Disruption of the outer mitochondrial membrane as a result of large amplitude swelling: the impact of irreversible permeability transition

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Abstract Upon induction of permeability transition with different agents (Ca^{2+}, *tert*-butyl hydroperoxide, atractyloside), mouse hepatocyte mitochondria manifest a disruption of outer membrane integrity leading to the release of cytochrome *c* and apoptosis-inducing factor (AIF), two proteins which are involved in programmed cell death (apoptosis). Chelation of Ca^{2+} shortly (within 2 min) after its addition to isolated mitochondria reestablished the mitochondrial transmembrane potential ($\Delta\Psi_m$), prevented induction of large amplitude swelling and release of both cytochrome *c* and AIF. In contrast, late Ca^{2+} chelation (10 min after addition of Ca^{2+}) failed to affect these parameters. Cytochrome *c* appears to be released through a mechanically damaged outer mitochondrial membrane rather than via a specific release mechanism. These findings clarify the mechanisms through which irreversible permeability transition occurs with subsequent large amplitude swelling culminating in the release of intermembrane proteins from mitochondria. Moreover, they confirm the hypothesis formulated by Skulachev [FEBS Lett. 397 (1996) 7–10 and Q. Rev. Biophys. 29 (1996) 169–202] linking permeability transition to activation of the apoptogenic catabolic enzymes.

Key words: Cytochrome *c*; Mitochondrial transmembrane potential; Permeability transition; Swelling; Outer membrane disruption; Hypoploidy

1. Introduction

The mitochondrial permeability transition (MPT) is due to opening of the MPT pore (or mitochondrial 'megachannel') [1,2]. Although little is known about the physiology of the MPT pore, it appears that it can act at least at two different levels of conductance and reversibility. At low level of conductance (MPT is permeable to H^+ and Ca^{2+} but not to saccharose), the MPT pore opening is reversible and does not entail large amplitude swelling of the mitochondrial matrix, although it does cause a collapse of the $\Delta\Psi_m$. This function of the MPT may be involved in Ca^{2+} signaling and/or in the regulation of Ca^{2+} release of the mitochondrial matrix [3]. At high level of conductance (MPT is permeable to molecules up to 1.5 kDa), the MPT pore opening is irreversible and culminates in large amplitude swelling of the mitochondrial matrix. It has been shown that irreversible, high conductance MPT is involved in the mediation of cell death. Massive MPT is a rate-limiting factor of necrotic cell death in a variety of different systems ([4,5]). Moreover, MPT occurs during apoptotic cell death [6,7], which is a highly 'regulated' or 'programmed' process responsible for the removal of superfluous, aged or damaged cells [8]. Triggering of MPT can cause apoptosis [9,10], and its inhibition can prevent apoptosis [9,11]. It appears that the apoptosis-inhibitory proto-oncogene product Bcl-2, which localizes to mitochondrial membranes, acts as an endogenous inhibitor of the MTP pore [11,12].

During apoptosis, two different proteins are released from the mitochondrial intermembrane space: the 14-kDa cytochrome *c* [13–18] and the 50-kDa protein apoptosis inducing factor (AIF) [12]. Cytochrome *c* can interact with the protein Apaf-1 to activate caspase-9 [19], which in turn activates other caspases and a nuclease-activating factor (reviewed in [20]). In contrast, AIF can act as a direct activator of nuclear DNases, thereby causing the characteristic oligonucleosomal DNA fragmentation pattern observed during apoptosis [12,21].

Although it is known that induction of MPT causes the release of cytochrome *c* and AIF from isolated mitochondria [12,22], little information is available on the mechanisms linking both phenomena. We therefore studied the relationship between different MPT-triggered alterations using a multiparametric approach (quantitation of $\Delta\Psi_m$, swelling, oxygen consumption, cytochrome *c* and AIF release). Our data suggest that irreversible MPT leading to mitochondrial large amplitude swelling causes the release of cytochrome *c* and AIF due to mechanical disruption of the outer mitochondrial membrane.

2. Materials and methods

2.1. Preparation of mitochondria

Three- to four-week-old mice (BALB/c) or Lewis rats were killed by decapitation. Mitochondria were isolated from liver in a medium containing 0.3 M saccharose, 5 mM *N*-Tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid (TES) and 0.2 mM ethylene glycol-bis(β-aminoethyl ether) *N,N′,N″*-tetraacetic acid (EGTA) (pH 7.2) [23]. To separate intact from broken mitochondria, the organelles were layered on a Percoll gradient (10 min at $8500\times g$) consisting of three layers of 18%, 30% and 60% (w/v) Percoll in 0.3 M saccharose, 10 mM TES and 0.2 mM EGTA (pH 6.9). Mitochondria were collected from the 30%/60% interface and washed with 0.3 M saccharose, 10 mM TES and 0.2 mM EGTA (pH 7.2). Mitochondria (0.5–1 mg/ml) were left untreated or were incubated with different combinations of atractyloside (10 μM), *tert*-butyl hydroperoxide (*tert*-BHP) (50 μM), $CaCl_2$

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Abbreviations: AIF, apoptosis inducing factor; Cyt *c*, cytochrome *c*; $DiOC_6(3)$, 3,3′-dihexyloxacarbocyanine iodide; mClCCP, carbonyl cyanide *m*-chlorophenylhydrazone; PT, permeability transition; $\Delta\Psi_m$, mitochondrial membrane potential; EGTA, ethylene glycol-bis(β-aminoethyl ether) *N,N′,N″*-tetraacetic acid; FS_{peak}, forward angle light scatter peak; PCD, programmed cell death; *tert*-BHP, *tert*-butyl hydroperoxide; TPP^+, tetraphenylphosphonium

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(75–375 nmol/mg protein), and/or 500 μM EGTA (added 2 or 10 min after $CaCl_2$) for the indicated period. When the outer membrane was altered, the mitochondria were recovered at the 18%/30% interface.

2.2. Oxygen uptake and membrane potential

Membrane potential and oxygen uptake were simultaneously monitored in an oxygen-electrode chamber (Hansatech, UK) using tetraphenylphosphonium cation (5 μM) as a membrane potential probe. The transmembrane potential ($\Delta\Psi_m$) was measured at 25°C in a final volume of 1.5 ml respiratory medium consisting of 0.3 M sucrose, 10 mM KCl, 5 mM $MgCl_2$, 10 mM TES, 10 mM KH_2PO_4 (pH 7.2) according to Kamo et al. [24]. Tetraphenylphosphonium (TPP^+) binding was corrected according to Rottenberg [25].

2.3. Flow cytometry analysis of the mitochondrial characteristics

Flow cytometry was performed on an Epics Elite ESP flow cytometer (Coultronics, Miami, FL, USA) equipped with a 488-nm argon laser. The optical filters were 515 nm long-pass interference, 515 nm long-pass absorbance and a 525 nm bandpass for green fluorescence [23,26,27]. For mitochondrial membrane potential measurements, mitochondria were stained for 15 min at 37°C with 20 nM $DiOC_6(3)$. 5×10^5 mitochondria devoid of debris or aggregates were counted for each histogram. The light scattering properties of the mitochondria were analyzed through the forward low angle light scatter (at 488 nm) taken as a peak measurement (FS_{peak}).

2.4. Large amplitude swelling

Mitochondrial swelling was estimated from the decrease in absorbance measured at 520 nm in a Uvikon 930 spectrophotometer (Kontron Instruments). The medium was sucrose 250 mM, 20 mM Tris-MOPS (pH 7.2), 2 μM rotenone, 10 μM EGTA, 300 μM P_i-Tris. The addition of P_i was to ensure an optimal activity of the succinate transport and the subsequent succinate dehydrogenase activity. The presence of EGTA at low concentration (10 μM) ensures better experimental reproducibility.

2.5. Determination of cytochrome c concentrations

Quantitative measurements of cytochrome *c* were performed according to Chance and Williams [28]. Spectra of different solutions were recorded at room temperature using an Aminco DW2A spectrophotometer (USA) operated in the beam-split mode (quartz cell 200 μl). The absolute spectrum of oxidized sample was automatically subtracted from the reduced spectrum in the presence of dithionite.

2.6. Western blots

Proteins in the supernatant (15 μl) of different mitochondrial preparations were subjected to SDS-PAGE (10–15%). Immunoblots for the determination of cytochrome *c* release were performed using a specific monoclonal antibody (Pharmingen).

2.7. Characterization of the factors contained in the mitochondrial supernatant

After incubation of mitochondria for 30 min at 25°C in the swelling medium in the presence of succinate-Na 5 mM and the indicated inducers and inhibitors of MPT, the mitochondria were subjected to a two step centrifugation ($8500\times g$, 10 min; then $1.5\times10^5\times g$, 60 min, at 4°C for the supernatant). Supernatants were mixed with purified HeLa cell nuclei (10^3/ml) in the presence of anti-oxidants and anti-proteases [12], and incubated for 90 min at 37°C. Nuclei were stained with propidium iodide (PI) for the cytofluorometric determination of subdiploid nuclei, as described [12,29].

3. Results and discussion

3.1. Induction of MPT permeabilizes the outer mitochondrial membrane to cytochrome c

Although most studies of the MPT have been performed on rat liver mitochondria [1,30], we chose mouse mitochondria based on the fact that most studies of apoptosis have been performed with material from humans or mice rather than from rats. As shown in Fig. 1 and Table 1, both mouse and rat hepatocyte mitochondria undergo a transient, then permanent decrease in the $\Delta\Psi_m$ after addition of Ca^{2+}. Mouse mitochondria require higher amounts of Ca^{2+} (approx. 375 nmol/mg protein) than rat mitochondria (approx. 100 nmol/mg) to reach a permanent state of $\Delta\Psi_m$ dissipation indicative of high conductance MTP opening. This latter Ca^{2+} effect is mediated by the MTP pore, because it is inhibited in the presence of 1 μM cyclosporin A (not shown). We consider that most of the literature available on rat hepatocyte mitochondria also applies to mouse material, although some species differences may exist. In the presence of antimycin A, a specific inhibitor of the respiratory chain, mitochondria from mouse hepatocytes fail to respire, and this effect is not reversed by exogenous reduced cytochrome *c* (Fig. 1C), suggesting that cytochrome *c* cannot access the inner membrane of the mitochondrion. In contrast, after induction of MPT, cytochrome *c* can trigger oxygen consumption in antimycin A-

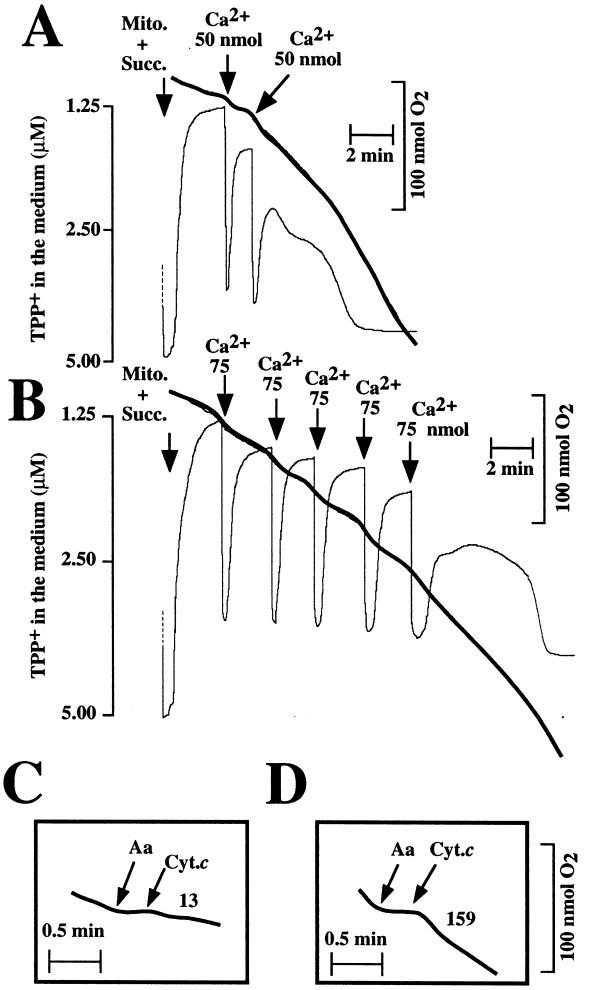

Fig. 1. Calcium-induced MPT in rat and mice liver mitochondria. Successive additions of Ca^{2+} were used for PT induction in rat (Ca^{2+}, 50 nmol/mg mitochondrial protein) (A) or in mouse liver mitochondria (Ca^{2+}, 75 nmol/mitochondrial protein) (B,C,D). Simultaneous recording of the oxygen consumption (thick line) and of the mitochondrial membrane potential (thin line) was achieved with a Clark electrode and a TPP^+ electrode, respectively. The oxygen consumption was measured after addition of antimycin A (Aa) and exogenous reduced cytochrome *c* (Cyt. c) before (C) or after induction of MPT (D, after cumulative addition of 375 nmol Ca^{2+} nmol/mg mitochondrial protein, as in B). Numbers refer to the oxygen consumption (nmol O_2/min/mg protein) determined under these conditions.

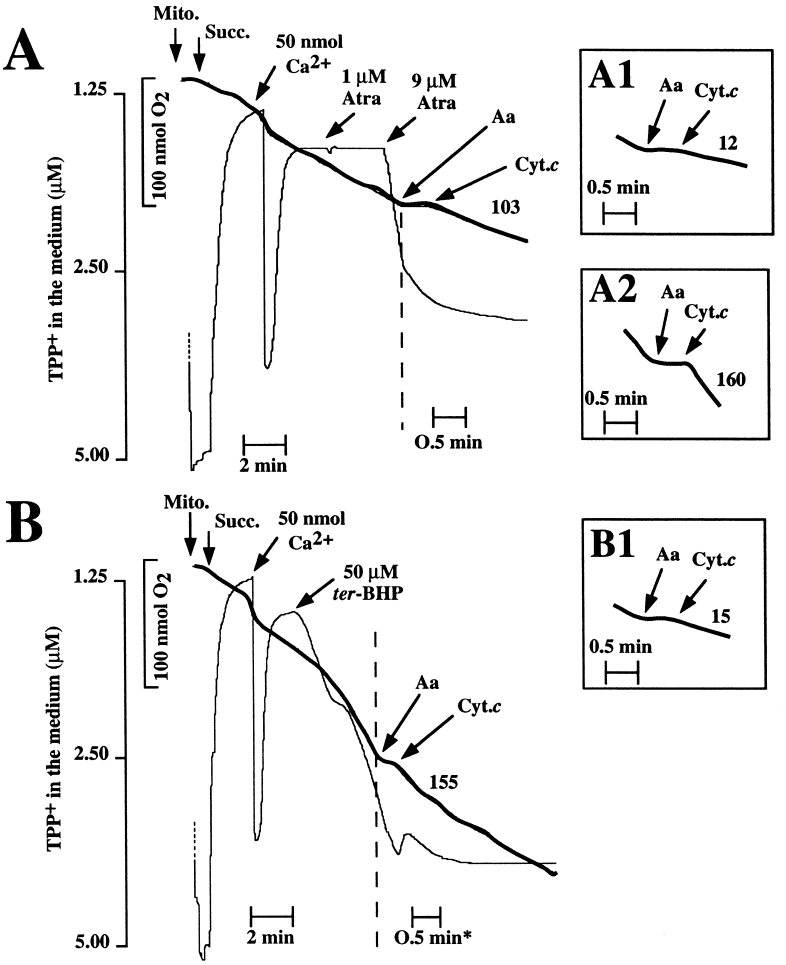

Fig. 2. Changes in mitochondrial respiration, membrane potential, and cytochrome *c* oxidase activity after incubation with MPT inducers. Mouse liver mitochondria were exposed to succinate (Succ., 5 mM), Ca^{2+} (50 nmol/mg protein), atractyloside (Atra, 10 μM, A) or *tert*-butyl hydroperoxide (*ter*-BHP, 50 μM, B), and the transmembrane potential (thin line) as well as the oxygen concentration (thick line) were monitored as in Fig. 1. Numbers refer to the oxygen consumption (nmol O_2/min/mg protein) determined after addition of antimycin A (Aa) and cytochrome *c* (Cyt. *c*) either before (A1, B1) or after induction of MPT (A, A2 and B). In A2, the Cyt. *c* addition was at 10 min. Note that neither Ca^{2+} nor Atra (or *ter*-BHP) induces MPT when used alone at these concentrations (not shown).

treated mitochondria, indicating that cytochrome *c* readily enters the intermembrane space (Fig. 1D). These results suggest that mitochondria which have undergone Ca^{2+}-induced MPT possess an outer membrane which has lost its barrier function for cytochrome *c*. Similar results have been obtained when MPT was induced by a combination of low dose Ca^{2+} (50 nmol/mg protein) and atractyloside (Atr) (Fig. 2A), a ligand of the mitochondrial adenine nucleotide translocator, or *tert*-BHP, a prooxidant (Fig. 2B). In each case, exogenous reduced cytochrome *c* triggers increased oxygen consumption in antimycin A-treated mitochondria only after induction of MPT (Fig. 2A,A_2,B). After MPT induction, the oxygen consumption increases with time as the mitochondria swell and the outer membrane permeability increases and finally the membrane breaks (Fig. 2A,A_2).

3.2. Correlation between MPT-induced mitochondrial swelling and cytochrome c release

As pointed out above, the outer mitochondrial membrane only becomes permeable for cytochrome *c* after permanent disruption of the $\Delta\Psi_m$. Accordingly, stimulation of mitochondria with a dose of Ca^{2+} able to induce MPT (375 nmol/mg protein) followed by chelation of Ca^{2+} with an excess of EGTA can give rise to two different responses. If EGTA is

Table 1
Main characteristics of Percoll purified rat and mouse mitochondria

Mitochondria (Percoll purified)	Integrity (%)	Respiratory rate (nmol O_2/min/mg protein)	Respiratory control	ADP/O	Membrane potential (mV)	Pore opening with calcium in nmol Ca^{2+}
Rat liver	96 ± 3	140 ± 6	5.8 (7.1)[a]	1.5 ± 0.1	181 ± 15[b]	80–100
Mouse liver	95 ± 4	145 ± 8	5.6 (7.0)[a]	1.5 ± 0.1	182 ± 18[b]	350–400

[a]The respiratory control (CR) is the state 3/state 4 respiration. In parentheses the respiratory control is taken as the ratio of the respiration in state 4 versus the respiration uncoupled in the presence of mClCCP.
[b]The values are corrected for the non-specific binding of the probe.

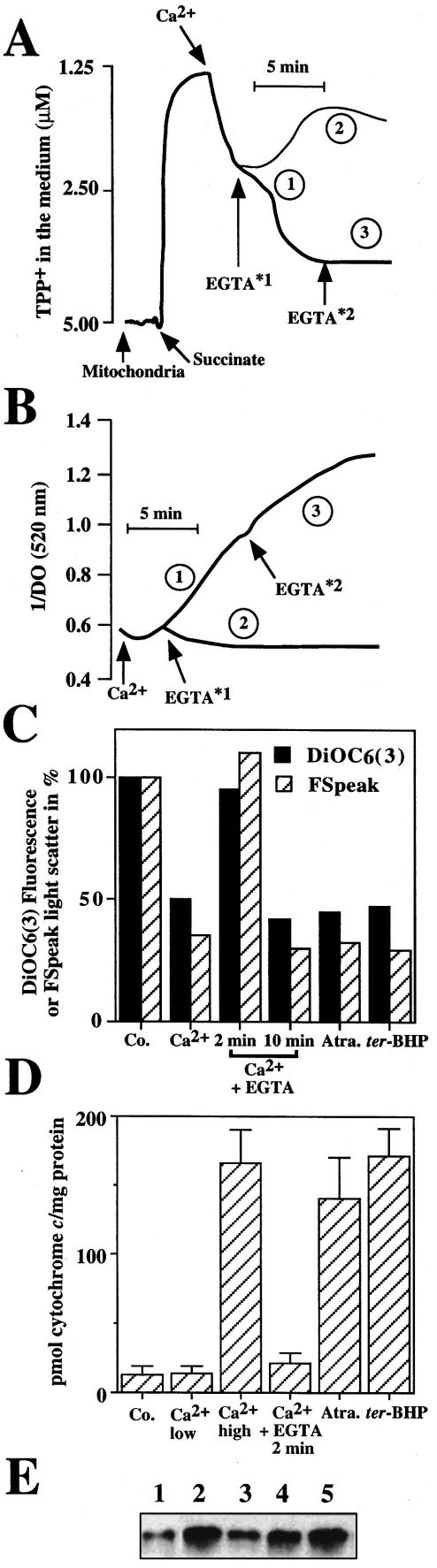

Fig. 3. Cytochrome *c* release as a consequence of irreversible MPT. $\Delta\Psi_m$ (measured with the TPP^+ electrode, A) and mitochondrial volume (measured as large amplitude swelling at 520 nm, B) were measured after addition of 375 nmol Ca^{2+}/mg protein, either alone (1) or after addition of an excess of the Ca^{2+} chelator EGTA (500 μM), 2 min (2) or 10 min (3) after Ca^{2+}. Alternatively, these parameters were assessed by cytofluorometry (C) using the incorporation of $Di0C_6(3)$ as a measure of $\Delta\Psi_m$ and the forward scatter (FS) as an estimate of large amplitude swelling, as described in Section 2. *tert*-BHP was used at a final concentration of 100 μM and atractyloside at 10 μM, in combination with 50 nmol Ca^{2+}/mg protein, as in Fig. 2. The protonophore mC1CCP (2.5 μM) selectively decreases the $\Delta\Psi_m$ without affecting the light scatter, as an internal control. The amount of cytochrome *c* released into the supernatant of mitochondria was quantified by subtracted absorbance spectra obtained in conditions of cytochrome *c* oxidation and reduction (D). Alternatively, supernatants were subjected to immunoblot for detection of cytochrome *c* (E). Lane 1: control; 2: Ca^{2+}; 3: Ca^{2+}+EGTA after 2 min; 4: Ca^{2+}+*tert*-BHP; 5: Ca^{2+}+Atr (same doses as above). Results are representative of seven independent experiments.
←

added within 2 min, the $\Delta\Psi_m$ dissipation is transient (Fig. 3A), and no large amplitude swelling (monitored as a decrease in the absorbance at 520 nm in Fig. 3B or as a reduction in the forward scatter measured by cytofluorometry in Fig. 3C) occurs. In contrast, when EGTA is added after 10 min, mitochondrial swelling has begun and will continues until completion of the process (Fig. 3A–C). It appears that cytochrome *c* release, as measured by subtractive (oxidized minus reduced) absorption spectra (Fig. 3D), strictly correlates with the induction of irreversible MPT involving large amplitude swelling. The estimated cytochrome *c* content of mice liver mitochondria of 200 ± 32 pmol/min/mg protein is close to the content determined by Schwerzmann et al. [31] for rat liver mitochondria or described by Bourgeron et al. [32] for human B lymphoblastoid cell line mitochondria. Assuming that approximately 10–13% of the cytochrome *c* is strongly bound to the inner mitochondrial membrane [33], the amount of cytochrome *c* release in presence of *tert*-BHP or Ca^{2+} appears to be close to the maximum amount that mitochondria can release. Immunoblots (Fig. 3E) of cytochrome *c* confirm the

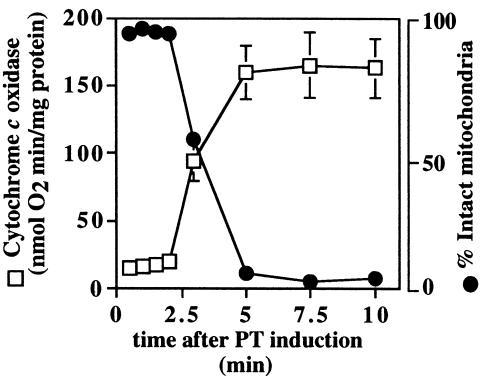

Fig. 4. Correlation between cytochrome *c*-stimulatable cytochrome *c* oxidase activity and changes in mitochondrial buoyant density after Ca^{2+}-induced MPT. Mitochondria were treated with 375 nmol Ca^{2+}/mg protein. After the indicated interval, mitochondria were loaded on a Percoll step gradient (see Section 2) and the amount of mitochondrial protein recovered from the 30%/60% Percoll interface (normal buoyant density) was estimated. In parallel, aliquots were treated with antimycin A+cytochrome *c*, followed by determination of oxygen consumption as in Figs. 1 and 2.

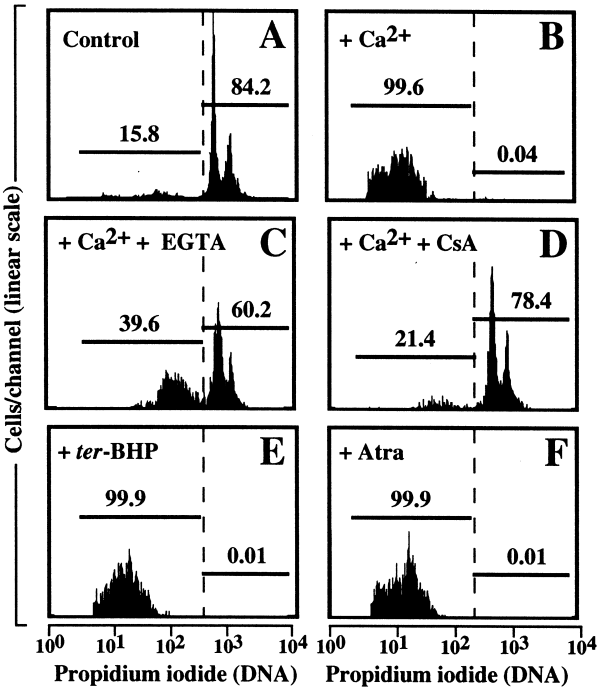

Fig. 5. Release of AIF activity from mitochondria after induction of MPT. Mouse liver mitochondria (0.5–1 mg/ml) were left untreated (A) or incubated for 30 min at 25°C with $CaCl_2$ (375 nmol/mg protein) (B), cyclosporin A (2 μM) plus $CaCl_2$ (375 nmol/mg protein) (C), $CaCl_2$ (375 nmol/mg protein) with EGTA (500 μM, 2 min after Ca^{2+}) (D), $CaCl_2$ (50 nmol/mg plus 50 μM *tert*-BHP (E), or $CaCl_2$ (50 nmol/mg protein) plus atractyloside (5 μM) (F) as in Figs. 1 and 2. Supernatants of these mitochondria were then incubated with purified nuclei from HeLa cells for 60 min at 37°C. Nuclei were then stained with PI and analyzed by flow cytometry for DNA content.

above described data. Note that the slight positivity of the control and of the Ca^{2+}-EGTA samples is certainly due to the small amount of damaged mitochondria present in all the mitochondrial preparations (integrity approximately 95 ± 4%, Table 1).

Thus cytochrome *c* is not released when Ca^{2+}-treated mitochondria are 'rescued' by early EGTA addition (2 min), but does leave the mitochondria when EGTA is added late (10 min). This correlates with the time frame of mitochondrial swelling (measured by monitoring the absorbance at 520 nm, Fig. 3B) and with the gradual loss of intact mitochondria (measured by application of mitochondria to a Percoll gradient, Fig. 4). Again, it appears that only mitochondria which have undergone irreversible MPT after Ca^{2+} (starting after approximately 2.5 min) are accessible to exogenous cytochrome *c* (Fig. 4).

3.3. Correlation between MPT-induced mitochondrial swelling and AIF release

AIF is a labile 50 kDa protein that is released from mitochondria of apoptotic cells. Since immunodepletion of cytochrome *c* does not affect the AIF activity (not shown), it is considered to act independently of cytochrome *c*. If added to purified nuclei, AIF (which has no DNase activity) [12] activates pre-formed nucleases and thereby causes a gradual loss of nuclear DNA content which can be measured in a cytofluorometer after staining with the DNA-intercalating dye PI. As shown in Fig. 5, AIF activity is released from mitochondria treated with the MPT inducer Ca^{2+}. Again, treatment with EGTA within 2 min after Ca^{2+} or pretreatment of mitochondria with the MTP inhibitor cyclosporin A prevent AIF release, thus expanding and confirming the notion that AIF is released as a consequence of large amplitude swelling [11]. In addition to Ca^{2+}, Atr and *tert*-BHP trigger MPT opening (Fig. 3C) and the release of both cytochrome *c* (Fig. 3D,E) and AIF (Fig. 5).

3.4. Concluding remarks

The data reported in this paper suggest that large amplitude swelling occurring as a consequence of MPT leads to the release of the intermembrane proteins cytochrome *c* and AIF. These findings suggest that colloidosmotic swelling of the mitochondrial matrix resulting from high conductance MTP opening causes mechanical disruption of the outer mitochondrial membrane (which has a far smaller surface than the inner membrane), thereby causing the selective release of mitochondrial intermembrane (rather than matrix) proteins. In accordance with this notion, electron microscopic pictures of cells undergoing apoptosis reveal a transient swelling of the mitochondrial matrix accompanied by local disruption of the outer membrane during early apoptosis [34]. Since apoptotic cells extrude small ions (potassium, sodium, chloride and water) and shrink during the post-mitochondrial phase of the apoptotic process, the colloidosmotic pressure of the cytosol may be expected to increase dramatically [35,36]. This may be the reason why mitochondrial matrix swelling is only observed during early apoptosis, before cells shrink (unpublished observations).

If our data indicate that MTP can be one mechanism leading to cytochrome *c* release, they do not prove that this is the only mechanism of cytochrome *c* release. Indeed, a so far unique transport pathway is utilized for the *import* of cytochrome *c* from the cytosol into the mitochondrion. Apo-cytochrome *c* is synthesized in the cytosol as heme-free apoprotein lacking a typical mitochondrion-targeting N-terminal presequence. It binds specifically to a mitochondrial outer membrane component, which is unrelated to the TOM complex and allows its translocation [37]. As found for presequence-containing preproteins [38], membrane passage of apoprotein is reversible [37]. Unidirectional transport is only achieved by stable binding of the apoprotein to cytochrome *c* heme lyase, the enzyme responsible for covalent attachment of heme to the apoprotein [33]. The mature holo-cytochrome *c* then remains in the intermembrane space. It is unlikely that such a sophisticated import mechanism would be implicated in cytochrome *c* release induced by MPT opening. Nevertheless, at present it cannot be ruled out that proteins involved in cytochrome *c* import or other specific export mechanisms may be perturbed during apoptosis and contribute to the extrusion of cytochrome *c*. Several authors have reported that cytochrome *c* is released from mitochondria before the $\Delta\Psi_m$ dissipates [34,39,40]. Although some of these data [34,39] may be criticized [41] because rhodamine-123 was used to quantify the $\Delta\Psi_m$ [34,39], these findings may suggest that cytochrome *c* release can occur independently of MPT. Alternatively, it remains possible that prolonged exposure of mitochondria to conditions favoring the reversible low conductance function of the MPT pore [3] leads to the periodic influx of ions and water into the mitochondrial matrix, thereby resulting in dis-

ruption of the outer membrane before the net $\Delta\Psi_m$ is disturbed. These possibilities are currently under investigation in our laboratory.

Apart from these details, our data reinforce the link between mitochondrial regulation and cell death and thus confirm the hypothesis initially formulated by Skulachev [42,43] that mitochondria have a major impact on cell demise, in both physiology and pathology. Thus, although neglected for long, it appears now that mitochondria 'decide' between death and life, and that maintenance of the barrier function of mitochondrial membranes, rather than constituting a passive contribution to cellular homeostasis, is a regulated process which key implications for cell biology.

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