced the opening of the K^+_{ATP} channel, caused a stronger release of this protein. Conversely to this result, no release of adenylate kinase (another protein of the intermembrane space) was detected (result not shown).

DISCUSSION

The presence of K^+_{ATP} channels on the inner membrane of mammalian mitochondria is well recognized (Bernardi, 1999; Garlid *et al*., 1995). Similarly to plasma membrane K^+_{ATP} channels, their molecular structure is composed of a channel protein and a sulphonylurea receptor with a high affinity for ATP (Garlid, 1996). The channel protein has also been solubilized and reconstituted into a bilayer lipid membrane (Mironova *et al*., 1999). Besides γ, to be inhibited by ATP, this channel is regulated by several physiological and pharmacological agents. In particular, it is inhibited by long-chain acyl-CoA esters and activated by guanine nucleotides (GDP or GTP) (Paucek *et al*., 1996), whose regulatory effects are exerted on sites of K^+_{ATP} channel facing the cytosol (Yarov-Yarovoy *et al*., 1997). In addition, the inhibition by ATP is relieved not only by GTP, but also by diazoxide (another K^+_{ATP} channel opener), whereas two well-known K^+_{ATP} channel blockers (glyburide and 5-hydroxydecanoate) inhibit both the diazoxide and GTP-induced opening (Jabůrek *et al*., 1998). The K^+_{ATP} channel of pea stem mitochondria, as shown in this paper, responds in the same way to the latter openers and blockers, thus suggesting that there may be molecular similarities between this channel and that of mammalian mitochondria.

The K^+_{ATP} channel of mammalian mitochondria is regulated by redox agents, involving thiol groups as target for redox-active ligands (Grigoriev *et al*., 1999). As previously suggested (Petrussa *et al*., 2001), the K^+_{ATP} channel of pea stem mitochondria, in the light of the results here presented, is also modulated through dithiol–disulfide interconversion. This evidence arises from the observation that two oxidizing agents (diamide and phenylarsine

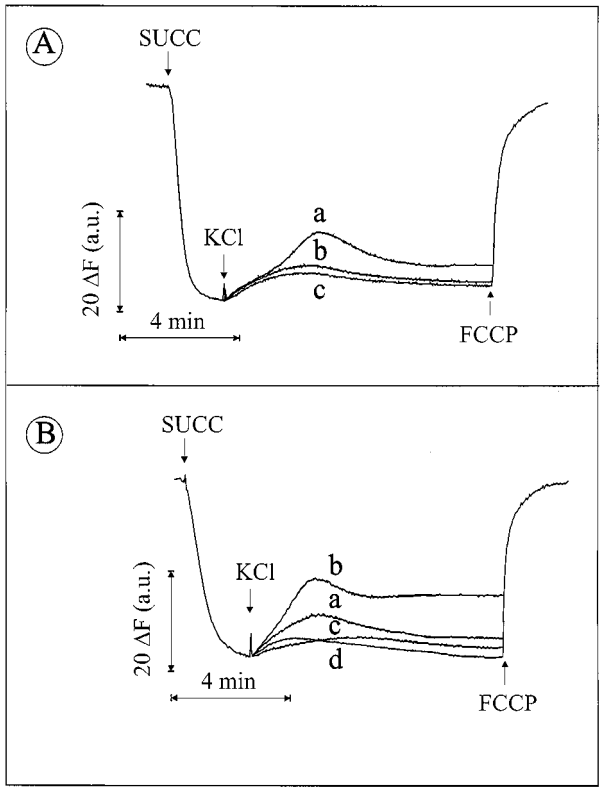

Fig. 4. Effect of H_2O_2 (A) and NO (B) on KCl-induced dissipation of $\Delta\Psi$ in pea stem mitochondria. (A): a, 40 mM KCl; b, 40 mM KCl plus 40 μM H_2O_2 (added after succinate); c, 40 mM KCl plus 0.5 mM glucose/ 0.5 IU/mL glucose oxidase (present in the incubation mixture). (B): a, 40 mM KCl; b, 40 mM KCl plus 500 μM SNP (present in the incubation mixture); c, 40 mM KCl plus 500 μM SNP and 50 μM carboxy-PTIO (both present in the incubation mixture); d, 40 mM KCl plus 500 μM SNP and 1 mM ATP (1 μg/mL oligomycin, 0.2 mM $MgCl_2$) (both present in the incubation mixture). Other additions: SUCC, 5 mM succinate; FCCP, 1 μM.

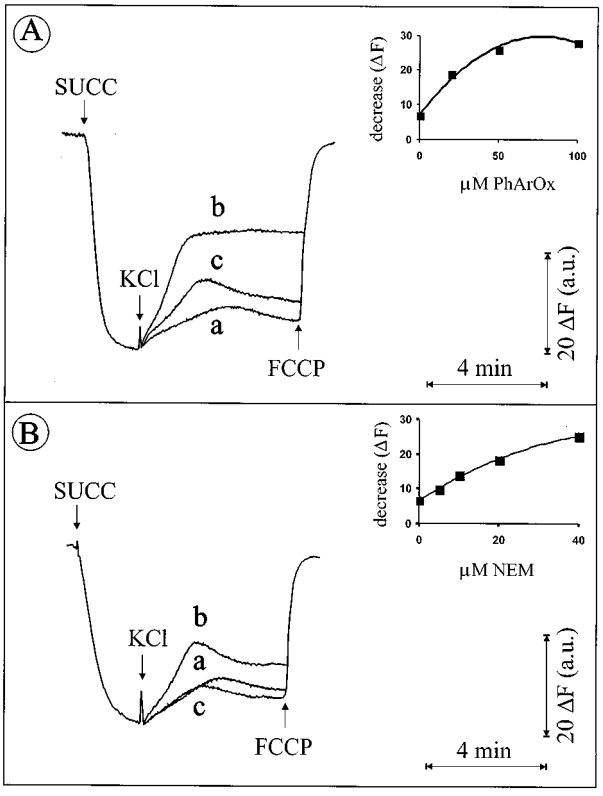

Fig. 5. Effect of phenylarsine oxide (A) and *N*-ethyl maleimide (B) on KCl-induced dissipation of $\Delta\Psi$ in pea stem mitochondria. (A): a, 40 mM KCl; b, 40 mM KCl plus 20 μM PhArOx (present in the incubation mixture); c, 40 mM KCl plus 20 μM PhArOx and 50 μM DTE (added after succinate). (B): a, 40 mM KCl; b, 40 mM KCl plus 20 μM NEM (present in the incubation mixture); c, 40 mM KCl plus 20 μM NEM and 50 μM DTE (added after succinate). Insets represent the relationship between PhArOx or NEM concentrations and the KCl-induced dissipation of $\Delta\Psi$. Other additions: SUCC, 5 mM succinate; FCCP, 1 μM.

oxide) stimulate channel opening, while a reducing agent (DTE) prevents this effect. In addition, it has been shown that a sulfhydryl group blocker (NEM) mimics the effect of diamide and phenylarsine oxide by a mechanism which is again blocked by DTE. This result is surprising, but can be rationalized by considering that, as previously suggested for PTP, potentiation by NEM can be mediated by an oxidative event rather than by a substitution as such (Costantini *et al*., 1998). It seems that NEM is able to unmask cryptic sites that are then easily auto-oxidizable.

Programmed cell death is a well-recognized phenomenon involved in several physiological processes related to both the development and the interaction of plants with the environment (Greenberg, 1996; Jones and Dangl, 1996; Pennell and Lamb, 1997). It appears to proceed through steps that are in part similar to those occurring in mammalian cells (Danon *et al*., 2000; Havel and Durzan, 1996). As known, PCD in the latter type of cells is characterized by a mitochondrial step, regulated by antiapoptotic and proapoptotic proteins of the Bcl-2 family (Tsujimoto and Shimizu, 2000), that implies the release of cytochrome *c* and other proteins (AIF and "smac-diablo") (Bernardi *et al*., 2001; Gottlieb, 2000; Green and Reed, 1998; Pedersen, 1999; Susin *et al*., 1998). The mechanism of release of these intermembrane proteins has been explained by two models. In the first Bax, a death-promoting member of the Bcl-2 family of proteins, joins with porin to form a large channel on the outer mitochondrial membranes that allows the exit of cytochrome *c*. In the second, the opening of the PTP results in depolarization of the inner membrane and swelling of the matrix which is responsible for cytochrome *c*

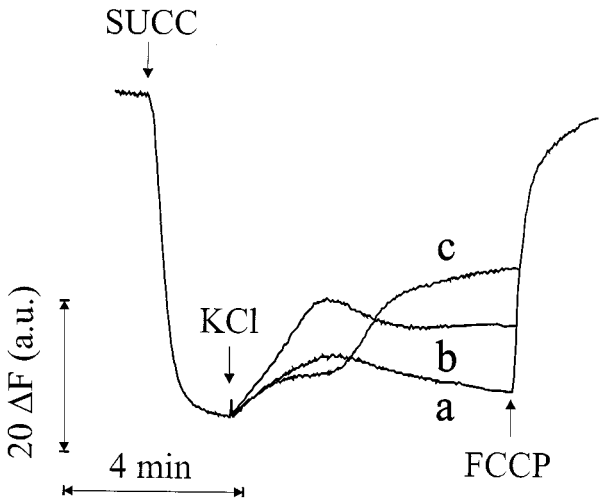

Fig. 6. Effect of DTE on the stimulation by NO on KCl-induced dissipation of $\Delta\Psi$ in pea stem mitochondria: a, 40 mM KCl; b, 40 mM KCl plus 500 μM SNP (in the incubation mixture); c, 40 mM KCl plus 500 μM SNP and 1 mM DTE (added after succinate). Other additions: SUCC, 5 mM succinate; FCCP, 1 μM.

release (Bernardi *et al*., 2001; Gottlieb, 2000). This release induces the subsequent activation of cysteine proteases (caspases) which then leads to condensation of the nucleus and the cytoplasm, DNA fragmentation, and packaging of the cell corpse in vesicles that are ingested by phagocytosis (Cohen, 1997). This picture is still fragmentary in plant cells. The involvement of plant mitochondria in this chain of events leading to PCD has been suggested (Jones, 2000). There is evidence for a release of cytochrome *c* in plant cells after the induction of PCD (Balk *et al*., 1999; Sun *et al*., 1999) and this effect, in some cases, implies caspase activation (del Pozo and Lam, 1998; Sun *et al*., 1999). In addition, it has been shown that Bax triggers cell death when expressed in plants (Lacomme and Santa Cruz, 1999). Only very recently, by an immunocytochemical analysis, it has been demonstrated that cytochrome *c* is partially released from mitochondria of plant cells before gross morphological changes associated to PCD (Balk and Leaver, 2001). However, very little is known about the mechanism underlaying this release. It has been suggested that it may occur through porin (Lam *et al*., 1999), because only very recently a cyclosporin A-sensitive PTP has been shown in potato tuber mitochondria (Arpagaus *et al*., 2001), albeit a cyclosporin A-insensitive permeability transition has also been described (Fortes *et al*., 2001). Therefore, their physiological roles, at the moment, can be only matter of speculation.

A well-known example of PCD in plants is represented by the hypersensitive response (HR), a localized cell death induced by an invading pathogen which hinders the next diffusion of the pathogen itself (Greenberg, 1997). The hypersensitive response seems to be the result of the activation of independent signal transduction pathways (Sasabe *et al*., 2000) and to be modulated by both H_2O_2 and NO (Delledonne *et al*., 1998; Hausladen and Stamler, 1998; Van Camp *et al*., 1998). In this context, H_2O_2, generated at the cell wall/plasma membrane interface (oxidative burst), leads to the expression of defence genes (Sasabe *et al*., 2000), while NO could have a more direct role in the mitochondrial step associated to cell death implying cytochrome *c* release. As shown in this paper, the opening of the K^+_{ATP} channel of plant mitochondria is inhibited by H_2O_2, but stimulated by NO. The latter observation is corroborated by the findings showing that NO directly activates the K^+_{ATP} channel of mitochondria from rabbit ventricular myocytes (Sasaki *et al*., 2000) and

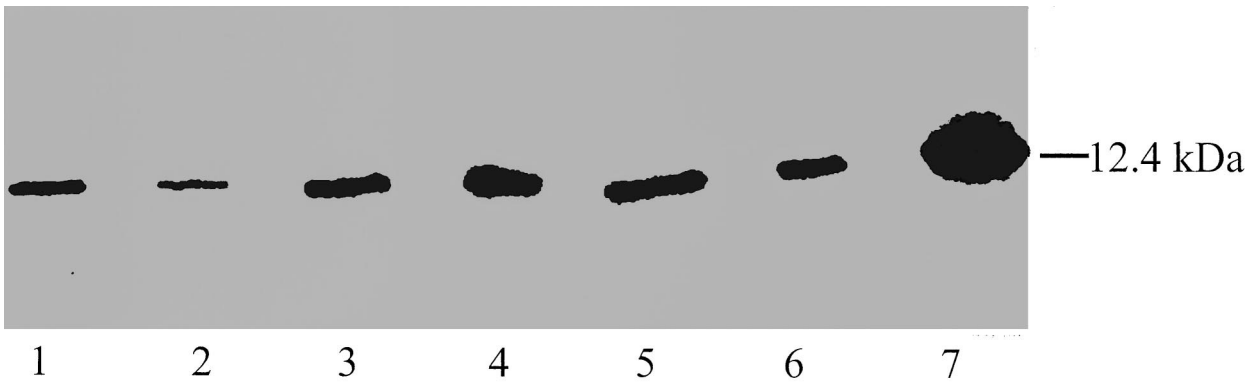

Fig. 7. Western blot analysis of cytochrome *c* released from pea stem mitochondria treated by different modulators of K^+_{ATP} channel. Lane 1, control (0.2 M KCl); lane 2, 1 mM ATP; lane 3, 500 μM SNP; lane 4, 1 mM diamide; lane 5, 200 μM phenylarsine oxide; lane 6, 40 μM NEM. Lanes 1–6 contained 15 μg protein from supernatants. Lane 7, 1 μg commercial cytochrome *c*.

that of mitochondria from rat liver posterior pituitary nerve terminals (Ahern *et al*., 1999). The stimulatory effect of NO on plant mitochondrial K^+_{ATP} channel seems to involve a nitrothiosylation reaction between NO^+ (the form of NO released by SNP) and specific sulfhydryl groups in the protein forming the channel. In other words, it appears to be performed by a mechanism already described for the K_{Ca} channel of airway smooth muscle (Abderrahmane *et al*., 1998). The plant K^+_{ATP} channel opening leads to a release of cytochrome *c* by a mechanism which implies only a slight swelling of mitochondria due to K^+ entry, similarly to what happens in cardiac mitochondria (Holmuhamedov *et al*., 1998), where modulation of the K^+_{ATP} channel also determines a release of cytochrome *c*. Considering that the mechanism of cytochrome *c* release in plant mitochondria is poorly understood, it is suggested that the K^+_{ATP} channel can constitute a reliable candidate in mediating the mitochondrial step of PCD in plant cells.

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