Mitochondrial cytochrome *c* release may occur by volume-dependent mechanisms not involving permeability transition

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The mechanisms regulating mitochondrial outer-membrane permeabilization and the release of cytochrome *c* during apoptosis remain controversial. In the present study, we show in an *in vitro* model system that the release of cytochrome *c* may occur via moderate modulation of mitochondrial volume, irrespective of the mechanism leading to the mitochondrial swelling. In contrast with mitochondrial permeability transition-dependent release of cytochrome *c*, in the present study mitochondria remain intact and functionally active.

Key words: apoptosis, cytochrome *c*, mitochondria, permeability transition, swelling, valinomycin.

INTRODUCTION

Apoptosis in almost all cells involves the release of cytochrome *c* (and other proteins) from the mitochondrial intermembrane space. In fact, an argument could be made that in certain instances, the release of mitochondrial proteins is as important for apoptosis as the activation of procaspases. In spite of this, however, the mechanisms regulating mitochondrial outer-membrane permeabilization and the release of cytochrome *c* remain controversial [1].

Using isolated liver mitochondria, we demonstrated previously that cytochrome *c* release can occur via distinct mechanisms that are either Ca^{2+}-dependent or -independent [2]. In one case, an increase in mitochondrial matrix Ca^{2+} promoted the opening of the permeability transition pore, rupture of the outer mitochondrial membrane and the release of cytochrome *c*. In contrast, Ca^{2+}-independent cytochrome *c* release was induced by oligomeric Bax protein and occurred without mitochondrial swelling. However, our findings also indicated that Bax possessed the capacity to hasten the onset of permeability transition-induced cytochrome *c* release.

That mitochondria of apoptotic cells undergo permeability transition was believed originally to be the mechanism responsible for cytochrome *c* release [3]. Induction of MPT (mitochondrial permeability transition) leads to an impairment of mitochondrial functional activity, including an uncoupling of oxidative phosphorylation and a decrease in mitochondrial membrane potential. This decrease in membrane potential, in turn, stimulates mitochondrial ATP hydrolysis, a disruption of Ca^{2+} homoeostasis and permeability transition pore opening that leads to swelling in the entire population of mitochondria (reviewed in [4]).

Importantly, however, swelling of mitochondria can occur without MPT induction. Specifically, Halestrap et al. [5] reported a K^+-selective, ATP-dependent swelling process, occurring even when MPT is inhibited by the inclusion of the Ca^{2+} chelator EGTA in the incubation medium. The aim of the present study was to extend our earlier findings [2] and more specifically to determine whether mitochondrial cytochrome *c* release occurs under conditions neither involving MPT induction nor insertion of pro-apoptotic proteins into the outer mitochondrial membrane. The results indicate that osmotic changes, triggered either by hypotonic shock or treatment with the K^+-selective ionophore valinomycin, were sufficient to induce mitochondrial swelling and cytochrome *c* release in the absence of MPT. Moreover, mitochondria that were loaded with Ca^{2+} under strict non-MPT conditions also released cytochrome *c*. Together, these findings indicate that mitochondrial cytochrome *c* release can occur via volume-dependent mechanisms that do not necessarily involve the induction of a permeability transition.

EXPERIMENTAL

Isolation of rat liver mitochondria

Male Harlan Sprague–Dawley rats (6–8 weeks old) were killed by CO_2 inhalation in accordance with the European Directive of Protection of Vertebrate Animals for Scientific Research. The liver was removed, minced on ice, resuspended in 50 ml of MSH buffer (210 mM mannitol/70 mM sucrose/5 mM Hepes, pH 7.5) supplemented with 1 mM EDTA and homogenized with a glass Dounce homogenizer and Teflon pestle. Homogenates were centrifuged at 600 ***g*** for 8 min at 4 °C. The supernatant was decanted and re-centrifuged at 5500 ***g*** for 15 min to form a mitochondrial pellet that was resuspended in MSH buffer without EDTA and centrifuged again at 5500 ***g*** for 15 min. The final mitochondrial pellet was resuspended in MSH buffer at a protein concentration of 80–100 mg/ml.

Measurement of functional activity of isolated mitochondria

Mitochondria (1 mg/ml) were incubated in MSH buffer or a buffer containing 150 mM KCl, 1 mM KH_2PO_4, 5 mM succinate and 5 mM Tris (pH 7.4) at 25 °C. Rotenone (2 μM) was added to maintain nicotinamide nucleotides in a reduced form. Estimation of $\Delta\psi$ was performed using an electrode sensitive to the lipophilic cation TPP^+ (tetraphenylphosphonium). Energized mitochondria rapidly accumulate TPP^+ from the incubation buffer and release this cation as $\Delta\psi$ decays. Ca^{2+} fluxes across the inner mitochondrial membrane were monitored using a Ca^{2+}-sensitive electrode (Thermo-Orion, Beverly, MA, U.S.A.). Mitochondrial swelling was monitored continuously by noting the changes

Abbreviations used: CsA, cyclosporin A; MPT, mitochondrial permeability transition; TPP^+, tetraphenylphosphonium.

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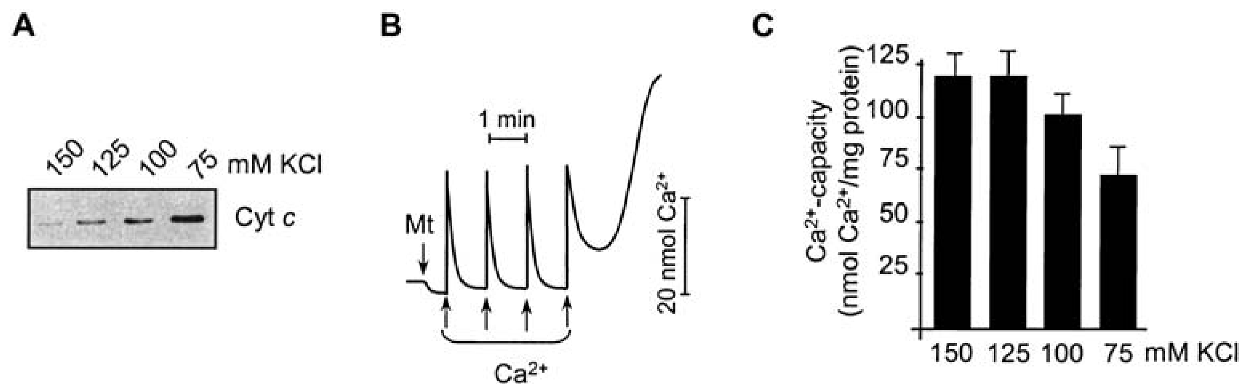

Figure 1 Short-time hypotonicity stimulated cytochrome *c* release from mitochondria

Mitochondria (1 mg/ml) were incubated in KCl-base buffer in the presence of respiratory substrate as described in the Experimental section. (**A**) Stimulation of cytochrome *c* release from mitochondria. After a 1 min stabilization period, an hypotonic state was created by adding an incubation buffer with different concentrations of KCl. After 1 min, the initial concentration was restored by hypertonic KCl solution and samples were taken for determination of cytochrome *c* release. (**B**) Estimation of the Ca^{2+} capacity of mitochondria. After restoration of the tonicity of the buffer, as described in (**A**), mitochondria were sequentially loaded with Ca^{2+} until spontaneous release was started. Ca^{2+} capacity was calculated as a sum of all additions per mg of mitochondrial protein. (**C**) Quantitative estimation of Ca^{2+} capacity of mitochondria after treatment as described in (**A**).

in absorbance A_{540}. Oxygen consumption by isolated rat liver mitochondria was measured using a Clark-type oxygen electrode (Yellow Springs Instruments, Yellow Springs, OH, U.S.A.) at 25 °C. Mitochondria with a respiratory control ratio (defined as the rate of respiration in the presence of ADP divided by the rate obtained after the expenditure of ADP) above 4 were used for all experiments. In some instances, mitochondrial respiration was uncoupled by the addition of 1 μM carbonyl cyanide *m*-chlorophenylhydrazone. Fresh mitochondria were prepared for each experiment and used within 4 h. At the end of the incubation period, mitochondrial suspensions were centrifuged at 10 000 ***g*** for 5 min and the resulting supernatants or pellets were used for Western-blot analysis.

Western-blot analysis

Samples were mixed with Laemmli's loading buffer, boiled for 5 min and subjected to SDS/PAGE (15 % gel) at 130 V followed by electroblotting on to nitrocellulose membranes for 2 h at 100 V. Membranes were blocked for 1 h with 5 % (w/v) non-fat milk in PBS at room temperature (22 °C) and subsequently probed overnight with an anti-cytochrome *c* antibody (1:2500). The membranes were rinsed and incubated with a horseradish-peroxidase-conjugated secondary antibody (1:10 000). After the secondary antibody incubation, the membranes were rinsed and bound antibodies were detected using enhanced chemiluminescence according to the manufacturer's instructions.

Flow-cytometric analysis of mitochondria

Permeabilization of the outer mitochondrial membrane was investigated by monitoring staining of mitochondrial cytochrome oxidase using anti-OxPhos Complex IV subunit I antibody, conjugated with Alexa Fluor® 488 (A-21296, Molecular Probes, Eugene, OR, U.S.A.). Briefly, mitochondria (50 μg of protein) were preincubated with 1 μg of anti-OxPhos antibody in a buffer containing 150 mM KCl, 2 mM KH_2PO_4, 5 mM succinate, 5 mM Tris (pH 7.4) for 10 min. Before FACS analysis, valinomycin (0.1 nM) was added to mitochondria for 2 min. Mitochondrial staining was assessed with a 488 nm argon laser in the FL-1 channel. The results were analysed by WinMDI 2.8 software.

RESULTS AND DISCUSSION

Hypotonic shock-induced release of cytochrome *c*

It is well established that MPT-dependent release of cytochrome *c* from the intermembrane space of mitochondria involves matrix swelling and subsequent permeabilization of the outer mitochondrial membrane. Since mitochondrial volume is determined by a balance of osmotic pressure across the inner mitochondrial membrane, another mechanism of swelling induction can involve hypotonic shock triggered by a lowering of cytosolic K^+ concentration.

To determine whether mitochondria release cytochrome *c* at this instance, these organelles were subjected to a 1 min bout of hypotonic shock in KCl solutions ranging in concentration from 75 to 125 mM. Importantly, initial tonicity (150 mM KCl) was restored immediately after the 1 min shock by the addition of concentrated KCl solution. As illustrated in Figure 1(A), incubating mitochondria for 1 min in either 100 or 75 mM KCl induced significant cytochrome *c* release when compared with control conditions (150 mM KCl). In addition, when mitochondrial functional activity (Ca^{2+}-buffering capacity) was evaluated on the restoration of initial tonicity (Figure 1B), it was clear that these organelles were intact and capable of accumulating this bivalent cation. However, the amount of Ca^{2+} that was needed to induce MPT was lower after hypotonic shock (Figure 1C).

The fact that even a short-term (1 min) hypotonic shock (75 mM KCl) induced considerable cytochrome *c* release is striking, since it is more than twice the concentration of K^+ observed in the cytosol of cells undergoing apoptosis [6–9]. Furthermore, evidence indicates that assembly of an active apoptosome complex requires a pronounced lowering of cytosolic K^+ concentration [10]. Taken together, a decrease in cytosolic K^+ may play two roles during apoptosis: (i) to stimulate cytochrome *c* release and (ii) to permit the formation of an active apoptosome complex.

Valinomycin-induced mitochondrial swelling and the release of cytochrome *c*

When the concentration of solute particles on each side of the inner mitochondrial membrane is equal, the net movement of water is inhibited and these organelles maintain their shape. However, certain compounds or conditions can disturb the balance of solutes

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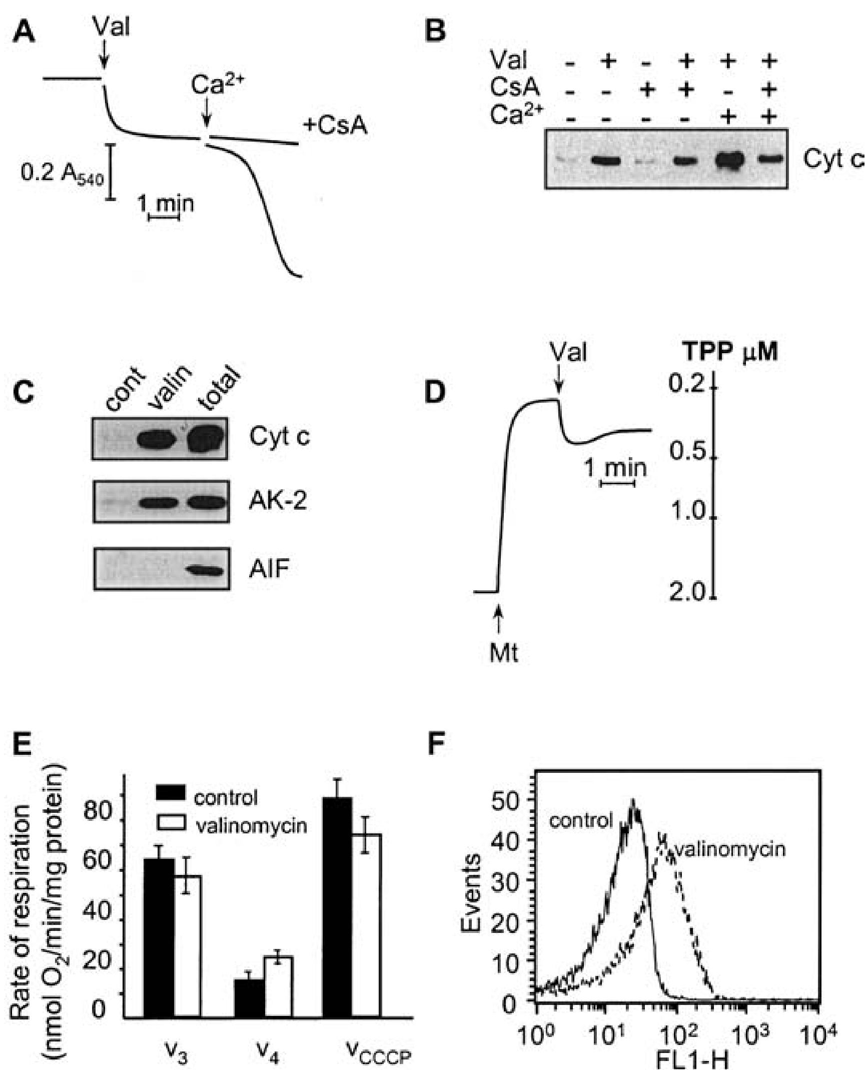

Figure 2 Effect of valinomycin on mitochondrial volume and functional parameters

(**A**) Mitochondria were incubated in KCl-based buffer at a concentration of 0.5 mg/ml. After a 1 min stabilization period mitochondrial swelling was initiated by 2 nM valinomycin (Val). After 4 min, samples were taken for cytochrome *c* release determination and MPT was induced by loading of mitochondria with Ca^{2+} (100 nmol/mg of protein). After that, samples for cytochrome *c* were taken again. CsA, 1 μM (where indicated). (**B**) The release of cytochrome *c* (Cyt *c*) from mitochondria under non-MPT and MPT conditions. Incubation conditions were the same as in (**A**). (**C**) Valinomycin (valin)-induced release of different mitochondrial proteins [cytochrome *c* (Cyt *c*), adenylate kinase-2 (AK-2) and apoptosis-inducing factor (AIF)]. Cont, control. (**D**) Estimation of mitochondrial membrane potential in the presence of valinomycin. Mitochondria (1 mg/ml) were incubated in the presence of 2 μM TPP^+. After the stabilization period, 2 nM valinomycin was added. (**E**) Effect of valinomcin on mitochondrial respiration. Mitochondria were incubated as described in the Experimental section. Phosphorylation was induced by the addition of 400 nmol of ADP and uncoupled respiration was measured in the presence of 1 μM carbonyl cyanide *m*-chlorophenylhydrazone. (**F**) FACS analysis of valinomycin-induced permeabilization of the outer membrane of mitochondria.

and specifically allow different particles to enter the mitochondrial matrix.

As seen in Figure 2(A), the addition of the K^+-selective ionophore valinomycin to mitochondria induced swelling as determined by a decrease in the overall absorbance of the suspension. Moreover, swelling was accompanied by an impressive release of cytochrome *c* (Figure 2B). Next, to evaluate the extent of valinomycin-induced swelling and cytochrome *c* release, MPT was induced by the addition of Ca^{2+} (Figure 2A). Since the MPT inhibitor CsA (cyclosporin A) completely prevented the second phase of mitochondrial swelling induced by Ca^{2+} (Figure 2A) but had no effect on valinomycin-induced swelling and cytochrome *c* release (Figures 2A and 2B), these results demonstrate that valinomycin-induced release of cytochrome *c* occurs without MPT and these organelles maintain their membrane integrity. Moreover, because of the submaximal swelling that was observed in the presence of valinomycin alone (Figure 2A), the amount of cytochrome *c* released from mitochondria was lower than that during MPT induction (Figure 2B). In addition to cytochrome *c*, mitochondrial swelling triggered the release of adenylate kinase-2 (AK-2), which is present in the intermembrane space, but not apoptosis-inducing factor (AIF) (Figure 2C). Importantly, the concentration of valinomycin (2 nM) was not sufficient to induce a complete depolarization of mitochondria (Figure 2D). Mitochondria also maintained controlled respiration; however, due to the partial loss of cytochrome *c*, the rate of state 3 and uncoupled respiration were suppressed (Figure 2E). Finally, valinomycin stimulated state 4 respiration (Figure 2E) that resulted in a decrease of the respiratory control ratio (results not shown).

To test whether permeabilization of the outer mitochondrial membrane occurs in all mitochondria or only in a subpopulation of these organelles, mitochondria were incubated with an antibody against cytochrome oxidase, which is bound to the inner

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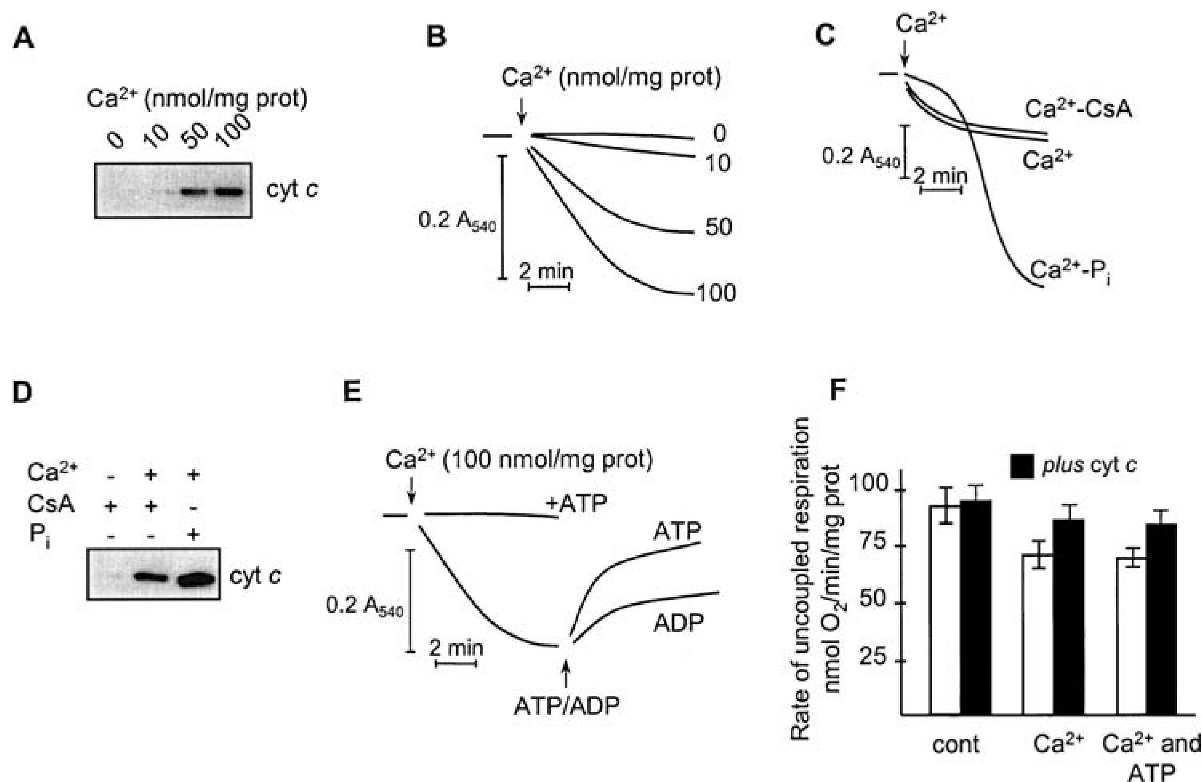

Figure 3 Stimulation of cytochrome *c* release by Ca^{2+} under non-MPT conditions

Mitochondria were incubated in a buffer containing 110 mM KCl, 10 mM $KHCO_3$, 5 mM succinate, 5 mM Tris (pH 7.4). (**A**) Mitochondria were loaded with different amounts of Ca^{2+} and after 10 min aliquots were withdrawn for cytochrome *c* determination. (**B**) Estimation of mitochondrial swelling induced by Ca^{2+}. (**C**) Ca^{2+}-induced MPT-dependent and -independent swelling and corresponding release of cytochrome *c* (**D**). (**E**) Reversal of Ca^{2+}-induced mitochondrial swelling by ATP and ADP. (**F**) Simulation of uncoupled mitochondrial respiration by exogenous cytochrome *c* (100 μg/ml; black bars) after swelling (Ca^{2+}) or contraction (Ca^{2+} and ATP) had occurred. Cont, control (white bars).

mitochondrial membrane, and analysed by flow cytometry. The results indicated that permeabilization of the outer mitochondrial membrane occurs equally in the presence of valinomycin, as evidenced by the increased staining of cytochrome oxidase within the entire population of mitochondria (Figure 2F).

Ca^{2+}-regulated MPT-independent release of cytochrome *c*

Normally, any K^+ that passively diffuses across the inner mitochondrial membrane is extruded by an electroneutral K^+/H^+ exchanger. In addition, mitochondria possess specific channels that normally allow K^+ penetration into the matrix. An example is the ATP-dependent K^+ channel [11–13]. Evidence indicates that the activity of these transport systems may be regulated by bivalent cations, including Ca^{2+}. Indeed, it has been shown that depletion of endogenous bivalent cations induces electroneutral K^+/H^+ exchange activity [14,15]. Additions of Ca^{2+} to mitochondria might suppress the activity of K^+/H^+ exchange and uncompensated K^+ entry will cause the net movement of water and stimulate mitochondrial swelling. It also cannot be excluded that Ca^{2+} directly stimulates the entry of K^+ via the Ca^{2+}-stimulated, voltage-dependent K^+ channel (K_{Ca} channel) in the inner mitochondrial membrane [16]. The mitochondrial-K_{Ca} channel significantly contributes to mitochondrial K^+ uptake in myocytes and an opener of the channel protects against myocardial infarction [17].

To determine whether Ca^{2+} can stimulate the release of cytochrome *c* without pore opening, experiments were performed under strict non-MPT conditions. That is, mitochondria were incubated in a phosphate-free buffer containing CsA. The results indicated that the addition of micromolar concentrations of Ca^{2+} to mitochondria incubated in 110 mM KCl plus potassium acetate or potassium hydrocarbonate (10 mM) stimulated cytochrome *c* release. The amount of cytochrome *c* that was released from mitochondria hinged on the extent of Ca^{2+} loading (Figure 3A). Furthermore, under these conditions, Ca^{2+}-induced cytochrome *c* release was linked with mitochondrial swelling (Figure 3B) and all of these effects were insensitive to CsA (Figure 3C). Swelling and cytochrome *c* release were inhibited by exogenous ATP and, to a lesser extent, ADP (Figure 3E), which is consistent with the ability of ATP to block a K^+-ATP channel [18]. Although the amount of cytochrome *c* released from mitochondria was significantly less than that induced by MPT (Figures 3C and 3D), even a moderate release resulted in a deterioration of the respiratory chain function, assessed by estimation of the rate of uncoupled respiration (Figure 3F). Finally, uncoupled respiration could be restored by adding exogenous cytochrome *c* (black bars) to the mitochondrial suspension after swelling or ATP/ADP-stimulated contraction occurs, which indicates that permeabilization of the outer membrane is irreversible.

Concluding remarks

Volume-dependent mechanisms of cytochrome *c* release from the intermembrane space are based on swelling of mitochondria, leading to permeabilization of the outer mitochondrial membrane. The swelling that is observed in these instances is most often MPT-dependent. Opening of the MPT pore requires at least two

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obligatory components, namely high intramitochondrial Ca^{2+} and significantly lowered ATP levels (reviewed in [4]); low ATP level is uncharacteristic of cells committed to undergo apoptosis. Induction of MPT leads, in turn, to a severe impairment of Ca^{2+} and ATP homoeostasis. Thus one might surmise that during apoptosis the probability of persistent pore opening must be relatively low, and MPT-independent volume changes could play an important role in the regulation of cytochrome *c* release. In contrast to MPT, relatively moderate increases in mitochondrial volume stimulated by osmotic changes would not affect organelle functional activity or the intracellular ATP level.

Regulation of the mitochondrial volume by modulation of the uptake and release systems represents a different route of outer-membrane permeabilization and may be of importance during apoptosis. Recently, stimulation of potassium uptake by mitochondria via a K-uniporter (K_{ATP} channel) was shown in HL-60 cells undergoing etoposide-induced apoptosis [19]. As a result, mitochondrial swelling was initiated, which was responsible for the release of cytochrome *c*. Although MPT was also observed [20], the authors propose that it was not the primary cause of mitochondrial swelling. Interestingly, K^+ uptake and swelling of a mitochondria were blocked by Bcl-2 overexpression whereas tBid stimulated potassium uptake and subsequent cytochrome *c* release. It should be mentioned, however, that the existence of a mitochondrial K_{ATP} channel has been questioned [21]. The authors, however, do not rule out the presence of other mitochondrial K^+ channels. Studies of mitochondrial volume regulation led Halestrap and coworkers to suggest that Ca^{2+}-dependent increase in matrix volume is caused by K^+ entry, and that the responsible protein is adenine nucleotide translocase, which, under normal conditions, translocates ATP and ADP across the inner mitochondrial membrane. According to Halestrap and co-workers [5,22,23], low (micromolar) concentrations of Ca^{2+} stimulate formation of pyrophosphate, which displaces adenine nucleotides from adenine nucleotide translocase and converts the latter into a potassium channel.

In conclusion, our results show that, in an *in vitro* model system, the release of cytochrome *c* may occur via modulation of mitochondrial volume regardless of the mechanisms leading to the mitochondrial swelling. In contrast with MPT-dependent release of cytochrome *c*, under these circumstances the mitochondria remain intact and functionally active.

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