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Occurrence and Characteristics of the Mitochondrial Permeability Transition in Plants*

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Silvio Arpagaus‡, André Rawyler, and Roland Braendle

From the Institute of Plant Sciences, University of Bern, Altenbergrain 23, Bern CH-3013, Switzerland

The behavior of purified potato mitochondria toward the main effectors of the animal mitochondrial permeability transition has been studied by light scattering, fluorescence, SDS-polyacrylamide gel electrophoresis, and immunoblotting techniques. The addition of Ca^{2+} induces a phosphate-dependent swelling that is fully inhibited by cyclosporin A if dithioerythritol is present. Mg^{2+} cannot be substituted for Ca^{2+} but competes with it. Disruption of the outer membrane and release of several proteins, including cytochrome *c*, occur upon completion of swelling. Ca^{2+}-induced swelling is delayed and its rate is decreased when pH is shifted from 7.4 to 6.6. It is accelerated by diamide, phenylarsine oxide, and linolenic acid. In the absence of Ca^{2+}, however, linolenic acid (≤20 μM) rapidly dissipates the succinate-driven membrane potential while having no effect on mitochondrial volume. Anoxic conditions favor *in vitro* swelling and the concomitant release of cytochrome *c* and of other proteins in a pH-dependent way. These data indicate that the classical mitochondrial permeability transition occurs also in plants. This may have important implications for our understanding of cell stress and death processes.

Since the late 1970s, it has been known that animal mitochondria can experience a sudden increase in the permeability of their inner membrane to low and medium molecular weight compounds via the opening of a pore (1–3). This mitochondrial permeability transition pore (PTP)[1] is viewed as a multiprotein complex composed at least of the voltage-dependent anion channel, the adenine nucleotide translocator (AdNT), and cyclophilin-D, at the contact sites between outer and inner membranes (4). When the pore opens, solutes up to about 1.5 kDa can pass through the inner membrane, a process known as the mitochondrial permeability transition (MPT). Subsequently, the membrane potential (ΔΨ) decays, oxidative phosphorylation is uncoupled from electron flow, intramitochondrial ions and metabolites are released, and a large amplitude swelling can occur, disrupting the outer membrane and releasing intermembrane compounds.

Although pore opening primarily requires the accumulation of Ca^{2+} in the mitochondrial matrix, it is also modulated by numerous factors. For instance, P_i, low ΔΨ, thiol-oxidizing reagents, low ATP level, fatty acids, anoxia, and reaeration stress all favor pore opening, whereas thiol-reducing agents, low pH, high ΔΨ, and divalent cations other than Ca^{2+} counteract it (5). Inhibition of MPT is readily achieved with submicromolar concentrations of cyclosporin A (CsA) (6, 7). This highly specific effect has decisively contributed to the acceptance of the pore theory (6, 7) and is used today as the primary diagnostic trait of the classical MPT (5). The implication of mitochondria and PTP in mammalian cell death gave a new impetus to the research. For instance, cytochrome *c* has been shown to be released from the mitochondrial intermembrane space into the cytosol (8, 9), where it can trigger apoptosis (10). How cytochrome *c* is released into the cytosol is still unclear, but a probable way is via PTP opening and subsequent swelling and disruption of the outer membrane (4, 11–13).

Among the steps shared by apoptosis in animals and programmed cell death in plants (14, 15), cytochrome *c* release and caspase activation are early and crucial events (16–18). In particular, questions remain as to the mechanism of cytochrome *c* release into the cytosol of plant cells and how mitochondria are involved in this process. MPT might be one underlying mechanism, but to date no evidence is available for the occurrence of PTP in plants. Jones (19) recently discussed the possible role of mitochondria and PTP as stress sensors and dispatchers of programmed cell death in animal and plant cells. Working with pea stem mitochondria, Vianello *et al.* (20) showed that the lag phase preceding the ΔΨ collapse induced by carbonyl-cyanide-4-trifluoromethoxyphenylhydrazone (FCCP) or by carboxyatractylate was increased by CsA. However, their mitochondria did not exhibit high amplitude swelling, which was suggested to be an intrinsic characteristic of pea stem mitochondria (20). This first attempt to substantiate the occurrence of MPT in plants remained thus inconclusive.

Here we present evidence for the existence of MPT in plant mitochondria isolated from potato (*Solanum tuberosum* L.) tubers. First, Ca^{2+} (but not Mg^{2+}) induces swelling in the presence of P_i, and this process is completely inhibited by CsA if dithioerythritol is present. Second, swelling of mitochondria causes the complete disruption of the outer (but not of the inner) membrane and a subsequent release of cytochrome *c* and of several other polypeptides. Third, PTP opening can be delicately modulated by (bio)chemical and physiological factors such as thiol-oxidizing reagents, pH, free fatty acids, and anoxia. We conclude that MPT does occur in plant mitochondria in a very similar way as in animal mitochondria and discuss the implication of these findings for stress physiology and cell death in plants.

EXPERIMENTAL PROCEDURES

Plant Material—Only young, nonsprouting potato tubers (*S. tuberosum* L.) were used. According to the seasonal offer, the cultivars Agria,

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‡ To whom correspondence should be addressed. Tel.: 41 31 631 49 57; Fax: 41 31 332 20 59; E-mail: silvio.arpagaus@ips.unibe.ch.

[1] The abbreviations used are: PTP, permeability transition pore; AdNT, adenine nucleotide translocator; MPT, mitochondrial permeability transition; Δψ, membrane potential; CsA, cyclosporin A; FCCP, carbonyl-cyanide-4-trifluoromethoxyphenylhydrazone; MOPS, 4-morpholinepropanesulfonic acid.

Stella, and Charlotte were selected.

Isolation of Potato Tuber Mitochondria—Mitochondria were isolated and purified in a self-generated Percoll gradient essentially as described by Moore *et al.* (21), except that all media contained 5 mM pyruvate and 1 mM succinate. Purified mitochondria were resuspended in storage medium (200 mM sucrose, 5 mM pyruvate, 1 mM succinic acid, 5 mM MOPS/Tris, pH 7.2), kept on ice, and used within 5–6 h.

Protein Quantitation—It was achieved with a dye-binding microassay using the Bio-Rad Reagent (Bio-Rad) and bovine serum albumin as a standard.

Mitochondrial Swelling and Protein Release—Swelling was determined by monitoring the changes in 90° light scattering (22) at 540 nm with a PerkinElmer Life Sciences 1000 spectrofluorimeter. Neutral density filters were inserted into the light pathways so as to shield the photomultiplier from any excess of scattered light. A suspension volume equivalent to 500 μg of mitochondrial protein was delivered to a stirred and thermostated cuvette (25 °C) containing air-saturated incubation medium (200 mM sucrose, 10 mM MOPS, 5 mM succinic acid, 10 μM EGTA, and, except where indicated, 2 μM rotenone, 1 μg/ml oligomycin and 1 mM H_3PO_4). The pH was adjusted to 7.4 (with Tris base) except where indicated (see legends to Figs. 5 and 7). The final volume was 2 ml. The signal was plotted on a chart recorder.

At the end of the optical measurements, suspensions were centrifuged for 10 min at 20,000 × *g*. Supernatants were used to determine the amount of protein released from mitochondria. They were also treated with ice-cold trichloroacetic acid (10% (w/v), final concentration) and centrifuged for 10 min at 10,000 × *g*. Precipitated proteins were taken up in a suitable volume of 1 M NaOH, separated by SDS-PAGE, and analyzed as follows.

SDS-PAGE and Immunoblotting—Protein extracts (1 volume) were mixed with ¼ volume of sample buffer (60 mM Tris/HCl, pH 6.8, 25% glycerol, 2% SDS, 14.4 mM β-mercaptoethanol, 0.1% bromphenol blue) and boiled for 5 min. SDS-PAGE was carried out with a Mini Protean II Dual Slab Cell (Bio-Rad) according to Laemmli (23), using precasted 10–20% linear Tris-HCl acrylamide gels (Bio-Rad). After electrophoresis, gels were stained with Coomassie Brilliant Blue R-250 to scan the polypeptide patterns or blotted onto 0.45-μm nitrocellulose membranes (Bio-Rad). Immunodetection was performed as described by Arpagaus and Braendle (24). The selectivity of the monoclonal antibody against cytochrome *c* (clone 7H8.2C12; Pharmingen) was verified with purified horse heart cytochrome *c* and with total mitochondrial proteins.

Anoxia Pretreatment of Purified Mitochondria—Pretreatment was carried out in cylindrical glass vials (50 × 14 mm) fitted with rubber septa and screw caps, in a total volume of 2 ml. Hypoxic conditions were first obtained by bubbling argon in the incubation medium. Mitochondria (500 μg of protein) were then added, and the cuvette was closed under argon. The residual O_2 was consumed within 1 min, according to parallel measurements with a Clarke O_2 electrode (Rank Brothers). Normoxic controls were treated similarly except that the medium was saturated with air and the cuvette was not closed. During both pretreatments, suspensions were kept under constant stirring and temperature (25 °C). All subsequent additions (from N_2-saturated stock solutions) were made through the septum with a microsyringe.

Estimation of Mitochondrial Intactness—After completion of the treatments, the samples were centrifuged for 5 min at 18,000 × *g*. Pellets were delicately resuspended with a brush in storage medium and used for the measurements of membrane intactness (0.250 mg/ml protein in each case). Outer membrane intactness was determined with the method of Neuburger *et al.* (25), based on the impermeability of the intact outer mitochondrial membrane to exogenous cytochrome *c*. Inner membrane intactness was estimated from the activity levels of the matrix enzyme isocitrate dehydrogenase (26).

$\Delta\Psi$—The membrane potential was monitored with the dye safranine in the fluorometric mode (27). Measurements were performed in 2 ml of a medium made of 200 mM sucrose, 10 mM MOPS, 10 μM EGTA, 2 μM rotenone, 1 μg/ml oligomycin, and 1 mM H_3PO_4 (pH 7.4 with Tris base) under the same conditions and with the same equipment as for the light scattering experiments, using a dye/protein ratio of 1:20 (5 μM safranine, 100 μg/ml protein).

RESULTS

Immediately after isolation, the Percoll-purified mitochondria isolated from Agria, Stella, and Charlotte potato tubers exhibited a high degree of intactness, with an average value of 94.3% ± 1.5 (n = 5) for their outer membrane. Mitochondria kept their volume constant for prolonged periods, as indicated by the stability of the light scattering signal in controls (Fig. 1*a*). We could ascertain an essentially linear relationship between the swelling capacity and the intensity of the light scattering signal under our conditions, as already reported by Petronilli *et al.* (22). The addition of $CaCl_2$ (5 mM final concentration) first induced a fast shrinkage, followed by a lag phase and a pronounced swelling (Fig. 1*b*). This effect was completely abolished by preliminary additions of dithioerythritol and CsA (Fig. 1*c*). However, neither CsA nor dithioerythritol alone could inhibit the Ca^{2+}-induced swelling; in this case, the only effect was a slight increase of the lag phase (Fig. 1, *d* and *e*). When $CaCl_2$ was replaced by an equal concentration of $MgCl_2$, shrinkage still appeared, but no swelling occurred (Fig. 1*f*). However, at 5 mM Ca^{2+}, raising the Mg^{2+} level up to 20 mM increased the time required for completion of swelling in a linear fashion (10% increase per mM Mg^{2+}; r = 0.997), while leaving unaffected both the lag phase and the swelling amplitude (not shown). No swelling could be obtained after the Ca^{2+} addition if P_i was omitted from the medium (Fig. 1*g*). According to tuber variety and physiological age, the Ca^{2+} concentration required to induce swelling in potato mitochondria ranged from

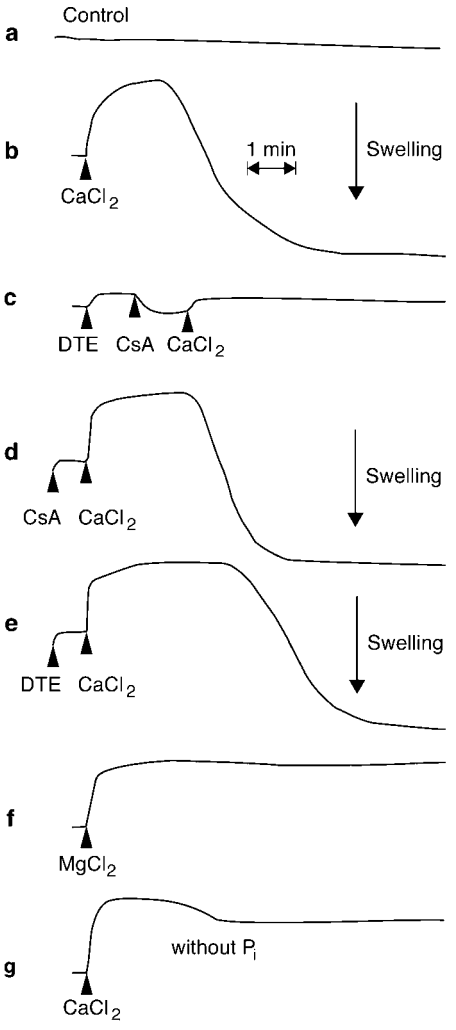

FIG. 1. **Ca^{2+}-induced swelling of potato mitochondria.** The *arrow* indicates the increase in swelling, and the *double-headed arrow* indicates a 1-min time scale. Additional details are given under "Experimental Procedures." *Trace a*, control mitochondria, no additions; *trace b*, after the addition of 5 mM $CaCl_2$; *trace c*, pretreatment with dithioerythritol (*DTE*; 1 mM) and CsA (1.6 μM), followed by the addition of $CaCl_2$; *trace d*, pretreatment with CsA alone, followed by $CaCl_2$; *trace e*, pretreatment with dithioerythritol alone, followed by $CaCl_2$; *trace f*, after the addition of 5 mM $MgCl_2$ instead of $CaCl_2$; *trace g*, as in *trace b*, but P_i was omitted from the medium.

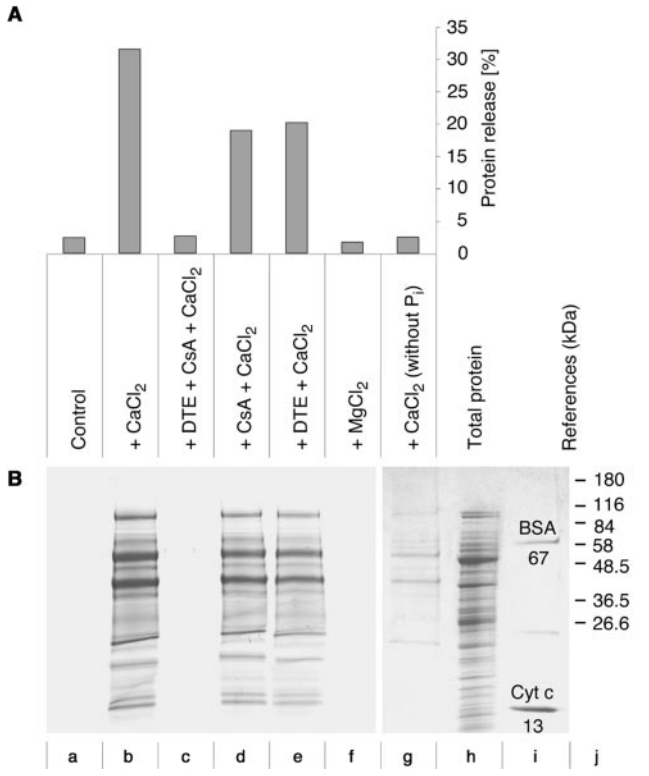

FIG. 2. **Protein release from potato mitochondria after swelling.** The incubation conditions and treatments were as in Fig. 1. *A*, the concentrated supernatants were analyzed for protein content, expressed as a percentage of the initial mitochondria amount (*bars*). *B*, equal volumes were loaded on a 10–20% linear acrylamide gel and separated by SDS-PAGE, so as to allow a direct comparison between lanes *a–g*. *Lane a*, control mitochondria, no addition; *lane b*, after the addition of 5 mM $CaCl_2$; *lane c*, pretreatment with dithioerythritol (*DTE*; 1 mM) and CsA (1.6 μM), followed by $CaCl_2$; *lane d*, pretreatment with CsA alone, followed by $CaCl_2$; *lane e*, pretreatment with dithioerythritol alone, followed by $CaCl_2$; *lane f*, after the addition of 5 mM $MgCl_2$ instead of $CaCl_2$; *lane g*, as in *b*, but P_i was omitted from the medium; *lane h*, total mitochondrial proteins (10 μg); *lane i*, horse heart cytochrome *c* (13 kDa) and bovine serum albumin (*BSA*; 67 kDa); *lane j*, molecular weight markers.

0.5 to 5 mM but gave similar results.

Mitochondrial swelling is usually interpreted as reflecting an expansion of the matrix that may culminate in the physical rupture of the outer membrane (28). The amount of protein released into the medium was thus measured after the removal of mitochondria by centrifugation (Fig. 2*A*). In addition, SDS-PAGE was employed to visualize the polypeptide pattern of the released protein material (Fig. 2*B*). Control mitochondria hardly released any proteins (Fig. 2*A*, *lane a*), and all of those treatments that did not promote swelling (Fig. 1, *c*, *f*, and *g*) also failed to release proteins (Fig. 2*A*, *lanes c*, *f*, and *g*). Conversely, significant protein amounts (Fig. 2*A*, *lanes b*, *d*, and *e*) were found in the medium only if swelling occurred (Fig. 1, *a*, *d*, and *e*). Among the released proteins, and regardless of the treatments, several polypeptide bands were noticeably enriched, and a few others were either depleted or absent as compared with the pattern of total mitochondrial proteins (Fig. 2*B*, *lane h*). These differences suggest that the outer and inner membranes are not affected to the same extent by swelling. This was confirmed by determining the intactness of control mitochondria and of those having experienced Ca^{2+}-induced swelling, which show that the outer membrane of the latter was completely disrupted (Table I). In contrast, the inner membrane still exhibited about 60% intactness (Table I), which essentially reflects the increased fragility of the inner membrane upon swelling in an Mg^{2+}-free medium (29).

TABLE I

Effect of swelling on the intactness of outer and inner membrane of potato mitochondria

Swelling was induced by 5 mM $CaCl_2$ and monitored by light scattering as in Fig. 1, except that rotenone and oligomycin were absent from the incubation medium. Control and Ca^{2+}-treated mitochondria were then collected by centrifugation and resuspended in storage medium. The intactness of the outer and inner membranes was assayed according to Neuburger *et al.* (25) and Bergman *et al.* (26), respectively. Data represent the mean ± S.D. of four independent measurements.

Mitochondria	Outer membrane intactness	Inner membrane intactness
	%	%
Control	94 ± 3	>98
Ca^{2+}-treated	<1	58 ± 3

Interestingly, the patterns of released proteins (Fig. 2*B*, *lanes b*, *d*, and *e*) show the presence of a polypeptide with an electrophoretic mobility similar to that of cytochrome *c* (Fig. 2*B*, *lane i*) and which was absent in the patterns from nonswollen samples (Fig. 2*B*, *lanes c*, *f*, and *g*). A Western blot analysis showed that cytochrome *c* was effectively released from potato mitochondria and that this release was strictly dependent on Ca^{2+}-induced swelling (Fig. 3, compare *b* and *d* with *a*, *e*, and *f*). Moreover, inhibition of swelling by the couple dithioerythritol plus CsA abolished cytochrome *c* release (Fig. 3*c*).

The redox state of mitochondrial sulfhydryl groups was modulated by a pretreatment of organelles during 1 min either with 1,1′-azo-bis-*N*,*N*-dimethylformamide (diamide), which oxidizes thiols to disulfides or with phenylarsine oxide, which bridges two thiol groups (Fig. 4). A subsequent addition of Ca^{2+} led to a dramatic decrease of the lag phase preceding swelling and to a faster swelling rate, as compared with the control. In addition, on a molar basis, phenylarsine oxide (Fig. 4*c*) was more efficient than diamide (Fig. 4*b*).

Next, the pH dependence of the Ca^{2+}-induced swelling was studied over a physiologically relevant range of pH values (Fig. 5). Since it was rapidly apparent that the time required for completion of swelling (see *inset* of Fig. 5) could vary between different mitochondria preparations assayed under the standard pH 7.4 condition, this time was always taken as 100%, and the other time values were expressed as percentages of this standard time before being plotted against pH. We have compared the Ca^{2+}-induced swelling in two different mitochondria preparations exhibiting extreme values (6.6 and 21.6 min) of this standard time. The two curves were remarkably similar, indicating that they reflect a mechanism that depends on the pH but not on the standard time value. The time required for completion of swelling was 3.5–4-fold longer at pH 6.6 than at 7.6 (Fig. 5). In any case, the two components of the light scattering response displayed a high degree of sensitivity to $[H^+]$; the lag phase was prolonged, and the swelling rate diminished when pH decreased (not shown). It is worth mentioning that within this narrow pH range, swelling was never completely inhibited, even at low pH values; rather, its expression was considerably slowed down with time.

Fatty acids are commonly used to alter the structural and bioenergetic properties of mitochondrial membranes. In a first set of experiments, the amplitude of $\Delta\Psi$ in energized mitochondria was monitored by measuring the change in the safranine fluorescence signal upon a 5-min treatment with linolenic acid (Fig. 6, *solid lines*). In a second set of experiments with the same mitochondria preparation (but in the absence of safranine), the same fatty acid treatment was applied, but then $CaCl_2$ was added to induce swelling, which was monitored by light scattering (Fig. 6, *dashed lines*). Since mitochondria were isolated in the presence of pyruvate and succinate as stabiliz-

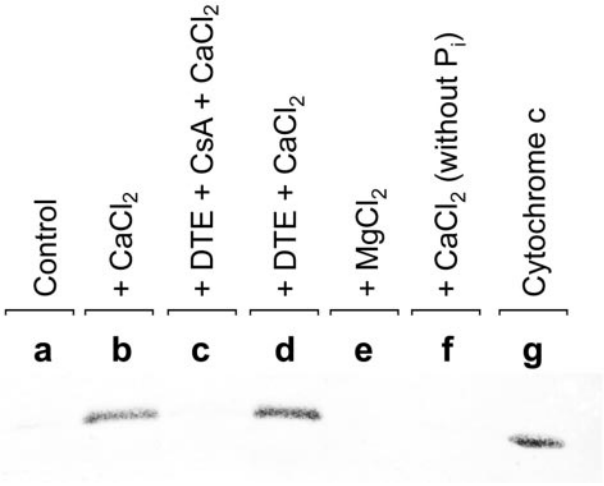

FIG. 3. **Western blot of the proteins released from potato mitochondria during swelling.** Swelling was induced by 0.5 mM $CaCl_2$, and the released proteins were probed with a monoclonal antibody against cytochrome *c*. Similar results were obtained with 5 mM $CaCl_2$. At the end of the light scattering measurement period (see Fig. 1), mitochondria were sedimented, and the supernatants were treated, loaded, and separated by SDS-PAGE as in Fig. 2. *Lane a*, control mitochondria, no addition; *lane b*, after the addition of $CaCl_2$ (0.5 mM); *lane c*, pretreatment with dithioerythritol (*DTE*; 1 mM) and CsA (1.6 μM), followed by $CaCl_2$; *lane d*, pretreatment with dithioerythritol alone, followed by $CaCl_2$; *lane e*, after the addition of 0.5 mM $MgCl_2$ instead of $CaCl_2$; *lane f*, as in *lane b*, but P_i was omitted from the medium; *lane g*, horse heart cytochrome *c* as marker.

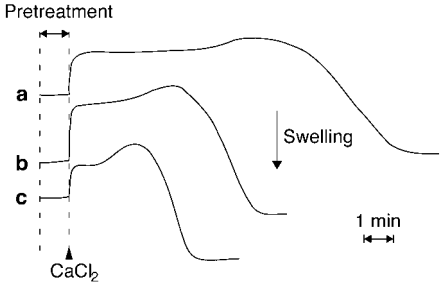

FIG. 4. **Effect of thiol reagents on Ca^{2+}-induced swelling.** The experimental conditions are those of Fig. 1. Mitochondrial suspensions were preincubated for 1 min without any addition (*a*) or pretreated during 1 min with either 100 μM diamide (*b*) or 100 μM phenylarsine oxide (*c*) before swelling was induced by the addition of 5 mM $CaCl_2$.

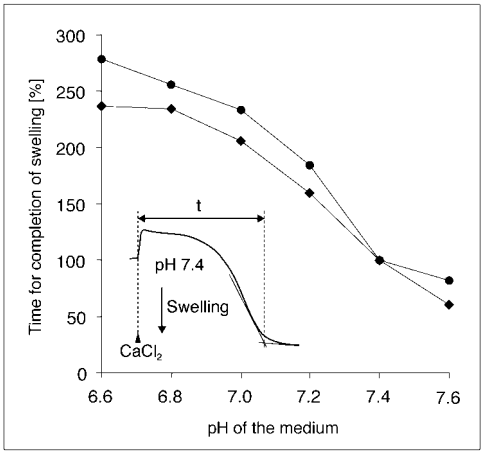

FIG. 5. **Ca^{2+}-induced swelling at different external pH values.** A suspension volume equivalent to 0.5 mg of mitochondrial protein was added to the incubation medium as described in the legend to Fig. 1. The pH values of these media were set with MOPS/Tris buffers. Mitochondrial swelling was monitored by light scattering and induced with 5 mM $CaCl_2$, and the time elapsed from the Ca^{2+} addition until completion of swelling was measured in dependence on pH. The *inset* illustrates how this time was extracted from the light scattering signal obtained at pH 7.4. The *graph* shows the results of two experiments made with mitochondria preparations exhibiting a swelling completion time of 6.6 min (●) and 21.6 min (♦) at pH 7.4. The swelling completion time at pH 7.4 was taken as 100%, and all other time values were expressed as percentages.

ing agents, they could obviously sustain an already large $\Delta\Psi$. The addition of succinate thus had only a slight effect and was primarily aimed at developing a maximal $\Delta\Psi$ that could remain stable during the whole treatment (Fig. 6*a*). The addition of increasing amounts of linolenic acid (5, 10, and 20 μM) progressively decreased $\Delta\Psi$, both in rate and amplitude (Fig. 6, *b* and *c*) and eventually collapsed it (Fig. 6*d*). At this point, the complete collapse of $\Delta\Psi$ was ascertained by the signal insensitivity to the addition of 1 μM FCCP (not shown). The mitochondrial volume remained unaffected during the pretreatment period with linolenic acid. However, striking differences could be observed upon Ca^{2+} addition (Fig. 6, *a–d*). Compared with the control (Fig. 6*a*), the lag phase already decreased after pretreatment with 5 μM linolenic acid (Fig. 6*b*) and completely disappeared at higher concentrations (Fig. 6, *c* and *d*). Simultaneously, the swelling rate was noticeably enhanced with increasing fatty acid concentrations (Fig. 6, compare *a* with *b–d*). Separate control experiments in the absence of mitochondria have ascertained that no sizable light scattering signal was generated under our conditions when $CaCl_2$ was added to the buffered medium containing linolenic acid. When dithioerythritol and CsA were supplied before the pretreatment with 10 μM linolenic acid, swelling was completely inhibited, whereas depolarization was hardly affected (Fig. 6*e*). On the other hand, the addition of dithioerythritol alone before fatty acid pretreatment could not prevent swelling, while again having no effect on $\Delta\Psi$ decay (Fig. 6*f*).

Incubation under anoxia is another way to modulate the metabolic status of mitochondria, which will thus experience conditions that are as close as possible to those encountered for example in underground organs of flooded plants or in ischemic animal tissues. However, an important preliminary issue was to know whether an appreciable $\Delta\Psi$ still existed across the inner mitochondrial membrane under anoxia, as already shown for instance in hepatocytes and endothelial cells (30–32). When potato mitochondria were incubated under anoxia in the presence of safranine, its fluorescence signal persisted for more than 120 min at its maximum level (= 100%) if bovine serum albumin (3.6 μM) was present. Without bovine serum albumin, the safranine fluorescence signal decreased over ~10 min and then tended to stabilize at an intermediary value (~40%) for a long period too. In both cases, these signals were rapidly and fully abolished by FCCP (data not shown). These results suggest that a sizable $\Delta\Psi$ could be maintained for the long term in potato mitochondria when the electron transport chain was inactive. After an anoxic pretreatment of about 100 min at pH 7.4, the lag phase between Ca^{2+} addition and the onset of swelling decreased by about 40%, and the swelling rate almost doubled (compare *traces a* and *b* in Fig. 7*A*). Preincubations of 10 min or more under both normoxia and anoxia were required to observe a clear difference in swelling kinetics between these two conditions. The addition of dithioerythritol and CsA shortly before Ca^{2+} again inhibited swelling (Fig. 7*A*, *trace c*), although with a slightly lessened efficiency that is attributable to anoxia itself rather than to the long preincubation period under this condition (compare also *lanes b* and *c* in Fig. 7*B*). It is also known that anoxia shifts the cytoplasmic pH of plant cells from about 7.5 under normoxia to as low as 6.2 under anoxia (33). When the anoxia treatment was carried out at pH 6.6 (rather than 7.4), swelling did not occur after the

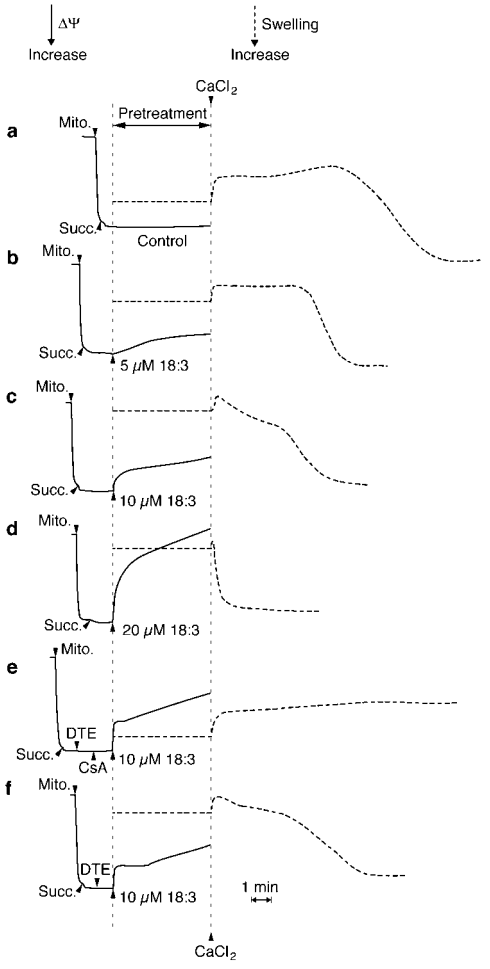

FIG. 6. **Effect of free linolenic acid on ΔΨ and Ca^{2+}-induced mitochondrial swelling.** Mitochondria were introduced as indicated (*Mito.*) into the incubation medium (see Fig. 1) to monitor the fluorescence signal of safranine associated with ΔΨ (*continuous lines*). Succinic acid (*Succ.*; 5 mM), dithioerythritol (*DTE*; 1 mM), CsA (1.6 μM), and linolenic acid (18:3) were added as indicated. Mitochondria having received exactly the same pretreatment were then analyzed for swelling (*dashed lines*). In control mitochondria (*a*), no addition was made during the pretreatment period. Mitochondria were pretreated during 5 min with 5 (*b*), 10 (*c*), or 20 μM linolenic acid (*d*) before inducing swelling with 5 mM $CaCl_2$. In mitochondria pretreated with 10 μM linolenic acid, the Ca^{2+}-induced swelling was monitored after the addition of both 1 mM dithioerythritol and 1.6 μM CsA (*e*) or of dithioerythritol alone (*f*). The *continuous* and *dashed arrows* indicate increasing ΔΨ and swelling, respectively. The *double-headed arrow* represents a 1-min time scale.

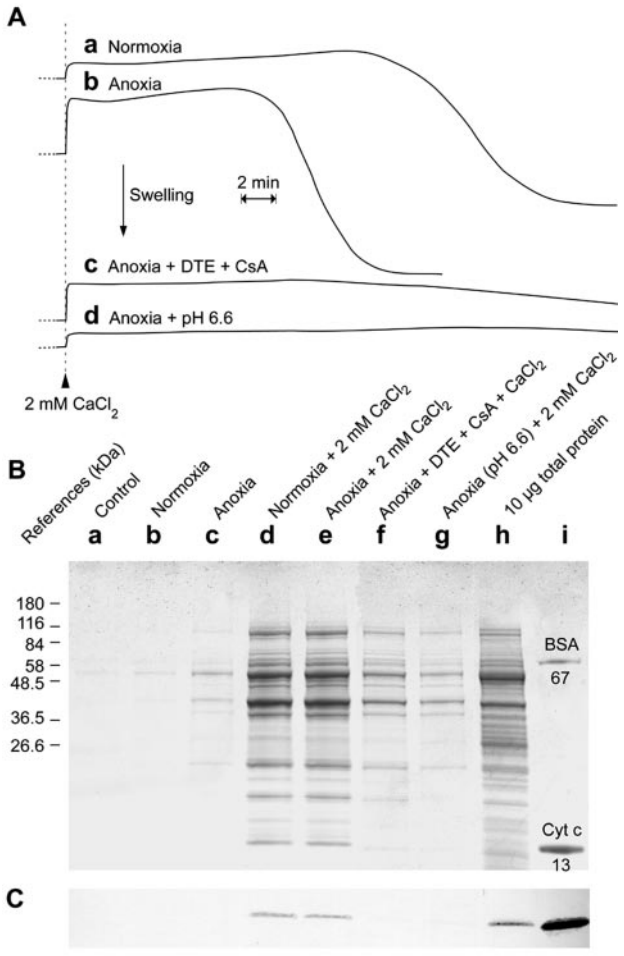

FIG. 7. **Effects of anoxia on Ca^{2+}-induced mitochondrial swelling and protein release.** Mitochondria were suspended in the same medium as in Fig. 1, except that rotenone and oligomycin were omitted. They were then preincubated under normoxic or anoxic conditions for 100 min (*dotted lines*). Mitochondrial swelling was induced by the addition of 2 mM $CaCl_2$. At the end of the treatments, mitochondria were sedimented, and the supernatants were used for analysis of protein release by SDS-PAGE as in Figs. 2 and 3. *A*, light scattering. *Trace a*, control (normoxia pretreatment for 100 min and then the addition of $CaCl_2$); *trace b*, anoxia pretreatment for 100 min and then the addition of $CaCl_2$; *trace c*, anoxia pretreatment for 100 min and then the addition of 1 mM dithioerythritol (*DTE*) and 1.6 μM CsA, followed by $CaCl_2$; *trace d*, anoxia pretreatment for 100 min at pH 6.6 and then the addition of $CaCl_2$. The *vertical arrow* indicates increasing swelling, and the *double-headed arrow* represents a 2-min time scale. *B*, proteins released from mitochondria (Coomassie Blue). *C*, Western blot of the released polypeptides, probed with monoclonal antibody against cytochrome *c*. *Lane a*, control mitochondria suspended in incubation medium (see above) and immediately sedimented; *lane b*, mitochondria incubated for 100 min under normoxia; *lane c*, as in *lane b* but under anoxia; *lane d*, mitochondria incubated for 100 min under normoxia and then treated with $CaCl_2$, postincubated as described in the *upper panel*, and finally sedimented; *lane e*, as in *lane d* but under anoxia; *lane f*, as in *lane e*, but with the addition of dithioerythritol (1 mM) and CsA (1.6 μM) shortly before $CaCl_2$; *lane g*, as in *lane e*, but with an incubation medium buffered at pH 6.6; *lane h*, total mitochondrial proteins (10 μg); *lane i*, horse heart cytochrome *c* (13 kDa) and bovine serum albumin (*BSA*; 67 kDa).

addition of Ca^{2+} (Fig. 7*A*, *lane d*), again suggesting that protons are implicated in counteracting the Ca^{2+}-induced swelling process. SDS-PAGE analysis of mitochondrial supernatants showed that protein release was important when swelling occurred (Fig. 7*B*, *lanes d* and *e*) but was much smaller in its absence (Fig. 7*B*, *lanes a*, *b*, *c*, *f*, and *g*). The electrophoretic patterns of the material released under normoxia (Fig. 7*B*, *lane d*) and anoxia (Fig. 7*B*, *lane e*) were remarkably similar. In particular, cytochrome *c* was among the polypeptides released upon swelling (Fig. 7*C*, *lanes d* and *e*). This suggests that swelling of normoxic and anoxic mitochondria stem from a common mechanism. However, at pH 7.4 but in the absence of Ca^{2+}, the anoxia pretreatment alone was not sufficient, even after 100 min or more, to promote swelling (not shown) as well as protein (Fig. 7*B*, *lane c*) and cytochrome *c* (Fig. 7*C*, *lane c*) release. This emphasizes once again the central role of Ca^{2+} in these processes.

DISCUSSION

The first tenet of animal MPT is its requirement for Ca^{2+} accumulation in the mitochondrial matrix (5), and this rule suffers only rare exceptions (34). However, the amount of Ca^{2+} necessary for pore opening depends, besides species and tissues, on inducers such as P_i (5). While the synergetic effect of P_i and Ca^{2+} was recognized long ago (35), the absolute requirement of Ca^{2+}-induced MPT for P_i has been established only

recently (36). The second tenet is that the extremely potent MPT inhibitor CsA provides the sharpest diagnosis tool in the field (5).

The swelling response of isolated potato tuber mitochondria to these compounds (Fig. 1) presents characteristics that are typical of animal MPT. First, the Ca^{2+} dependence is ubiquitous: swelling occurs only if Ca^{2+} is present (Fig. 1, *b*, *d*, and *e*), and neither linolenic acid (Fig. 6) nor anoxia (Fig. 7) alone suffices to induce such events. Second, the Ca^{2+} effect seems to be specific. Indeed, the other physiologically important divalent metal cation Mg^{2+} does not induce swelling (Fig. 1*f*). This complete lack of effect of Mg^{2+} and the fact that it competes with Ca^{2+} (see "Results"), as already reported by Hunter *et al.* (1), suggests that Mg^{2+} antagonizes Ca^{2+} in a purely electrostatic way. The Ca^{2+} concentration range necessary to trigger the swelling of potato mitochondria (0.5–5 mM), higher than in animal mitochondria (<0.5 mM), suggests that the former have an increased accumulation threshold, which might reflect the shielding of plant cell mitochondria by the high Ca^{2+} pumping efficiency of vacuolar and endoplasmic stores (37). Third, the swelling kinetics do not differ noticeably in animal and plant mitochondria. For instance, after the addition of 10 μM Ca^{2+}, beef heart mitochondria also show a lag phase of several minutes before swelling starts, and the transition is usually completed in about 10–20 min (2). This again suggests that the Ca^{2+}-induced swelling mechanisms of animal and plant mitochondria are similar although differing in their own responsiveness to Ca^{2+}. Fourth, P_i seems to be absolutely required for the Ca^{2+}-induced swelling of potato mitochondria (Fig. 1*g*), in agreement with Sokolove and Haley (36), rather than to play the accelerator role usually conceded to that inducer in animal mitochondria (5). Fifth, the highly efficient inhibitory effect of CsA on Ca^{2+}-induced swelling can be observed not only under standard conditions (Fig. 1*c*) but also after pretreatment with the $\Delta\Psi$-collapsing linolenic acid (Fig. 6*e*) and under anoxia (Fig. 7*A*, *trace c*), suggesting that CsA acts on the same pore element in each case. CsA is known to inhibit mammalian MPT indirectly by binding to cyclophilin-D, the pore component that modulates its opening (38). With the discovery of cyclophilins in plant mitochondria (39), we know that three important components of the PTP in animal mitochondria (voltage-dependent anion channel, AdNT, and cyclophilin-D) are present in plants. However, a clear dithioerythritol dependence of the CsA inhibition of MPT has not been reported in animal systems but might be a typical trait of plant mitochondria. This is supported by the observation of Vianello *et al.* (20) that the presence of dithioerythritol was necessary to obtain a CsA modulation of $\Delta\Psi$ in pea mitochondria. Finally, the Ca^{2+}-induced swelling is accompanied by the preferential disruption of the outer membrane (Table I) and the release of intermembrane proteins (Figs. 2 and 7), including cytochrome *c* (Figs. 3 and 7), as already reported for animal mitochondria (4, 11). Altogether, these data demonstrate that fundamental aspects of the classical MPT phenomenology in animals are also present in isolated potato mitochondria.

Among the chemicals able to modulate the PTP gating state of animal mitochondria (5), thiol reagents favor pore opening. This has been explained by the oxidation of a critical dithiol, the so-called "S-site," which in its reduced state confers a low open probability to the pore (40). The earlier PTP opening promoted by phenylarsine oxide and diamide (Fig. 4) with the same differential efficiency already observed by Halestrap *et al.* (41) strongly suggests that thiol oxidation affects in potato tuber mitochondria a target equivalent to the S-site in animal mitochondria.

Long chain free fatty acids are known to modulate the PTP gating state of animal mitochondria (42). This occurs when they interact with AdNT to stabilize its "cytosolic" conformation (43). Alternatively, the protonophoric effect of fatty acid cycling mediated by AdNT (42) and other mitochondrial carriers decreases $\Delta\Psi$, thereby favoring pore opening (44). Although $\Delta\Psi$ also decreases in potato mitochondria pretreated with linolenic acid (Fig. 6, *b–d*), we cannot decide yet whether this occurs via a direct interaction of the fatty acid with the pore component AdNT (45) and/or with the plant uncoupling mitochondrial protein (46, 47). However, if used below its critical micellar concentration, linolenic acid does not induce swelling (Fig. 6), in contrast to higher concentrations (48). At any rate, the increasing fatty acid-induced depolarization progressively accelerates the onset and the rate of Ca^{2+}-triggered pore opening at a given [Ca^{2+}]. This is the typical behavior of an inducer. Future experiments will show whether depolarization also lowers the threshold [Ca^{2+}] necessary to trigger pore opening (1).

The inhibition of plant MPT at pH <7.0 (Fig. 5) has long been known in animal mitochondria (3), and both are similarly controlled over the same narrow pH range (see also Ref. 49). It is thus tempting to explain the inhibition of plant MPT at acidic pH by a decreased thiol reactivity related to the protonation of histidyl residues in membrane proteins, as proposed by Teixeira *et al.* (50).

In mammals, anoxia leads to metabolic injury and cell death (51, 52) via accelerated ATP depletion, increased P_i level, and dysregulated ion homeostasis (53). Since these metabolic alterations all favor pore opening but are efficiently counteracted by CsA (51, 52, 54), MPT has been implicated in the lethal cell injury caused by ischemia and reperfusion (55). In plants, O_2 deprivation is a very frequent stress imposed by flooding. The submerged underground organs of anoxia-intolerant species rapidly experience similar dysfunctions of their metabolic homeostasis as do ischemic animal tissues (56, 57), and this is also true of cultivated plant cells submitted to anoxia and reaeration (58–60). The accelerated onset and rate of Ca^{2+}-induced swelling observed in O_2-deprived mitochondria (Fig. 7*A*, *trace b*) and their inhibition by the couple dithioerythritol plus CsA (Fig. 7*A*, *trace c*) indicate that, at least *in vitro*, these plant organelles undergo a faster MPT (at pH 7.4) in the absence than in the presence of O_2. This conclusion is in agreement with the work of Krasnikov *et al.* (61) showing that an incubation of rat liver mitochondria at pH 7.4 under anoxia sensitizes them to Ca^{2+} and lowers the threshold [Ca^{2+}] that triggers MPT. The most likely explanation for this facilitated MPT would be the smaller amplitude of the $\Delta\Psi$ established and maintained under anoxia by the Mg^{2+}-dependent, H^+-pumping pyrophosphatase bound on the matrix side of the inner mitochondrial membrane (62). This interpretation is based on the data of Fig. 6 showing that depolarization strongly facilitates Ca^{2+}-induced swelling and on our observation that, even in the absence of bovine serum albumin, isolated potato mitochondria are able to sustain an intermediate $\Delta\Psi$ during a long time (see "Results"). Pyrophosphate is estimated to be present in mitochondrial matrices at about 0.1 mM (62), and the evidence for its involvement as a substitute to ATP under conditions of oxygen and energy deprivation is increasing (63). Moreover, the long term maintenance of a sizable $\Delta\Psi$ would allow an electrophoretic influx of Ca^{2+} into the matrix of anoxic potato mitochondria. The Ca^{2+} electrophoretic influx appears to be extremely variable among plant species (64–68) and between different organs and ages in a given species (69). This large inter- and intraspecific variability might explain the discrepancy between the claim that raw potato mitochondria are devoid of Ca^{2+} electrophoretic influx (68) and our results suggesting the existence of such a pathway in Percoll-purified

mitochondria isolated from young Agria, Stella, and Charlotte potato tubers. The remarkable inhibition of Ca^{2+}-induced swelling achieved under anoxia by lowering the pH to 6.6 (Fig. 7*A*, *trace d*) confirms the protective effect of protons depicted above (Fig. 5) and can be interpreted in terms of the pH paradox (53); anoxia lowers the cytoplasmic pH of plant cells to values that are low enough (33) to efficiently inhibit MPT, whereas reaeration would tend to restore a more alkaline pH and only then trigger pore opening.

Very recently, Fortes *et al.* (70) suggested that intramitochondrial Ca^{2+} is not required to induce a permeability transition in potato mitochondria. This process was induced by Ca^{2+} (>0.2 mM), enhanced by diamide, only partly inhibited by Mg^{2+} and acidic pH, and fully inhibited by dithiothreitol but not by CsA. These features appear to reflect a rather unusual permeability transition that contrasts with the MPT reported here at least on four issues, namely its sensitivity to CsA in the presence of dithioerythritol (Figs. 1*c*, 2 (*lane c*), 3 (*lane c*), 6*e*, and 7, *A* (*lane c*) and *B* (*lane f*)), its cytochrome *c*-releasing property (Figs. 3 and 7*C*), its occurrence in both energized (Figs. 1–3) and deenergized (Fig. 6*d*) mitochondria, and the fact that its occurrence under anoxia (Fig. 7, *A*, *lane b*, and *B*, *lane e*) rules out the requirement for reactive oxygen species (50). Kushnareva and Sokolove (71) argued that the mitochondrial permeability is also regulated by processes other than the classic Ca^{2+}-dependent, CsA-sensitive MPT and proposed the existence of a distinct, Ca^{2+}-independent, CsA-insensitive channel. The results of Fortes *et al.* (70) might better fit into this latter category.

The recognition that animal MPT is a critical mechanism underlying necrotic and apoptotic cell death is relatively recent (4, 72). One of the key events of mammalian apoptosis is the release of cytochrome *c* from the mitochondrial intermembrane space into the cytosol (73), where it binds to Apaf-1 (10). The resulting complex initiates the activation of a caspase cascade that amplifies the process leading to death (10, 74). Presently, the mechanism promoting cytochrome *c* release is still disputed, and two distinct models have emerged. In the first, the Ca^{2+}-dependent MPT results in mitochondrial swelling and rupture of the outer membrane, followed by the leakage of cytochrome *c* and of other intermembrane proteins (4, 11, 12). The Ca^{2+}-independent model states that a more selective protein release occurs without noticeable changes in mitochondrial volume (13) and in $\Delta\Psi$ (8).

At any rate, the presence of free cytochrome *c* in the cytosol is a common occurrence also in plant cells in which programmed cell death has been induced by various treatments (16–18) and conditions (75). Moreover, caspase-like proteases have been recently involved in the control of cell death in higher plants also (15). We have shown here that PTP opening in isolated potato mitochondria leads to swelling (Figs. 1 and 4–7) and outer membrane rupture (Table I) together with protein (Figs. 2 and 7) and cytochrome *c* (Figs. 3 and 7) release. With MPT, plant cells would therefore also possess a mechanism able to mediate the release of cytochrome *c* into the cytoplasm. It is tempting to suggest that in plants, cytochrome *c* might follow a similar translocation pathway to that occurring between mitochondria and Apaf-1 in animals.

Jones (76) proposed that subsequent to the role played by Ca^{2+} in all types of programmed cell death in plants, vacuole collapse is a central event in the execution pathway. However, the death program launched for instance in nonvacuolized but metabolically demanding meristematic tissues of root apices submitted to anoxia must obviously follow another way (77). Hence, we suggest that in plants also, mitochondria as well as MPT are involved in triggering cell death. The next step is now to provide *in vivo* evidence for the occurrence of MPT in plant cells. A new line of investigation is thus opened, in which MPT is expected to play a key role in the processes involved in plant cell death and to stand as a base piece for a unifying concept of cell death in animal and plant tissues.

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