Journal of Bioenergetics and Biomembranes, Vol. 33, No. 2, 2001

Cyclosporin A Induces the Opening of a Potassium-Selective Channel in Higher Plant Mitochondria[1]

Elisa Petrussa,[2] Valentino Casolo,[2] Enrico Braidot,[2] Elisa Chiandussi,[2] Francesco Macrì,[2] and Angelo Vianello[2,3]

Received August 10, 2000; accepted December 12, 2000

The immunosuppressive drug, cyclosporin A (CsA), induces the generation of a transmembrane electrical potential difference ($\Delta\Psi$) in deenergized plant mitochondria incubated in sucrose-based media. Build up of $\Delta\Psi$ is prevented by external monovalent cations in the order $K^+ > Rb^+ = Li^+ > Na^+$, or by the protonophore carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone, which also collapses the $\Delta\Psi$ generated by CsA. Entry of K^+ into mitochondria can be monitored as swelling by incubating the organelles in a medium containing KCl to maintain constant osmolarity. This swelling is inhibited by ATP and stimulated by CsA or valinomycin. In addition, in mitochondria energized by succinate, KCl causes a dissipation of $\Delta\Psi$, with sigmoidal kinetics, which is favored by CsA. Therefore, plant mitochondria appear to possess a K^+ selective, voltage-dependent channel, which is opened by CsA, regulated by the redox state, and inhibited by nucleotides. The hypothetical roles of this new K^+_{ATP} channel are discussed in relation to its potential involvement in mitochondrial volume regulation, thermogenesis, apoptosis, and/or prevention of reactive oxygen species formation in plants.

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

INTRODUCTION

The inner membrane of mammalian mitochondria is an ionic permeability barrier ensuring that redox energy, liberated by substrate oxidation, may be conserved in the form of an electrochemical proton gradient, which is then dissipated to synthesize ATP (via the F_0F_1-ATP synthase) (Mitchell, 1966). This membrane contains anion carriers responsible for shuttling metabolic substrates between matrix and cytosol (Palmieri *et al.*, 1996). It also contains some monovalent and divalent cation carriers and channels that regulate important aspects of cell and mitochondrial physiology (Bernardi, 1999; Garlid *et al.*, 1995). The main monovalent cation antiporters and channels hitherto identified are: a selective H^+/Na^+ exchanger, which appears to be distinct from an unselective H^+/K^+ (Na^+) exchanger; a selective Na^+ channel and both selective and nonselective K^+ channels mediating cation uniport (Bernardi, 1999).

These carriers and channels ensure K^+ transport cycles, driven by the vectorial proton-pumping enzymes, which are of particular importance to regulate mitochondrial volume (Garlid *et al.*, 1995; Garlid, 1988). Potassium is the major osmolyte of mitochondria. Its entry into the matrix is mediated by a selective K^+ channel inhibited by ATP (Paucek *et al.*, 1992) and is accompanied by the uptake of anions and water, which results in volume changes. Charge compensation for K^+ influx is provided by an H^+/K^+ antiporter, which catalyzes electroneutral H^+/K^+ exchange (Garlid, 1988).

Nevertheless, mammalian mitochondria from a variety of sources can undergo a sudden permeability increase to solutes with a molecular mass up to 1.5 kDa, which is facilitated by Ca^{2+} accumulation into the matrix. This phenomenon has been ascribed to the presence of an inner membrane channel, the permeability transition pore (PTP), which is modulated by several factors including

[1] Key to abbreviations: BSA, bovine serum albumin; CsA, cyclosporin A; DTE, dithioerythreitol; $\Delta\Psi$, transmembrane electrical potential difference; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; PTP, permeability transition pore; RCR, respiration control ratio; TEM, transmission electron microscopy.

[2] Department of Biology and Agro-industrial Economics, Section of Plant Biology, University of Udine, via Cotonificio 108, I-33100 Udine, Italy.

[3] To whom correspondence should be addressed at email: biolveg@dbea.uniud.it

0145-479X/01/0400-0107$19.50/0

membrane depolarization, matrix pH, and divalent cations (for reviews, see Bernardi *et al.*, 1994; Halestrap, 1994; Zoratti and Szabò, 1995). The most specific and selective inhibitor of PTP is cyclosporin A (CsA) (Crompton *et al.*, 1988), an immunosuppressive drug that binds tightly to a 20-kDa matrix protein, cyclophilin D (a peptidyl-prolyl *cis–trans* isomerase), to exert its effect (Halestrap and Davidson, 1990; Griffiths and Halestrap, 1991; Petronilli *et al.*, 1994b).

The inner mitochondrial membrane of yeast has a large-conductance channel regulated by nucleotides and phosphate (Manon *et al.*, 1998) that could accomplish the same function as mammalian PTP (Bernardi *et al.*, 1994; Zoratti and Szabò, 1995), although it appears to be differently regulated (Jung *et al.*, 1997).

Higher plant mitochondria are similar to mammalian ones in several respects, yet they possess unique features: (1) the presence of specific NAD(P)H dehydrogenases, (2) the size and complexity of DNA, (3) the rate of O_2 consumption, (4) the very low rate of oxidation of fatty acids, and (5) the presence of a cyanide-insensitive, salicylhydroxamic acid-sensitive, alternative oxidase (Douce and Neuburger, 1989). As in mammalian mitochondria, the inner membrane contains several anion carriers responsible for shuttling metabolic substrates between matrix and cytosol (Douce and Neuburger, 1989). Although passive and active monovalent cation transport takes place at this level, the knowledge of the mechanisms involved in these fluxes (channels or carriers) is scarce (Hanson, 1985). An electroneutral H^+/K^+ antiport (Hanson, 1985), inhibited by dibucaine (Klein and Koeppe, 1985), has been described, while evidence for the presence of PTP is circumstantial (Vianello *et al.*, 1995). Existence of a K^+_{ATP} channel has only recently been demonstrated (Pastore *et al.*, 1999).

The present paper shows that CsA induces the formation of a transmembrane electrical potential difference ($\Delta\Psi$) in deenergized plant mitochondria incubated in low K^+ media. This effect could depend on the closure of a PTP or to the opening of a CsA-sensitive channel. The results described here corroborate the latter hypothesis and confirm previous results (Pastore *et al.*, 1999), supplying further evidence for the presence of a voltage-dependent, K^+_{ATP} selective channel responsible for the CsA-induced $\Delta\Psi$ generation.

MATERIALS AND METHODS

Plant Material

Pea (*Pisum sativum* L., var. Alaska) seedlings were grown in sand for 5 days, in darkness, and 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 (Vianello *et al.*, 1995). The pellet (mitochondrial fraction) was suspended in 1 ml of resuspending medium composed of 0.4 M sucrose, 0.2% (w/v) bovine serum albumin (BSA) in 20 mM HEPES-Tris (pH 7.5). The suspension contained ca. 6 mg protein/ml and was stored on ice.

To repeat some basic experiments and to perform a transmission electron microscopy (TEM) analysis of mitochondria, these organelles were further purified on a discontinuous gradient formed by three layers (bottom to top) of 45, 21, 13.5% (v/v) Percoll in 20 mM MOPS-KOH (pH 7.2), 0.5 M sucrose, and 0.2% (w/v) BSA. The gradient was centrifuged at 20,000 $\times$ *g* for 40 min in a swinging bucket rotor (Sorvall HB-4) and the mitochondria collected at the 21/45% interface. Mitochondria were washed twice in 250 ml of 20 mM MOPS-KOH (pH 7.2), 0.3 M mannitol, 1 mM EDTA, and 0.1% (w/v) BSA to remove Percoll and centrifuged at 28,000 $\times$ *g* for 5 min. Purified mitochondria were resuspended in 1 ml of 20 mM HEPES-Tris (pH 7.5), 0.4 M sucrose, and 0.1% (w/v) BSA. The suspension, containing ca. 2 mg protein/ml, was stored on ice.

Membrane Potential Measurements

Safranin O was used to estimate $\Delta\Psi$ changes, as previously described (Vianello *et al.*, 1994). The fluorescence intensity change of safranin O was recorded at 25°C by a luminescence spectrometer, model LS 50 B (Perkin-Elmer, UK). The excitation and emission wavelengths were 495 and 586 nm, respectively. A slitwidth of 10 nm for both emission and excitation was used. The calibration of safranin O fluorescence intensity changes, as a function of K^+ diffusion potentials, was basically performed as described by Moore and Bonner (1982). In the incubation mixture used for $\Delta\Psi$ measurements, a K^+ diffusion potential was built up by the addition of 50 nM valinomycin. Then $\Delta\Psi$ was collapsed by increasing additions of KCl (0–16 mM range). The K^+ diffusion potential was calculated according to the Nernst equation, assuming an intramitochondrial concentration of 95 mM K^+ (Braidot *et al.*, 1998). At this dye:protein ratio, the K^+ diffusion potential versus fluorescence intensity changes was linear in the 60–200 mV interval. The data of Figs. 5 and 6

(see later) are presented as fluorescence intensity changes (arbitrary units).

Swelling Measurements

Swelling experiments were basically performed as described by Pastore *et al.* (1999). Absorbance changes at 540 nm of a suspension of mitochondria in sucrose, K-acetate, or KCl isotonic media were monitored at 25°C by a spectrophotometer, model λ15 (Perkin-Elmer, UK).

Microscopy Analysis

For TEM analysis, the purified mitochondrial suspension was left on ice for 20 min with 0.3 μM CsA and then centrifuged at 13,600 × *g* for 15 min in an Eppendorf centrifuge 5415 C. The samples were fixed for ca. 4 h in 2.5% (w/v) glutaraldehyde in 0.13 M phosphate buffer (pH 7.2). Mitochondria were then allowed to settle at 1 × *g* and rinsed several times with the same solution for 4 h. The rinsed samples were centrifuged at 12,000 × *g* for 10 min. Following centrifugation, the pellet was gently removed and postfixed in 2% (w/v) OsO_4 for 1 h. The samples were rinsed three times with buffer and dehydrated by a graded ethanol series. Finally, the mitochondria were embedded in pure Spurr resin for about 15 h at 70°C. Ultrathin sections (70–80 nm) were cut and stained with 3% (w/v) uranyl acetate and lead citrate. Specimens were examined with a Zeiss EM 902 transmission electron microscope at accelerating voltage of 80 kV.

The computer-assisted densitometric analysis of mitochondria images, obtained with the transmission electron microscope, was performed using NIH Image Version 1.62 on a Macintosh Performa 6200.

Protein Measurement

The mitochondrial protein was determined by the Bradford method, using the Bio-Rad protein assay.

Chemicals

All chemicals (the highest purity commercially available) were purchased from Sigma (St. Louis, MO, USA). CsA was a generous gift of Novartis Pharma AG (Switzerland).

Statistical Treatments

Experiments shown in Figs. 1–4 represent typical traces. Data of Figs. 5 and 6 are means of three replicates ± S.E., while those of Fig. 7 (two populations of 80 mitochondria of untreated and CsA-treated samples) were subjected to analysis of variance (ANOVA).

RESULTS

Effect of CsA on $\Delta\Psi$ in Deenergized Pea Stem Mitochondria

Figure 1 shows that the addition of CsA induced a time-dependent fluorescence quenching of safranin O, indicating the generation of a $\Delta\Psi$ (trace A). This potential was not collapsed by antimycin A (AA), while the addition of FCCP, when $\Delta\Psi$ had reached a steady-state, caused a rapid and complete dissipation. In addition, no oxygen consumption was observed in mitocondria supplemented with CsA as respiratory substrate (result not shown). Fluorescence quenching, comparable to that depicted in trace A, was also observed when CsA was added 15–20 min after mitochondria (trace B). The CsA-induced $\Delta\Psi$ was evaluated at pH 8.0 (trace A), because at this value it was higher than that detected at pH 7.0 (trace C). This effect did not depend on a different behavior of the probe at these two pH values. Figure 1 also shows that $\Delta\Psi$ was dissipated by salts in the following order: $KCl > MgCl_2 = NaCl$ (trace D), and that the addition of the selective ionophore valinomycin further increased $\Delta\Psi$ (trace E).

Effect of CsA on Mitochondrial Swelling

The results above reported demonstrate that the addition of CsA induced the formation of an outwardly directed K^+ diffusion potential and suggest the existence of CsA-modulated, K^+ selective mitochondrial channel. This notion is strongly supported by the experiments of Fig. 2. Pea mitochondria were not found to swell in sucrose (trace A), but, if they were resuspended in a medium containing KCl, they underwent a rapid swelling that was further increased by the addition of valinomycin (trace B). Swelling was also monitored in a K-acetate-based medium and nigericin induced a further strong increase (trace C). Considering that Cl^- may enter into the matrix of plant mitochondria (Hanson, 1985), the swelling, monitored in KCl medium, implies that also K^+, as previously suggested (Pastore *et al.*, 1999), may penetrate in the absence of valinomycin. This swelling was inhibited by ATP plus oligomycin (trace D) and strongly increased by valinomycin, added at the beginning (trace E). Therefore, these results confirm the presence of a K^+_{ATP} selective channel in the inner membrane of pea stem mitochondria.

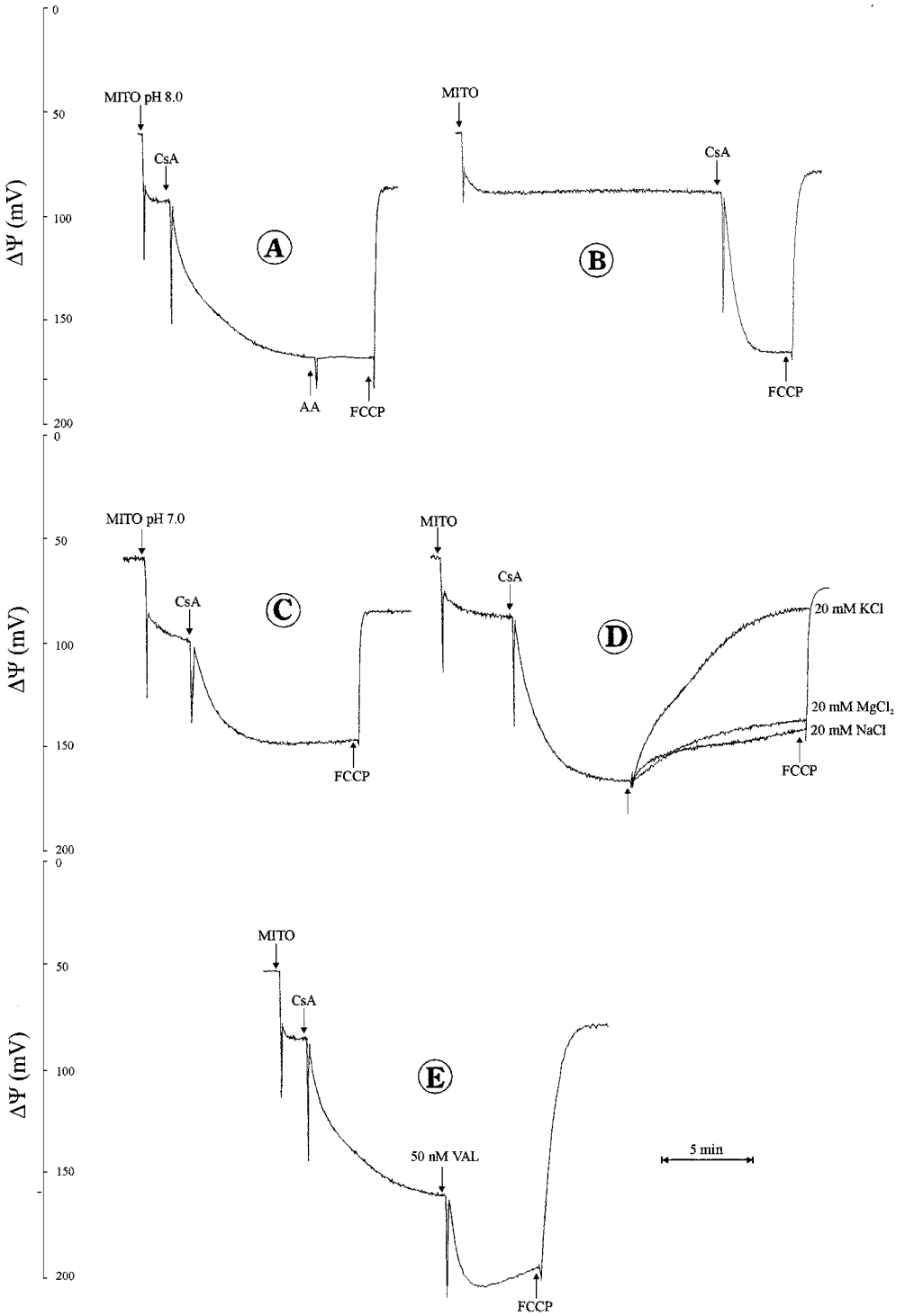

Fig. 1. CsA-induced $\Delta\Psi$ formation in isolated pea stem mitochondria. The incubation medium contained 0.4 M sucrose, 0.1% BSA, 5 μM safranin O in 20 mM HEPES-Tris (pH 7.0 or 8.0), and 0.1 mg/ml mitochondrial protein (MITO) in a final volume of 2 ml. The reaction was started by the addition of 0.3 μM CsA. Other additions include 100 nM FCCP and 1 μg/ml antimycin A (AA).

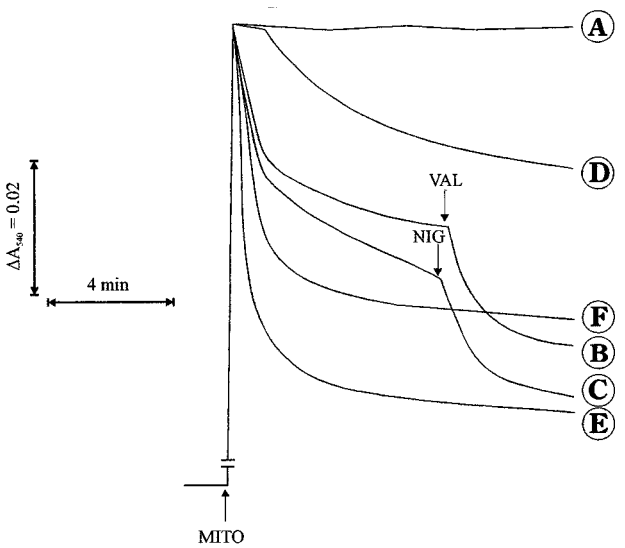

Fig. 2. Effect of CsA on swelling of isolated pea stem mitochondria. The incubation medium contained 0.4 M sucrose, 0.2 M KCl or 0.2 M K-acetate in 2 mM Tris-HCl (pH 7.0), and 0.1 mg/ml mitochondrial protein (MITO) in a final volume of 2 ml. Traces: (A) 0.4 M sucrose; (B) 0.2 M KCl; (C) 0.2 M K-acetate; (D) 0.2 M KCl plus 1 mM ATP and 1 μg/ml oligomycin; (E) 0.2 M KCl plus 50 nM valinomycin; (F) 0.2 M KCl plus 0.3 μM CsA. Other additions include 10 nM nigericin (NIG) and 50 nM valinomycin (VAL).

This channel, according to the above reported results, was opened by CsA, as indicated by the increase of swelling induced by this drug (trace F).

Effect of CsA on $\Delta\Psi$ of Energized Mitochondria

To verify if CsA could act on energized mitochondria, its effect was assayed on the $\Delta\Psi$ generated by the oxidation of succinate (Fig. 3). This substrate induced the building up of a $\Delta\Psi$, which was progressively collapsed by increasing additions of KCl (result not shown). The $\Delta\Psi$ was stable for several minutes (trace A) and was, after a lag phase, almost completely collapsed by the addition of KCl (trace B). The sigmoidal kinetic of this effect shows that this dissipation may be related to the aperture of the K^+ channel. CsA, favoring this aperture, caused a reduction of the lag phase, which reflected in a more rapid depolarization of the inner membrane (trace C). In the presence of ATP and oligomycin (trace D), the $\Delta\Psi$ was only partially and transiently collapsed by KCl. Trace E shows that, when the $\Delta\Psi$ was built up by NADH oxidation, similarly to trace D, the addition of KCl caused only a partial dissipation that was then followed by an almost complete recovery of $\Delta\Psi$. The subsequent addition of low concentrations of FCCP caused the complete dissipation of $\Delta\Psi$ with a sigmoidal kinetic. In agreement, the $\Delta\Psi$ generated by ATP was dissipated slowly by KCl (trace F). These results confirm the presence of a K^+ channel in the inner membrane, which is inhibitable by nucleotides and opened by CsA.

Effect of CsA on $\Delta\Psi$ and Swelling of Purified Mitochondria

Some basic results described in Figs. 1–3 were also repeated by using purified pea stem mitochondria (Fig. 4). As it can be seen, the addition of CsA to deenergized mitochondria, resuspended in a sucrose-based medium, induced the generation of a $\Delta\Psi$, which was collapsed by KCl and unaffected by NaCl (trace A). In a K^+-based medium, mitochondria underwent a swelling (trace B) that was stimulated by CsA (trace C) and inhibited by ATP plus oligomycin (trace D). In agreement, in succinate-energized mitochondria, KCl induced a dissipation of $\Delta\Psi$ (trace E), which was favored by CsA (trace F) and, again, inhibited by ATP plus oligomycin (trace G). The dissipation caused by KCl was only partial and smaller than that recovered in nonpurified mitochondria, requiring a higher concentration of salt. This difference can be rationalized considering that purified mitochondria were more coupled (RCR = ca. 3.5–4.0) than the nonpurified ones (RCR = ca. 2.5–3.0) and had thus a higher $\Delta\Psi$, which contributed to render the K^+ channel more difficult to open. These data are in strict agreement with the above described results and, hence, the effect of CsA can not be ascribed to a hypothetical K^+ channel present in membranes different from mitochondria.

Characterization of the K^+ Diffusion Potential Induced by CsA

The CsA-induced fluorescence changes were inhibited by increasing concentrations of KCl in the incubation medium. This effect showed a sigmoidal kinetic that became a hyperbolic kinetic, if the experiments were carried out after 2 h from the extraction of mitochondria (Fig. 5). However, when mitochondria were extracted and resuspended in 5 mM DTE, the sigmoidal kinetic was maintained even after 2 h (result not shown). Conversely, the effect of valinomycin, as a function of K^+ concentration, was linear. This suggests that the CsA-induced K^+ efflux could be mediated by a carrier or by a channel not completely specific for K^+, regulated by the redox state of mitochondria.

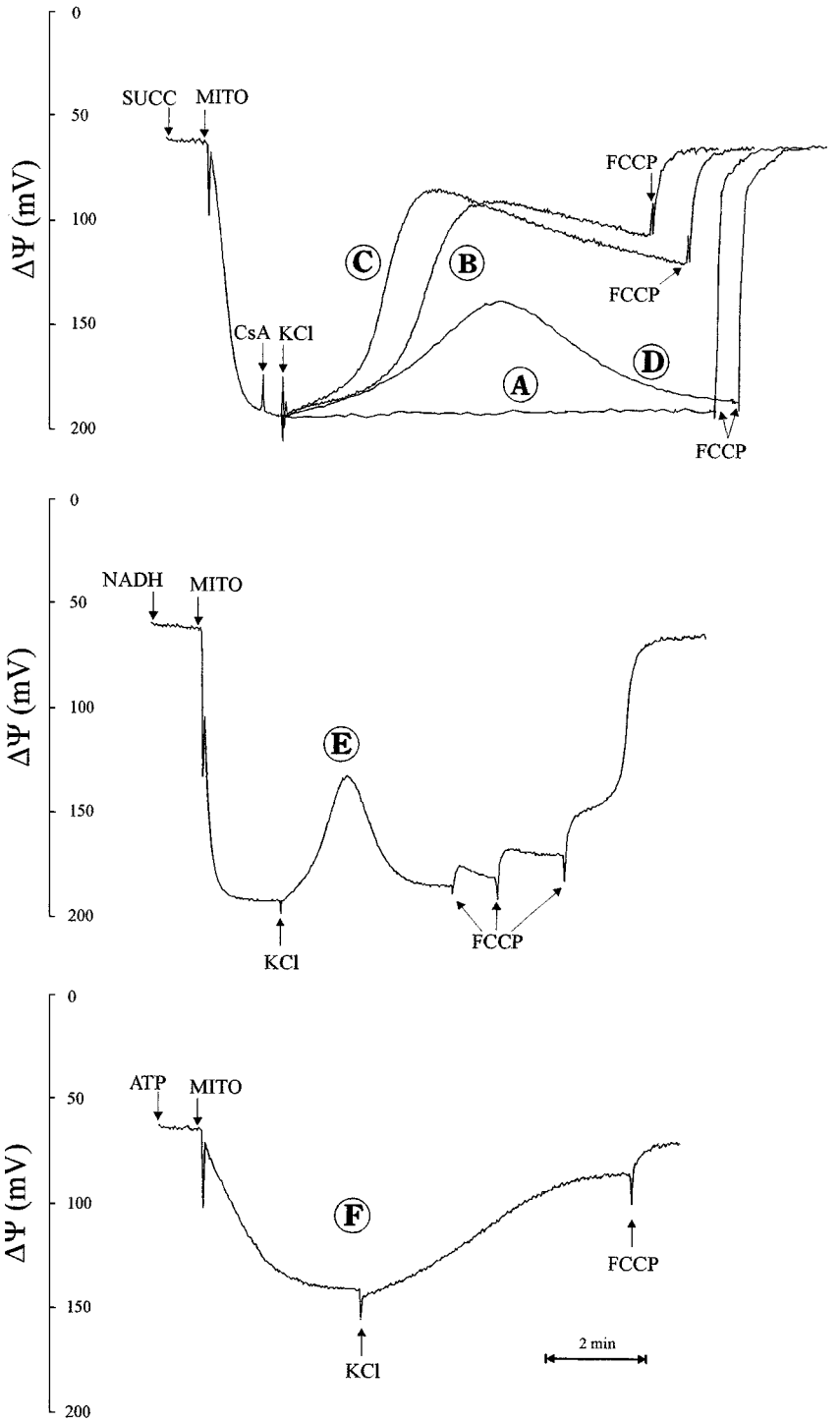

Fig. 3. Effect of K^+ and CsA on $\Delta\Psi$ of energized pea stem mitochondria. The incubation medium was as in Fig. 1. Traces (A–D), succinate-generated $\Delta\Psi$: (A) control; (B) KCl-induced dissipation; (C) effect of CsA on KCl-induced dissipation; (D) effect of ATP plus oligomycin on KCl-induced dissipation. Trace (E) effect of KCl on NADH-generated $\Delta\Psi$. Trace (F) effect of KCl on ATP-generated $\Delta\Psi$. Additions include MITO (0.1 mg/ml protein), 5 mM succinate, 1 mM NADH, 1 mM ATP, 40 mM KCl, 0.3 μM CsA, 1 μM FCCP, and 1 μg/ml oligomycin.

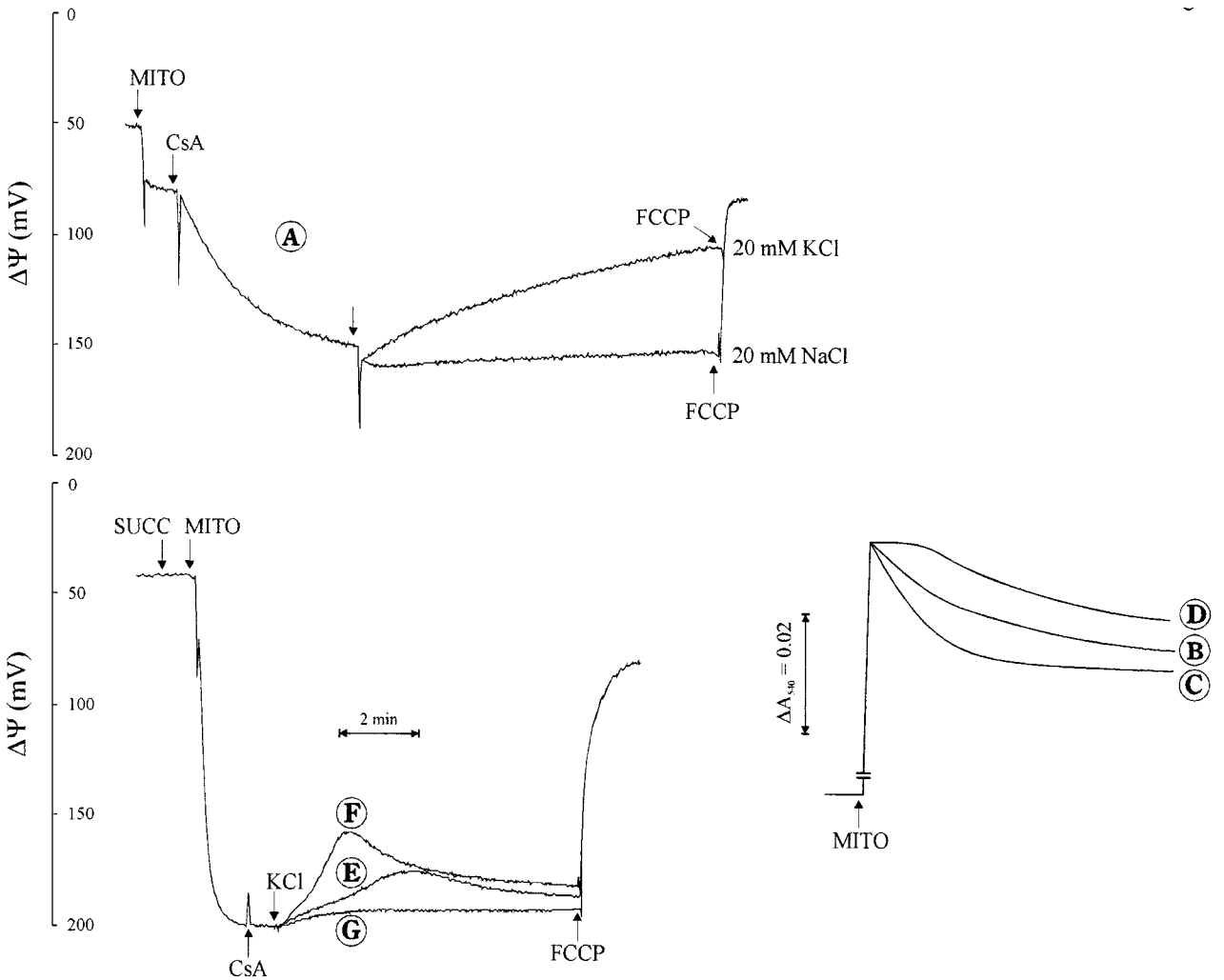

Fig. 4. Effect of CsA on $\Delta\Psi$ and swelling of purified pea stem mitochondria. Traces (A–D) refer to deenergized mitochondria, while traces (E–G) to succinate-energized mitochondria. Conditions and additions were as in Figs. 1–3, except that KCl added in traces (E–G) was 80 mM.

Figure 6 (Panel A) shows that the CsA-induced $\Delta\Psi$ formation (monitored as fluorescence intensity changes) was dependent on the monovalent cation, included as a component of the osmoticum. All the inhibitory effects caused by increasing concentrations of cations assumed a sigmoidal shape and K^+ was the most effective. Rubidium and Li^+ caused comparable effects, while Na^+ was the less effective. This result indicates that the channel exhibited a good degree of selectivity for K^+ and confirms that it was voltage dependent. The same figure also shows that the presence of different anions (Cl^-, F^-, and SO_4^{2-}) scarcely influenced the K^+ diffusion potential induced by CsA (Panel B).

TEM Analysis of CsA-Treated Pea Stem Mitochondria

Figure 7 shows the ultrastructure of a population of untreated (a) and CsA-treated (b) mitochondria. By comparing the mean area of the mitochondria in the two micrographs, it appears that CsA-treated mitochondria were ca. 30% smaller in size. The control and CsA-treated populations, evaluated by F test, were found to be significantly different ($p < 0.05$). In addition, when the mean volumes of mitochondria, calculated as the volume of a rotation elipsoid (around the major axis), were compared, this difference rose to 40%. This effect could be related to shrinkage of mitochondria as a consequence of a reduction

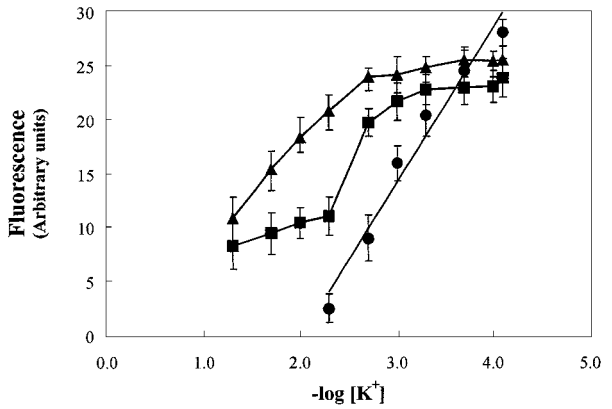

Fig. 5. Effect of K^+ concentration on $\Delta\Psi$ induced by CsA or valinomycin in isolated pea stem mitochondria. The conditions were as in Fig. 1. $\Delta\Psi$ was generated by the addition of 0.3 μM CsA or 50 nM valinomycin. $\Delta\Psi$ formation was prevented by the presence in the incubation medium of increasing concentrations of KCl. Inhibition, expressed in arbitrary fluorescence units, was calculated as residual FCCP-collapsed $\Delta\Psi$ with respect to the value obtained in the absence of salts. ■, mitochondria treated by CsA just after extraction; ▲, mitochondria treated by CsA after 2 h of incubation; ●, valinomycin-treated mitochondria.

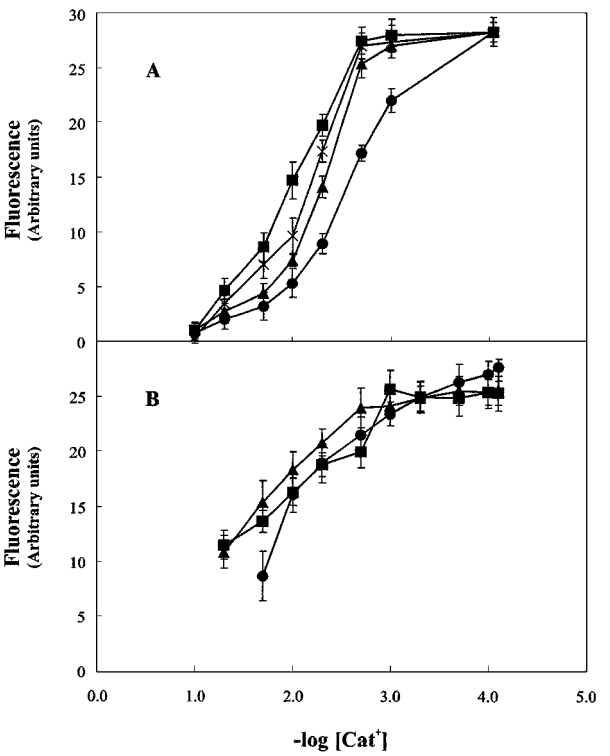

Fig. 6. Effect of different cations (panel A) and anions (panel B) on CsA-induced $\Delta\Psi$ in isolated pea stem mitochondria. In panel A the CsA-induced $\Delta\Psi$ was evaluated in the medium of Fig. 1, except that sucrose and increasing concentrations of KCl (●), NaCl (■), LiCl (×) or RbCl (▲) were used to maintain constant osmolarity. Experiments of panel B were also performed in the medium described in Fig. 1. Increasing concentrations of KF (▲), K_2SO_4 (■) or KCl (●) were supplied to the medium. Inhibition was calculated as described in Fig. 4.

in the osmotic pressure due to the K^+ efflux induced by CsA.

DISCUSSION

The results presented in this paper show that CsA induced opening of an ion channel in isolated plant mitochondria. In the presence of an outwardly directed K^+ gradient (deenergized mitochondria resuspended in a sucrose-based medium), channel opening caused an efflux of K^+, resulting in the establishment of a K^+ diffusion potential.

Valinomycin, a relatively selective K^+ ionophore that allows electrophoretic transport of K^+ with a net charge of 1, led to the establishment of a K^+ diffusion potential in deenergized mitochondria, which displayed the expected linear dependence on the logarithm of external $[K^+]$. In contrast to valinomycin, the cation flux through the CsA-dependent channel leveled off at high values of $\Delta\Psi$, suggesting that the channel is voltage-dependent and tends to close as the $\Delta\Psi$ increases (Fig. 5). Based on the experiments shown in Figure 6, the channel displayed a selectivity for monovalent cations in the following order: $K^+ > Rb^+ = Li^+ > Na^+$. Indeed, the measured $\Delta\Psi$ decreased in this order as the concentration of external cations was increased at constant osmolarity. On the other hand, no differences in measured $\Delta\Psi$ were observed after incubation with potassium salts of different anions. Because of the inside-negative $\Delta\Psi$, only matrix anions could contribute to charge compensation under these conditions and, therefore, we cannot make firm predictions about anion conductance through the channel at the present time.

The activity of this channel could also be followed as K^+ entry into mitochondria (swelling) that was inhibited by ATP plus oligomycin and stimulated by CsA and valinomycin (Figs. 2 and 4). In addition the channel functioned in energized mitochondria and its opening was again favored by CsA and inhibited by nucleotides (Figs. 3 and 4).

Taken together, these results indicate that a K^+ flux in plant mitochondria may occur in both directions through a voltage-dependent K^+_{ATP} channel. This channel possesses a remarkable ability to discriminate between K^+ and Rb^+, and appears to be modulated by the oxidoreductive state of mitochondria, possibly through thiol groups, since

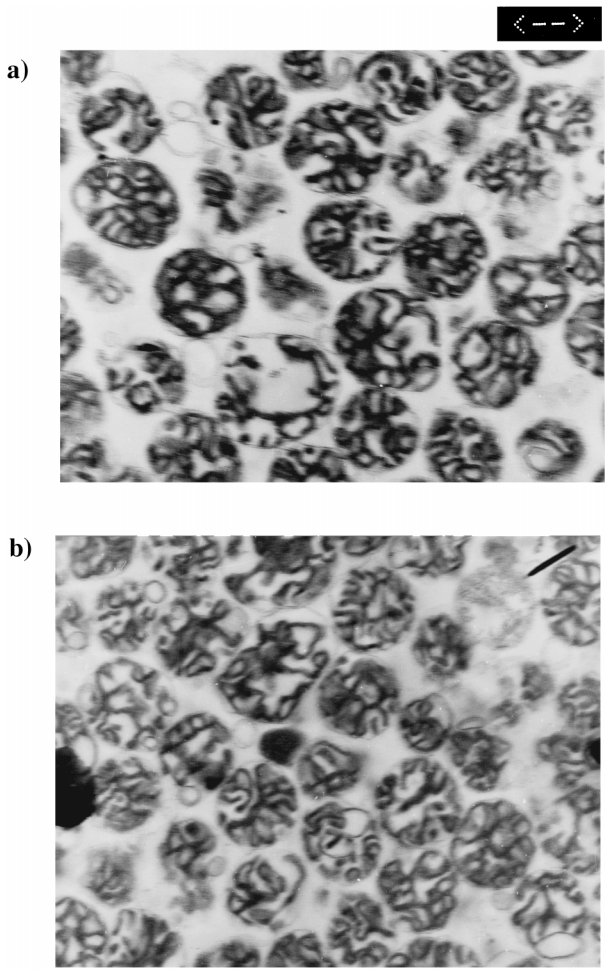

Fig. 7. Ultrastructure of purified pea stem mitochondria. (a) Control and (b) CsA-treated mitochondria. Magnification = 12,000 x; Bar = 0.6 μm. CsA was 0.3 μM. The mean area of mitochondria, calculated on two populations of 80 organelles, was 0.546 μm^2 in the control and 0.416 μm^2 in the CsA-treated sample, respectively.

different kinetics were obtained in the presence or absence of DTE. This mode of regulation is similar to that described for PTP (Petronilli *et al.*, 1994a), for the neuronal *N*-methyl-D-aspartate receptor channel (Moriyoshi *et al.*, 1991), the α/β K_v channels (Rettig *et al.*, 1994) and, as recently shown, for K^+_{ATP} channels purified from the inner mitochondrial membrane and reconstituted into lipid bilayer membranes (Grigoriev *et al.*, 1999). The sensitivity to CsA suggests that the open conformation of the channel depends on an interaction with cyclophilin (the ligand of CsA), which has also been identified in plant mitochondria (Breiman *et al.*, 1992). A similar mechanism operates in the case of the guard cells of *Vicia faba* (Kinoshita and Shimazaki, 1999) where a cyclophilin–CsA complex, by interacting with a protein phosphatase, blocks Ca^{2+}-induced inactivation of K^+ channels (Luan *et al.*, 1993).

Previously, it has been shown that FCCP, at low concentrations, dissipates the $\Delta\Psi$ of plant mitochondria and that this dissipation is delayed by CsA (Vianello *et al.*, 1995). This led to the suggestion that the effect could be related to the presence of a CsA-sensitive pore similar to the mammalian PTP (Bernardi *et al.*, 1994; Halestrap, 1994; Zoratti and Szabò, 1995). In principle, the CsA-dependent establishment of a K^+ diffusion potential could depend on the CsA-dependent closure of such a channel, acting in the substate at low conductance (Ichas and Mazat, 1998), which is able to mediate, at least in mammalian mitochondria, an electrogenic K^+ influx (Balakirev and Zimmer, 1998). This conclusion, however, appears to be unlikely. First, several PTP inducers (diamide, menadione, cumene hydroperoxide, *tert*-butyl hydroperoxide) did not modify the K^+ currents or cause high-amplitude swelling (results not shown); the second and most compelling evidence that the effect of CsA cannot be due to the closure of a high conductance channel is that prolonged incubation of mitochondria in K^+-free media did not hinder the generation of a $\Delta\Psi$ by CsA (Fig. 1).

The presence of voltage-dependent, K^+ selective channels responsible for volume regulation, has been demonstrated in the plasmalemma and tonoplast of higher plant cells (Hedrich and Dietrich, 1996; Maathuis *et al.*, 1997). Despite their enormous importance, the information concerning plant mitochondrial cation channels and exchangers is still scarce and only a H^+/K^+ exchanger has been identified (Hanson, 1985). Very recently, the existence of a K^+_{ATP} channel on the inner membrane of plant mitochondria has been reported (Pastore *et al.*, 1999). This channel resembles in several aspects that identified in the present work. Therefore, the results presented in this paper confirm and extend our knowledge on the presence of a voltage-dependent, K^+ selective channel in higher plant mitochondria. In addition, the K^+ channel described in this paper is similar to those identified in mammalian cells, where CsA induces hyperpolarization of the plasmalemma of different types of lymphocytes (Gergely and Aszalos, 1990; Vereb *et al.*, 1990; Weaver *et al.*, 1991; Aszalos, 1991; Tanner *et al.*, 1993). The effect is also dependent on a K^+ efflux, as indicated by the absence of hyperpolarization in the presence of KCl.

At present, the potential physiological role(s) of the CsA-sensitive K^+ channel can only be a matter of speculation. Plant cells contain high levels of K^+ that

accumulates into the vacuole (200 mM) and its concentration is maintained homeostatically around 80–100 mM in the cytosol (Leigh and Wyn Jones, 1984), while isolated plant mitochondria contain 120–140 mM K^+ (Hanson, 1985). The K^+_{ATP} channel described here may thus be a physiological mechanism regulating matrix K^+ concentration and, therefore, mitochondrial volume (Fig. 7). Since plant mitochondria also contain an electroneutral H^+/K^+ exchanger (Hanson, 1985), it is also possible that energy dissipation in K^+ cycling could lead to heat generation and, hence, to thermogenesis in plants. In addition, the depolarization of the inner membrane caused by opening of the K^+ channel may also constitute a means to prevent or minimize reactive oxygen species formation, as it has been suggested in different systems (Skulachev, 1998). In plants, mitochondria are a major site of generation of reactive oxygen species, which can be controlled by the engagement of non-coupled respiration (alternative oxidase), or by artificial uncouplers (Braidot *et al.*, 1999). Cation cycling thus provides a second line of defense against oxygen toxicity. Finally, increasing evidence suggests that mitochondria play an essential function, as an execution central, in the life and death of animal cells (Pedersen, 1999). In particular, they are involved in the regulation of programmed cell death (apoptosis), which occurs through cytochrome *c* and apoptosis-inducing factor release, as a consequence, in numerous cases, of PTP opening and mitochondrial swelling (Susin *et al.*, 1998; Bernardi *et al.*, 1999). Apoptosis is a well-recognized phenomenon also in plant cells, where a mitochondrial step seems to be crucial (Danon *et al.*, 2000; Jones, 2000). Since plant mitochondria do not appear to possess a PTP acting at high conductance (Vianello *et al.*, 1995), it is suggested that the K^+_{ATP} channel may substitute for PTP in inducing mitochondrial swelling and cytochrome *c* release in apoptotic plant cells.

ACKNOWLEDGMENTS

We are deeply indebted to Prof. P. Bernardi, Dipartimento di Scienze Biomediche, Università di Padova, for several helpful suggestions during the work, and for discussion and criticism on the final version of the paper. We also thank Prof. C.A. Beltrami M.D., S. Pizzolitto, M.D., and Mrs. M. Battistig, Policlinico, Istituto di Anatomia Patologica, Università di Udine, for TEM analysis.

This research was supported by the "Ministero dell'Università e della Ricerca Scientifica e Tecnologica" (Cofin 2000–2001) in the frame of the program entitled: Nitric Oxide and Plant Resistance to Pathogens, and by the University of Udine.

REFERENCES

Aszalos, A. (1991). *Eur. J. Pharmacol.* **205**, 125–133.
Balakirev, M. Y. and Zimmer G. (1998). *Arch. Biochem. Biophys.* **356**, 46–54.
Bernardi, P. (1999). *Physiol. Rev.* **79**, 1127–1156.
Bernardi, P., Broekmeier, K. M., and Pfeiffer, D. R. (1994). *J. Bioenerg. Biomembr.* **26**, 509–517.
Bernardi, P., Scorrano, L., Colonna, R., Petronilli, V., and Di Lisa, F. (1999). *Eur. J. Biochem.* **264**, 687–701.
Braidot, E., Petrussa, E., Macrì, F., and Vianello, A., (1998). *Biol. Plant.* **41**, 193–201.
Braidot, E., Petrussa, E., Vianello, A. and Macrì, F. (1999). *FEBS Lett.* **451**, 347–350.
Breiman, A., Fawcett, T. W., Ghirardi, M. L., and Matoo, A. K. (1992). *J. Biol. Chem.* **267**, 21293–21296.
Crompton, M., Ellinger, H., and Costi, A. (1988). *Biochem. J.* **255**, 357–360.
Danon, A., Delorme, V., Mailhac, N., and Gallois, P. (2000). *Plant. Physiol. Biochem.* **38**, 647–655.
Douce, R. and Neuburger, M. (1989). *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **40**, 371–414.
Garlid, K. D. (1988). In *Integration of Mitochondrial Function* (Lemasters J. J., Hackenbrock C. R., Thurman R. G., Westerhoff H. V., eds.), Plenum, New York, pp. 257–278.
Garlid, K. D., Sun, X., Paucek, P., and Woldegiorgis, G. (1995). *Methods Enzymol.* **260**, 331–348.
Gergely, P. and Aszalos, A. (1990). *Immunopharmacology* **20**, 191–200.
Griffiths, E. J. and Halestrap, A. P. (1991). *Biochem. J.* **274**, 611–614.
Grigoriev, S. M., Skarga, Y. Y., Mironova, G. D., and Marinov, B. S. (1999). *Biochim. Biophys. Acta* **1410**, 91–96.
Halestrap, A. P. (1994). In *Mitochondria: DNA, Proteins, Disease* (Darley-Usmar V. and Schapira A. H. V., eds.), Portland Press, London, pp. 113–142.
Halestrap, A. P. and Davidson, A. M. (1990). *Biochem. J.* **268**, 153–160.
Hanson, J. B. (1985). In *Encyclopedia of Plant Physiology. Higher Plant Cell Respiration* (Douce R., Day D. A., eds.), vol. 18. Springer Verlag, Berlin, pp. 248–280.
Hedrich, R. and Dietrich, P. (1996). *Botan. Acta* **109**, 94–101.
Ichas, F. and Mazat, J.-P. (1998). *Biochim. Biophys. Acta* **1366**, 33–50.
Jones, A. (2000). *Trends Plant Sci.* **5**, 225–230.
Jung, D. W., Bradshaw, P. C., and Pfeiffer, D. R. (1997). *J. Biol. Chem.* **272**, 21104–21112.
Kinoshita, T. and Shimazaki, K. (1999). *Plant Cell Physiol.* **40**, 53–59.
Klein, R. R. and Koeppe, D. E. (1985). *Plant Physiol.* **77**, 909–911.
Leigh, R. A. and Wyn Jones, R. G. (1984). *New Phytol.* **97**, 1–14.
Luan, S., W., Rusnak, F., Assmann, S. M., and Schreiber, S. L. (1993). *Proc. Natl. Acad. Sci. USA* **90**, 2202–2206.
Maathuis, F. J. M., Ichida, A. M., Sanders, D., and Schroeder, J. I. (1997). *Plant Physiol.* **114**, 1141–1149.
Manon, S., Roucou, X., Guérin, M., Rigoulet, M., and Guérin, B. (1998). *J. Bioenerg. Biomembr.* **30**, 419–429.
Mitchell, P. (1966). *Biol. Rev. Camb. Phil. Soc.* **41**, 445–502.
Moore, A. L. and Bonner, W. D. Jr. (1982). *Plant Physiol.* **70**, 1271–1276.
Moriyoshi, K., Masu, M., Ishii, T., Shigemoto, R., Mizuno, N., and Nakanishi, S. (1991). *Nature* (*London*) **354**, 31–37.
Palmieri, F., Bisaccia, F., Capobianco, L., Dolce, G., Fiermonte, V., Iacobazzi, V., Indiveri, C., and Palmieri, L. (1996). *Biochim. Biophys. Acta* **1275**, 127–132.
Pastore, D., Stoppelli, M. C., Di Fonzo, N., and Passarella, S. (1999). *J. Biol. Chem.* **274**, 26683–26690.

Paucek, P., Mironova, G., Mahdi, F., Beavis, A. D., Woldegiorgis, G., and Garlid, K. D. (1992). *J. Biol. Chem.* **267**, 26062–26069.
Pedersen, P. L. (1999). *J. Bioenerg. Biomembr.* **31**, 291–304.
Petronilli, V., Costantini, P., Scorrano, L., Colonna, R., Passamonti, S. and Bernardi, P. (1994). *J. Biol. Chem.* **269**, 16638–16642.
Petronilli, V., Nicolli, A., Costantini, P., Colonna, R., and Bernardi, P. (1994). *Biochim. Biophys. Acta* **1187**, 255–259.
Rettig, J., Heinemann, S. H., Wunder, F., Lorra, C., Parcej, D. N., Dolly, J. O., and Pongs, O. (1994). *Nature* (*London*) **369**, 289–294.
Skulachev, V. P. (1998). *Biochim. Biophys. Acta* **1363**, 100–124.
Susin, S. A., Zamzami, N., and Kroemer, G. (1998). *Biochim. Biophys. Acta* **1366**, 151–165.
Tanner, M. K., Wellhausen, S. R., and Klein, J. B. (1993). *Cytometry* **14**, 59–69.
Vereb, G., Jr., Panyi, G., Balázs, M., Mátyus, L., Matkó, J., and Damjanovich, S. (1990). *Biochim. Biophys. Acta* **1019**, 159–165.
Vianello, A., Petrussa, E., and Macrì, F. (1994). *FEBS Lett.* **349**, 407–410.
Vianello, A., Macrì, F., Braidot, E., and Mokhova, E. N. (1995). *FEBS Lett.* **371**, 258–260.
Weaver, J. L., Pine, P. S., and Aszalos, A. (1991). *Immunopharmacol. Immunotoxicol.* **13**, 563–576.
Zoratti, M. and Szabò, I. (1995). *Biochim. Biophys. Acta* **1241**, 139–176.