Apoptosis driven by IP$_3$-linked mitochondrial calcium signals

Gábor Szalai, Rajeshwari Krishnamurthy and György Hajnóczky[1]

Department of Pathology, Anatomy and Cell Biology, Room 253 JAH, Thomas Jefferson University, Philadelphia, PA 19107, USA

[1]Corresponding author
e-mail: Gyorgy.Hajnoczky@mail.tju.edu

Increases of mitochondrial matrix [Ca^{2+}] ([Ca^{2+}]$_m$) evoked by calcium mobilizing agonists play a fundamental role in the physiological control of cellular energy metabolism. Here, we report that apoptotic stimuli induce a switch in mitochondrial calcium signalling at the beginning of the apoptotic process by facilitating Ca^{2+}-induced opening of the mitochondrial permeability transition pore (PTP). Thus [Ca^{2+}]$_m$ signals evoked by addition of large Ca^{2+} pulses or, unexpectedly, by IP$_3$-mediated cytosolic [Ca^{2+}] spikes trigger mitochondrial permeability transition and, in turn, cytochrome c release. IP$_3$-induced opening of PTP is dependent on a privileged Ca^{2+} signal transmission from IP$_3$ receptors to mitochondria. After the decay of Ca^{2+} spikes, resealing of PTP occurs allowing mitochondrial metabolism to recover, whereas activation of caspases is triggered by cytochrome c released to the cytosol. This organization provides an efficient mechanism to establish caspase activation while mitochondrial metabolism is maintained to meet ATP requirements of apoptotic cell death.
Keywords: apoptosis/Ca^{2+}/IP$_3$/local signalling/mitochondria

Introduction

Increases of [Ca^{2+}] regulate a diverse range of cellular processes from fertilization to death (for review see Clapham, 1995; Thomas *et al.*, 1996; Berridge, 1997; Berridge *et al.*, 1998; Putney, 1998). The control of each process may also utilize multiple Ca^{2+}-regulated elements that sometimes appear to work in different directions. Regulation of cell death by Ca^{2+} is particularly complex. Large and sustained elevations of cytosolic [Ca^{2+}] ([Ca^{2+}]$_c$) can switch on a number of mechanisms that lead to the disintegration of cells, necrosis or to the ordered form of cell death, apoptosis (Ankarcrona *et al.*, 1995; Khan *et al.*, 1996; McConkey, 1996; Bian *et al.*, 1997; Lemasters *et al.*, 1998; Nicholls and Budd, 1998; Nicotera and Orrenius, 1998; Wang *et al.*, 1999). Alternatively, calcium ions have also been implicated in mechanisms that exert protection against cell death (Bian *et al.*, 1997; He *et al.*, 1997; Zhang *et al.*, 1998). It is not yet understood how Ca^{2+} turns from a signal for life to a signal for death and how selective activation of apoptotic

or necrotic death pathways is ensured by calcium signals. Evidence is emerging that spatial, temporal and amplitude patterns of calcium signals are important in encoding the specificity of cellular responses (for review see Clapham, 1995; Thomas *et al.*, 1996; Berridge, 1997; Berridge *et al.*, 1998; Putney, 1998). Recent studies have demonstrated that mitochondria may discriminate between different spatial and temporal patterns of cytosolic Ca^{2+} signals due to a local Ca^{2+} transfer between IP$_3$-regulated intracellular Ca^{2+} release sites and mitochondrial Ca^{2+} uptake sites (Rizzuto *et al.*, 1993, 1994, 1998; Pralong *et al.*, 1994; Hajnóczky *et al.*, 1995; Csordás *et al.*, 1999). At the same time, mitochondria were discovered to play an important role in apoptosis by releasing apoptotic factors (cytochrome c: Newmeyer *et al.*, 1994; Liu *et al.*, 1996; Kluck *et al.*, 1997; Yang *et al.*, 1997; and apoptosis-inducing factor: Susin *et al.*, 1996, 1999). Furthermore, a major target of mitochondrial [Ca^{2+}] regulation, the permeability transition pore (PTP), was shown to be involved in the process of releasing apoptotic factors (Petit *et al.*, 1996; Bradham *et al.*, 1998; Marzo *et al.*, 1998; Pastorino *et al.*, 1998). These results prompted us to put forward the hypothesis that the modulation of PTP by physiological Ca^{2+} signals may provide a means to utilize the spatial and temporal features of calcium signalling in the control of apoptotic cell death.

The central component of the apoptotic machinery is a proteolytic system that involves a family of proteases called caspases (Thornberry *et al.*, 1992; Yuan *et al.*, 1993). In cell-free systems induction of caspase activation (Liu *et al.*, 1996) and caspase-mediated apoptosis (Newmeyer *et al.*, 1994) were found to be dependent on the presence of cytochrome c released from mitochondria. Release of cytochrome c from mitochondria occurs during apoptosis and this process is inhibited by the presence of Bcl-2 on these organelles (Kluck *et al.*, 1997; Yang *et al.*, 1997). Cytosolic cytochrome c is an essential component of the apoptosome, which is composed of cytochrome c, Apaf-1 and procaspase-9 (Li *et al.*, 1997). This yields activation of caspase-9, which then processes and activates other caspases to orchestrate the biochemical execution of cells. Activation of caspases and caspase-dependent dismantling of the cell require ATP and so maintained ATP production by mitochondrial metabolism may be important to support execution of the steps of the apoptotic program distal to cytochrome c release.

Opening of PTP has been implicated in the release of cytochrome c and apoptosis-inducing factor from mitochondria (for recent review see Skulachev, 1996; Reed, 1997; Green and Kroemer, 1998; Green and Reed, 1998; Duchen, 1999) and in the effect exerted by proapoptotic (e.g. BAX) and anti-apoptotic members of the Bcl-2 family on the release (Hockenbery *et al.*, 1990; Oltvai *et al.*, 1993; Marzo *et al.*, 1998; Pastorino *et al.*, 1998).

Opening of PTP is controlled by Ca^{2+}, pH, adenine nucleotides, free radicals and mitochondrial membrane potential ($\Delta\Psi_m$) (for review see Bernardi and Petronilli, 1996), but it has not been elucidated how these factors are involved in PTP-dependent cytochrome *c* release. Recent data on mitochondrial transport of Ca^{2+} and solutes are in support of the idea that in health, increases in mitochondrial matrix $[Ca^{2+}]$ ($[Ca^{2+}]_m$) induced by Ca^{2+} mobilizing agonists do not evoke PTP opening associated with cytochrome *c* release (Rizzuto *et al.*, 1994; Ichas *et al.*, 1997; Ichas and Mazat, 1998). We postulated that in cells exposed to proapoptotic stimuli (e.g. ceramide, staurosporine) the Ca^{2+} sensitivity of PTP increases and, in turn, IP_3-mediated $[Ca^{2+}]_m$ spikes result in PTP opening and cytochrome *c* release. Since Ca^{2+} spikes are established by synchronized activation and deactivation of Ca^{2+} release channels (Hajnóczky and Thomas, 1997), the rise of $[Ca^{2+}]$ rapidly decays and so resealing of PTP may occur. Provided that residual cytochrome *c* is sufficient to support the electron transport, recovery of mitochondrial metabolism and ATP production may follow closing of the PTP.

Here we provide evidence that Ca^{2+} pulsing or IP_3-induced Ca^{2+} spikes yield PTP opening, cytochrome *c* release, caspase activation and nuclear apoptosis in cells exposed to proapoptotic stimuli, but not in naive cells. Our data demonstrate that IP_3 receptors utilize very small amounts of Ca^{2+} to evoke PTP opening and establish a transient opening of PTP followed by recovery of mitochondrial energy metabolism. Restoration of mitochondrial ATP production may account for the absence of necrotic cell death. Thus, IP_3-mediated $[Ca^{2+}]$ spikes can serve as an efficient and selective activator of the mitochondrial phase of the apoptotic machinery.

Results and discussion

Ca^{2+}-induced activation of PTP in permeabilized cells exposed to apoptotic agents

In order to study activation of PTP by $[Ca^{2+}]_m$ signals, we first established simultaneous fluorometric measurements of $\Delta\Psi_m$ and $[Ca^{2+}]_c$ in suspensions of permeabilized HepG2 cells. Figure 1A shows that addition of Ca^{2+} pulses (40 μM $CaCl_2$ each) to the permeabilized cells yielded increases of $[Ca^{2+}]_c$ which were transient (left panel, black trace). Endoplasmic reticulum (ER) and mitochondrial Ca^{2+} stores co-operate in intracellular Ca^{2+} accumulation, but in these experiments the ER Ca^{2+}ATPase inhibitor, thapsigargin (Tg) (2 μM), was added to permeabilized cells to abolish ER Ca^{2+} uptake. Under this condition the mitochondrial Ca^{2+} store is expected to play the major role in Ca^{2+} uptake. This was indeed shown to be the case as the falling phase of the $[Ca^{2+}]_c$ increases was reversed after addition of a Ca^{2+} ionophore, ionomycin or uncoupler (Figure 1A, left panel) and it was prevented by inhibitors of mitochondrial Ca^{2+} uptake sites, ruthenium red (RuRed; Figure 1A, right panel) or Ru360 (2 μM, $n = 3$). Figure 1A shows that relatively small and reversible decreases of $\Delta\Psi_m$ were associated with the falling phase of cytosolic $[Ca^{2+}]$ signals evoked by Ca^{2+} addition whereas complete dissipation of $\Delta\Psi_m$ was brought about by uncoupler (Figure 1A, left, black trace). In the presence of RuRed Ca^{2+} pulses

failed to evoke depolarizations, showing that Ca^{2+}-induced depolarizations are distal to mitochondrial Ca^{2+} uptake. Depolarizations can be explained by the fact that mitochondrial Ca^{2+} uptake occurs at the expense of $\Delta\Psi_m$. Activation of the PTP by accumulated Ca^{2+} may also yield loss of $\Delta\Psi_m$ but Ca^{2+}-induced depolarization in naive cells was not sensitive to inhibitors of PTP (Figure 1C, see below).

In order to study whether apoptotic stimuli affect $[Ca^{2+}]_m$ and $\Delta\Psi_m$ responses given to Ca^{2+} addition, cells were exposed to ceramide (C2) or staurosporine (Obeid *et al.*, 1993; Bertrand *et al.*, 1994). These agents have been described to involve mitochondria in the apoptotic program and to exert their effect in subcellular models as well (e.g. Bossy-Wetzel *et al.*, 1998; Garcia-Ruiz *et al.*, 1997; Gudz *et al.*, 1997; Yang *et al.*, 1997). Preincubation of the permeabilized cells with C2 for 180 s did not change the steady state $[Ca^{2+}]_c$ or $\Delta\Psi_m$ ($[Ca^{2+}]_c$: 0.75 ± 0.02 and 0.76 ± 0.01 ratio units; $\Delta\Psi_m$: 2.26 ± 0.17 and 2.26 ± 0.2 ratio units in cells incubated in the absence and presence of C2, $n = 6$) but elicited a large decrease of the Ca^{2+}-induced mitochondrial Ca^{2+} uptake and potentiated the Ca^{2+}-induced fall of $\Delta\Psi_m$ (Figure 1A left, purple traces). The enhancement of Ca^{2+}-induced depolarization by C2 was very small at the first addition of Ca^{2+} but it became large during the second and third pulses (Figure 1A). No depolarization was observed in C2-pretreated cells if mitochondrial Ca^{2+} uptake was inhibited by RuRed (Figure 1A right, purple traces). These results show that mitochondrial Ca^{2+} uptake caused larger depolarizations in C2-treated cells. Furthermore, this difference between naive and C2-treated cells is not due to stimulation of mitochondrial Ca^{2+} uptake by C2. Preincubation of the permeabilized cells with C2 for 10 s was sufficient to alter Ca^{2+}-induced $[Ca^{2+}]_c$ and $\Delta\Psi_m$ responses (rate of $[Ca^{2+}]_c$ decay measured after the second Ca^{2+} pulse: 69.7 ± 3.7% of control, $P < 0.001$; rate of depolarization: 184.3 ± 26.5% of control, $P < 0.01$, $n = 6$) and maximal effect of C2 was reached within 10 min of preincubation (Figure 1B). Figure 1 also shows that C2-dihydroceramide (iC2), a structural analog of ceramide that does not cause apoptosis, failed to affect Ca^{2+}-induced mitochondrial Ca^{2+} uptake and loss of $\Delta\Psi_m$ (Figure 1C), whereas a chemically unrelated activator of the apoptotic program, staurosporine, exerted C2-like effects (Figure 1D). Taken together, these data indicate that exposure of permeabilized cells to proapoptotic stimuli alters mitochondrial Ca^{2+} handling. Although prolonged treatment with apoptotic agents is known to yield mitochondrial damage including release of cytochrome *c* (e.g. Bossy-Wetzel *et al.*, 1998; Garcia-Ruiz *et al.*, 1997; Yang *et al.*, 1997), our data suggest that an effect of these agents on mitochondrial Ca^{2+} regulation occurs very early.

Impaired mitochondrial Ca^{2+} handling and large depolarizations could be due to a primary effect of apoptotic agents on mitochondrial metabolism, yielding decreased H^+ extrusion. Notably, inhibitory effects of C2 on mitochondrial electron transport chain complex III have been reported (García-Ruiz *et al.*, 1997; Gudz *et al.*, 1997). This mechanism could contribute to the effects of C2 and staurosporine that we observed in permeabilized cells supplemented with succinate (complex II substrate, Figure 1A–C) or malate/glutamate (complex I substrate, Figure 1D). To prevent the effects of C2 that are dependent
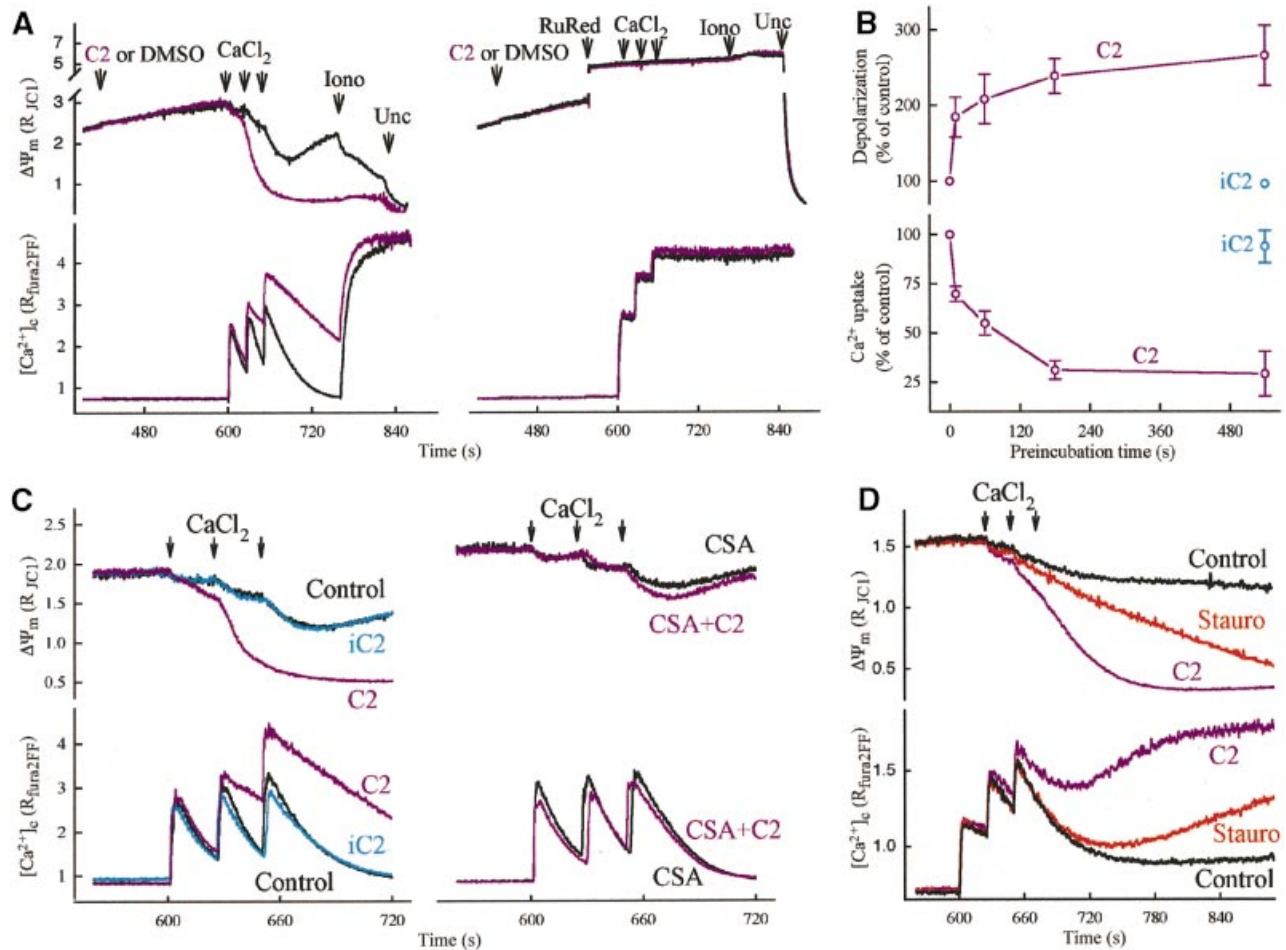
Fig. 1. Ca^{2+}-induced $\Delta\Psi_m$ and $[Ca^{2+}]_c$ responses in permeabilized cells exposed to proapoptotic stimuli. Simultaneous measurements of $\Delta\Psi_m$ and $[Ca^{2+}]_c$ were carried out in suspensions of permeabilized HepG2 cells using a membrane potential probe, JC1, and a Ca^{2+} tracer, fura2FF, respectively. (**A**) Effect of C2 on Ca^{2+}-induced mitochondrial depolarization and mitochondrial Ca^{2+} uptake. C2 (40 μM, purple) or solvent, DMSO (black), were added 180 s prior to Ca^{2+} pulsing to the permeabilized cells supplemented with succinate (2 mM). The same measurements were carried out in the absence (left panel) and presence of ruthenium red (2 μM RuRed added 60 s prior to Ca^{2+}, right panel). Other additions: $CaCl_2$, 40 μM $CaCl_2$ each; Iono, 10 μM ionomycin; Unc, 1 μM FCCP + 2.5 μg/ml oligomycin. Since RuRed resulted in a large, artifactual rise in R_{JC1} a compressed scale shows $\Delta\Psi_m$ after addition of RuRed. Traces are shown from separate incubations using the same cell preparation ($n = 3$). (**B**) Time course of C2 effect on rates of Ca^{2+}-induced depolarization and Ca^{2+} uptake. The effect of 10 min pretreatment with C2-dihydroceramide (iC2) is also shown (blue symbols). Experiments were carried out as shown in (A) and the rates of depolarization and Ca^{2+} uptake were calculated using the responses given to the third Ca^{2+} pulse. Rates represent the mean ± SEM of values from five or six separate experiments. (**C**) Effect of CSA on Ca^{2+}-induced mitochondrial depolarization and Ca^{2+} uptake. Measurement was carried out as described for (A) in permeabilized cells exposed to 40 μM iC2 (blue), 40 μM C2 (purple) or DMSO (black) for 180 s prior to Ca^{2+} addition. Incubations were carried out in the absence (left) and presence (right) of CSA (1 μM added 240 s prior to Ca^{2+}). Traces are shown from separate incubations using the same cell preparation ($n = 5$). (**D**) Effect of staurosporine (Stauro, 1 μM) on Ca^{2+}-induced mitochondrial depolarization and Ca^{2+} uptake. Pulses of $CaCl_2$ (10 μM each) were added to naive (black), C2-pretreated (180 s, purple) and Stauro-pretreated (60 min, red) cells supplemented with malate (1 mM)/glutamate (5 mM) and oligomycin (2.5 μg/ml). In the presence of oligomycin mitochondria could not utilize ATP added to the medium and Ca^{2+} release associated with Ca^{2+}-induced dissipation of $\Delta\Psi_m$ was augmented. Traces from three separate incubations using the same cell preparation ($n = 4$).

on respiratory chain activity, in subsequent experiments endogenous substrates were depleted by addition of ADP (100 μM) and then the cells were provided only with ATP and ATP regenerating system. Under this condition generation of $\Delta\Psi_m$ may be due to proton extrusion in the reverse mode of the ATP synthase. As expected, addition of an ATP synthase inhibitor, oligomycin (2.5 μg/ml), caused dissipation of $\Delta\Psi_m$ in permeabilized cells provided only with ATP and ATP regenerating system (half-life of depolarization was 33 ± 4 s, $n = 2$) whereas no oligomycin-induced depolarization was observed when mitochondria were energized with succinate or malate/glutamate ($n = 4$). Since C2 potentiated the Ca^{2+}-induced depolarization and inhibited Ca^{2+}-induced mitochondrial Ca^{2+} uptake in cells provided only with ATP (rate of depolariza-

tion: 218 ± 5% of control, $P < 0.001$; rate of $[Ca^{2+}]_c$ decay: 34 ± 5% of control, $P < 0.001$, $n = 3$), inhibition of the electron transport chain can not account for these effects. Alternatively, apoptotic agents may facilitate activation of PTP complex by accumulated Ca^{2+}. In contrast to the Ca^{2+}-induced depolarizations obtained in naive cells that were synchronized to the rapid falling phase of the $[Ca^{2+}]_c$ response and were reversed subsequently, the depolarizations in cells exposed to apoptotic agents carried on when $[Ca^{2+}]_c$ displayed a slow decrease or continuous increase (Figure 1, black versus purple or red traces). This result is consistent with the idea that Ca^{2+} led to activation of large pores, which allowed dissipation of the ionic gradients. Activation of PTP by $[Ca^{2+}]_m$ can be inhibited by cyclosporin A (CSA), a drug

causing dissociation of cyclophilin D from PTP on the luminal side (Broekemeier *et al.*, 1989; Halestrap and Davidson, 1990). Figure 1C shows that CSA (1 µM) did not exert a major effect on Ca^{2+}-induced changes of $\Delta\Psi_m$ and $[Ca^{2+}]_c$ in naive cells (compare black traces, left and right panels) but abolished the effects of C2 (purple traces, left and right panels). FK506 (10 µM), which affects extramitochondrial immunophilins but fails to affect mitochondrial cyclophilin D, did not decrease the effect of C2 (0 and 8% inhibition of depolarization in two experiments). An additional tool to target the permeability pore is bongkrekic acid, which binds to the adenine nucleotide translocator and, in turn, interferes with activation of the PTP complex. Since ADP supports the binding and inhibitory effect of bongkrekic acid (Klingenberg *et al.*, 1983; Brustovetsky and Klingenberg, 1996), the ATP regenerating system was omitted from the medium to prevent extramitochondrial phosphorylation of ADP. To our surprise, bongkrekic acid (300 nM–1 µM added 5 min before C2) enhanced Ca^{2+}-induced depolarization in naive cells ($\Delta\Psi_m$ after Ca^{2+} pulses: 50.9% of control) but this drug also inhibited Ca^{2+}-induced depolarization in C2-treated cells ($\Delta\Psi_m$ after Ca^{2+} pulses: 155.5% of control, $n = 2$). Measurements of $[Ca^{2+}]_c$ showed that bongkrekic acid facilitated the decay of the Ca^{2+}-induced $[Ca^{2+}]_c$ increases in C2-treated cells, but not in naive cells ($n = 2$), suggesting that sequestration of Ca^{2+} in the mitochondria was supported by the drug. Taken together, these data provide evidence that activation of PTP by accumulated Ca^{2+} accounts for the altered $\Delta\Psi_m$ and $[Ca^{2+}]_c$ responses of permeabilized cells exposed to C2.

Using the three-pulses protocol of Ca^{2+} addition shown in Figure 1, 30–40 µM pulses of $CaCl_2$ were required to obtain CSA-sensitive depolarization in C2-pretreated cells supplemented with succinate and ATP (Figure 1A–C). Opening of PTP required lower doses of Ca^{2+} (10–20 µM pulses of $CaCl_2$, Figure 1D) when electrons were provided to complex I (using malate/glutamate as substrate) rather than to complex II (using succinate as substrate) as described recently by Fontaine *et al.* (1998). Nevertheless, elevations of $[Ca^{2+}]_c$ evoked by addition of Ca^{2+} pulses were in the range 5–20 µM, which are substantially higher than the global increases of $[Ca^{2+}]_c$ in cells stimulated with Ca^{2+} mobilizing stimuli.

Recent reports suggest a model wherein activation of caspases is proximal to mitochondrial depolarization and cytochrome *c* release. Bossy-Wetzel *et al.* (1998) demonstrated that activation of a DEVD-specific caspase occurs before the dissipation of $\Delta\Psi_m$. Most recently, activation of death receptors was described to yield activation of caspase-8, which cleaves BID and, subsequently, truncated BID causes cytochrome *c* release from the mitochondria (Li *et al.*, 1998; Luo *et al.*, 1998). In our experiments washout of the cytosol prior to C2 addition did not prevent Ca^{2+}-induced PTP opening (an example is shown in Figure 3A), providing evidence that soluble cytosolic factors do not play an obligatory role in Ca^{2+}-induced PTP opening in cells pretreated with C2. Experiments were also carried out in the presence of caspase inhibitors, DEVD-CHO (50 µM), which inhibits most caspases including caspase-3, caspase-8 and caspase-9, or zVAD–FMK (50 µM), which inhibits all 10 known caspases. Caspase inhibitors failed to inhibit Ca^{2+}-induced depolar-

ization (C2 + DEVD-CHO: 112%; C2 + zVAD–FMK: 100 and 106% of C2 without caspase inhibitors, $n = 2$) or to decrease the duration of $[Ca^{2+}]_c$ transients induced by Ca^{2+} pulsing in C2-pretreated cells (C2 + DEVD-CHO: 104%; C2 + zVAD–FMK: 92 and 103% of C2 without caspase inhibitors, $n = 2$). These results suggest that activation of caspases is not required for facilitation of Ca^{2+}-induced opening of PTP by proapoptotic stimuli in this system.

Another fundamental question of the mechanism underlying Ca^{2+}-induced opening of PTP in cells exposed to apoptotic agents is whether resealing of PTP occurs after decay of the $[Ca^{2+}]$ rise. In living cells orchestrated Ca^{2+} transport through intracellular membranes and plasma membrane is responsible for deactivation of $[Ca^{2+}]_c$ transients. Since Ca^{2+} accumulation by the mitochondria represents the only mechanism to decrease $[Ca^{2+}]_c$ in permeabilized cells treated with Tg, to bring about the decay of the Ca^{2+} response, a Ca^{2+} chelator, EGTA (Figure 2A), or an inhibitor of mitochondrial Ca^{2+} accumulation, RuRed (Figure 2B) were added. Mitochondrial membrane potential that had been dissipated in response to Ca^{2+} was rapidly regenerated under both conditions, suggesting that PTP became closed and mitochondrial metabolism recovered. Repolarization of the mitochondria was not inhibited by oligomycin (rate of repolarization was 0.39 ± 0.06 ratio units/min, $n = 3$ and 0.39 ± 0.06 ratio units/min, $n = 5$ in cells incubated in the absence and presence of oligomycin), providing evidence that proton extrusion was not due to the reversed mode of the ATPase but to activation of the electron transport chain. These results show that a transient exposure to high Ca^{2+} leads to a transient activation of PTP in permeabilized cells exposed to apoptotic agents.

Calcium-induced activation of cytochrome c release and caspase activation in permeabilized cells exposed to apoptotic stimuli

The next set of experiments was carried out to determine whether activation of PTP by $[Ca^{2+}]_m$ yields cytochrome *c* release and caspase activation in C2-pretreated cells. To remove cytochrome *c* present in the cytosol before Ca^{2+} pulsing, the cells were washed after permeabilization. First, we repeated the measurements of $\Delta\Psi_m$ shown in Figure 1 using washed permeabilized cells. Figure 3A shows that Ca^{2+} addition induced extensive depolarization in C2-pretreated cells that was prevented by CSA. This result shows that washout of the cytosol did not prevent activation of PTP by Ca^{2+} pulses in permeabilized cells exposed to C2. It is noteworthy that addition of cytochrome *c* (400 nM) to the permeabilized cells was not found to evoke depolarization, showing that the PTP opening in response to Ca^{2+} is not due to cytochrome *c* release. At the end of each fluorescence measurement samples of the cytosolic fraction (14 000 *g* supernatant) were prepared to determine cytochrome *c* release corresponding to the $\Delta\Psi_m$ responses. In order to prevent Ca^{2+} from affecting cytochrome *c* release during isolation of the cytosol, the permeabilized cells were supplemented with Ca^{2+} chelator (1 mM EGTA) after exposure to Ca^{2+} pulses. EGTA resulted in resealing of PTP, and in turn, recovery of $\Delta\Psi_m$, as shown in Figure 2A. Figure 3B and C shows data from Western blot analysis of the cytosol samples. The
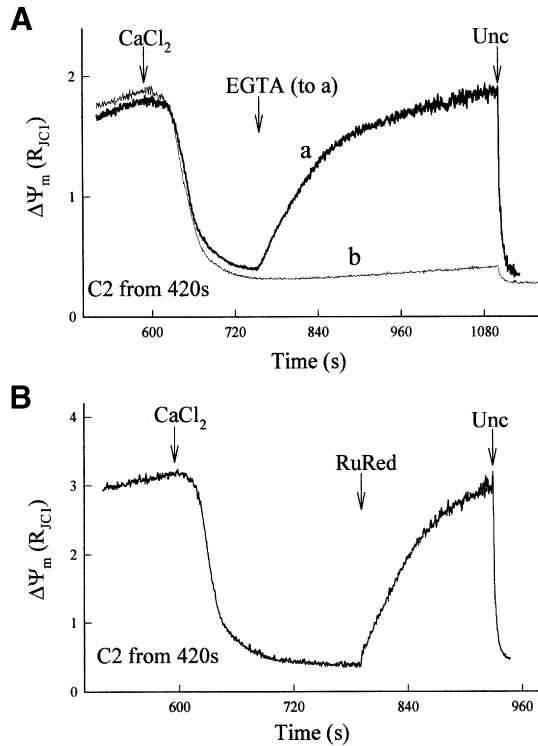
Fig. 2. Reversibility of Ca^{2+}-induced PTP opening in permeabilized cells exposed to C2. Experimental conditions were as in Figure 1(A). In the experiments shown in (**A**) five pulses of 30 μM $CaCl_2$ were added at 25 s intervals, in (**B**) 120 μM $CaCl_2$ was added continuously (75 s). Other additions: RuRed, 2 μM ruthenium red; C2, 40 μM C2-ceramide; EGTA, 200 μM EGTA; Unc, 1 μM FCCP + 2.5 μg/ml oligomycin. Data are representative of five (A) and three experiments (B).

cytochrome *c* band was identified using molecular weight markers and also with exogenous cytochrome *c* standard added to the permeabilized cells during the fluorescence measurement (Figure 3B). Cytosolic cytochrome *c* was present in the same amount in samples derived from suspensions of naive cells exposed to Ca^{2+} or from C2-pretreated cells without Ca^{2+} addition, whereas a several-fold larger level was detected in samples derived from C2-pretreated cells exposed to Ca^{2+} (Figure 3B and C). Cytochrome *c* release was inhibited by pretreatment with CSA (Figure 3C).

Cytochrome *c* is known to activate caspases in the cytosol (Kluck *et al.*, 1997; Yang *et al.*, 1997). A fluorometric assay (Nicholson *et al.*, 1995; Hampton *et al.*, 1998) was used to determine caspase activity in cytosol fractions prepared from suspensions of permeabilized cells exposed to C2 and Ca^{2+} as shown in Figure 1. Figure 3D shows the time course of DEVD–AMC cleavage and the inhibitory effect exerted by the caspase inhibitor, DEVD-CHO (IC$_{50}$ ~ 1 nM). Addition of exogenous cytochrome *c* (400 nM) to the cell suspension brought about a large increase in DEVD–AMC cleavage (Figure 3D and E). This result reconfirmed the observation that cytochrome *c* evokes caspase activation. Exposure of C2-pretreated permeabilized cells to Ca^{2+} pulses yielded stimulation of cytosolic DEVD–AMC cleavage (Figure 3D and E, $P < 0.001$, $n = 4$). This effect was as large as the effect of exogenous cytochrome *c* (Figure 3D and E). Taken together, these experiments demonstrate that Ca^{2+}-induced
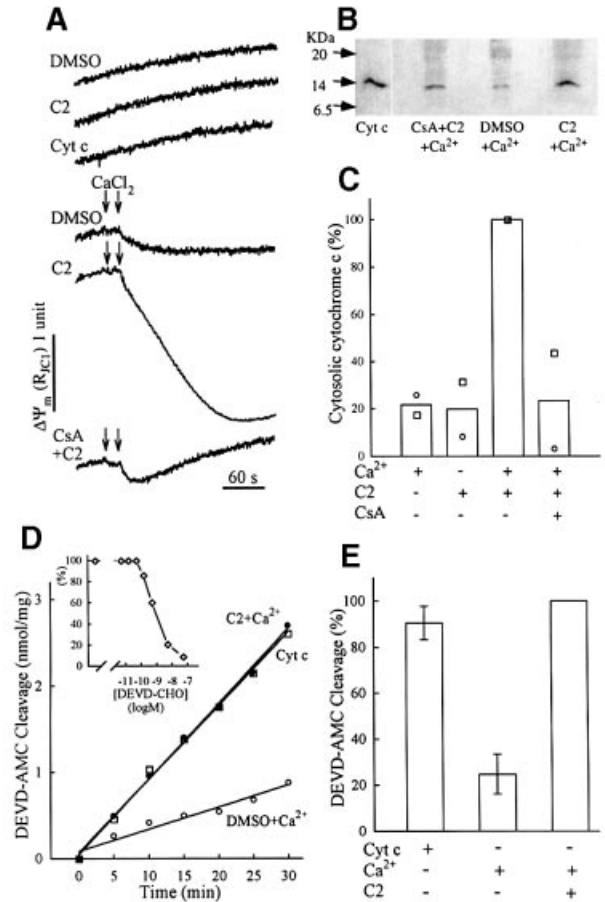

Fig. 3. Release of cytochrome *c* and activation of caspases evoked by Ca^{2+} pulses in permeabilized cells exposed to proapoptotic stimuli. (**A–C**) Cytochrome *c* release associated with Ca^{2+}-induced opening of PTP. Measurements of $\Delta\Psi_m$ in suspensions of washed permeabilized cells (A), immunoblots (B) and cumulative data of cytochrome *c* measurements (C) in 'cytosol' samples generated at the end of the $\Delta\Psi_m$ recordings shown in (A). Additions: C2, 40 μM C2-ceramide; Cyt c, 400 nM cytochrome *c*; CSA, 1 μM cyclosporin A; $CaCl_2$, 10 μM $CaCl_2$ each. Data are from experiments carried out in two separate cell preparations and each represents the mean of duplicates. (**D** and **E**) Caspase activation associated with Ca^{2+}-induced opening of PTP. Fluorometric assay of DEVD–AMC cleavage in cytosol extracts prepared at the end of measurements of $\Delta\Psi_m$ (shown in Figure 1D). Data in (D) are representative of three different experiments, whereas DEVD–AMC cleavage normalized to the activity obtained with C2+Ca^{2+} is shown as mean ± SEM in (E) ($n = 3–4$). The inset shows the dose–response for inhibition of DEVD–AMC cleavage by DEVD-CHO.

opening of PTP in C2-pretreated cells was associated with cytochrome *c* release and released cytochrome *c* led to activation of caspases.

As noted above (see Figure 2), regeneration of $\Delta\Psi_m$ follows the decay of the $[Ca^{2+}]$ rise, suggesting that release of cytochrome *c* does not prevent repolarization of the mitochondria. Thus, depolarizations preceding cytochrome *c* release may be hardly detectable at the level of the population average in cell populations that exhibit asynchronous $[Ca^{2+}]_c$ transients and PTP openings. Furthermore, it has been unresolved how ATP requirements of the energy-dependent apoptotic program would be satisfied if cytochrome *c* release was associated with a compromised metabolic function of the mitochondria. In addition to the impaired ATP production, opening of PTP
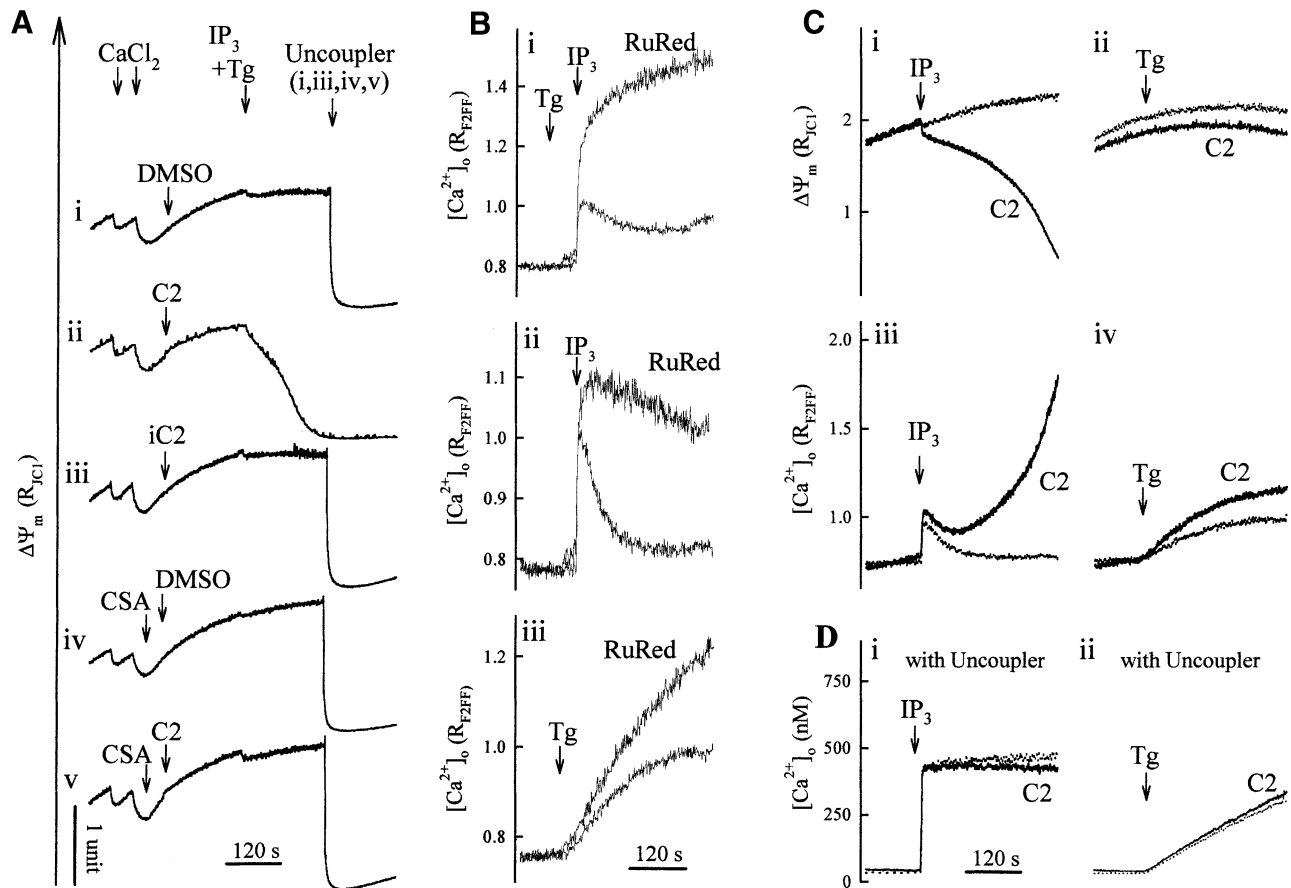
Fig. 4. Activation of PTP by IP$_3$-induced Ca^{2+} release in suspensions of permeabilized cells exposed to C2. (**A**) Comparison of $\Delta\Psi_m$ responses evoked by Ca^{2+} mobilization in naive and C2-pretreated permeabilized cells. Additions: CaCl$_2$, 50 μM CaCl$_2$; IP$_3$ + Tg, 10 μM IP$_3$ + 2 μM thapsigargin; Uncoupler, 1 μM FCCP + 2.5 μg/ml oligomycin; C2, 40 μM C2-ceramide; iC2, 40 μM C2-dihydroceramide; CSA, 1 μM cyclosporin A. Traces from five separate experiments with the same cell preparation are shown ($n = 4$). (**B**) Transmission of [Ca^{2+}]$_c$ changes into the mitochondria in permeabilized cells stimulated with 10 μM IP$_3$ and/or 2 μM Tg. In order to determine mitochondrial Ca^{2+} uptake associated with Ca^{2+} mobilization, measurements of [Ca^{2+}]$_c$ were carried out in the absence and presence of an inhibitor of mitochondrial Ca^{2+} uptake, ruthenium red (2 μM, RuRed). Traces from separate incubations using the same cell preparation are shown ($n = 3$). (**C**) Simultaneous measurements of $\Delta\Psi_m$ (panels i and ii) and [Ca^{2+}]$_c$ (panels iii and iv) during IP$_3$- (panels i and iii) and Tg-induced (panels ii and iv) Ca^{2+} release in naive and C2-pretreated (40 μM for 180 s) permeabilized cells. Data are representative of five experiments with IP$_3$ and three experiments with Tg. (**D**) Effect of C2 on Ca^{2+} release from ER induced by IP$_3$ or Tg. Comparison of [Ca^{2+}]$_c$ increases evoked by IP$_3$ (panel i) or Tg (panel ii) in naive and C2-pretreated (40 μM for 180 s) cells was carried out in the presence of uncoupler. Traces from four separate incubations using the same cell preparation are shown ($n = 2$).

would lead to mitochondrial ATP consumption due to the reverse operation of the F$_1$F$_0$-ATPase. Our findings that mitochondria reseal after the decay of the [Ca^{2+}] rise and that the residual cytochrome c content of the mitochondria is sufficient to maintain mitochondrial metabolism support the idea that mitochondria can provide fuels for the steps of the apoptotic program distal to cytochrome c release. As such, [Ca^{2+}] spikes may serve as an optimal signal to trigger the mitochondrial phase of apoptosis.

IP$_3$-induced activation of PTP in C2-treated permeabilized cells

As reported previously in other cell types (e.g. Rizzuto *et al.*, 1993, 1994, 1998; Hajnóczky *et al.*, 1995; Csordás *et al.*, 1999), large and rapid increases of [Ca^{2+}]$_m$ were found in HepG2 cells in association with [Ca^{2+}]$_c$ signals elicited by activation of the IP$_3$-linked calcium signaling pathway through P$_{2y}$ receptors with ATP (10–200 μM, data not shown). To determine whether the cells exposed to proapoptotic stimuli respond to IP$_3$-mediated [Ca^{2+}]$_m$ signals by mitochondrial permeability transition and

cytochrome c release, experiments were carried out to test IP$_3$-induced $\Delta\Psi_m$ responses in suspensions of naive and C2-treated permeabilized cells, respectively. Thapsigargin was omitted from the buffer in these experiments to allow ER Ca^{2+} uptake and IP$_3$-induced Ca^{2+} release, but to prevent re-uptake of released Ca^{2+} into the ER, and in turn, to promote mitochondrial uptake of released Ca^{2+}, IP$_3$ was added together with Tg. Figure 4A shows $\Delta\Psi_m$ responses associated with IP$_3$-induced Ca^{2+} release. Naive cells responded to IP$_3$ by rapid and very small depolarizations. This result supports the idea that PTP is relatively insensitive to IP$_3$-linked [Ca^{2+}]$_c$ signals under physiological conditions. In contrast to the result obtained in naive cells, C2-pretreated cells showed dissipation of $\Delta\Psi_m$ in response to IP$_3$. No large IP$_3$-induced depolarization was observed in cells preincubated with iC2 or with C2 in the presence of CSA (Figure 4A) or RuRed ($n = 3$). These results provide evidence that IP$_3$ receptor-mediated mitochondrial Ca^{2+} uptake brought about depolarization in cells pretreated with proapoptotic ceramide and also suggest that the depolarization was due to the opening of PTP.
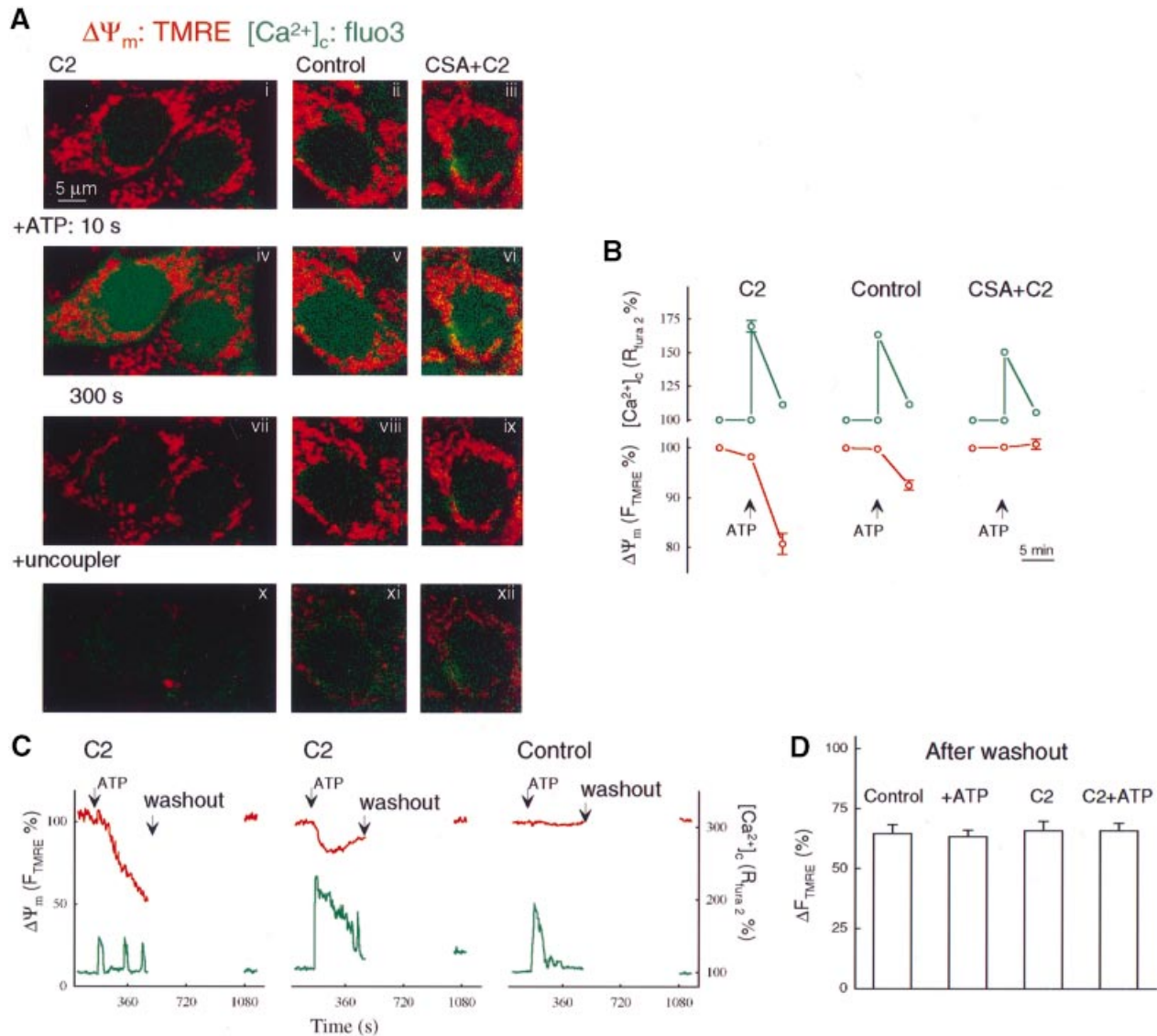
Fig. 5. Activation of PTP in intact cells stimulated with IP$_3$-linked stimuli. Simultaneous measurements of [Ca^{2+}]$_c$ and $\Delta\Psi_m$ carried out in intact HepG2 cells exposed to C2 (40 µM), solvent or CSA (1 µM) + C2. (**A**) Confocal images of cells loaded with fluo3 and TMRE showing [Ca^{2+}]$_c$ (green) and $\Delta\Psi_m$ (red) in C2-treated cells (left side), in naive cells (middle) and in CSA + C2-treated cells (right side) before addition of 200 µM ATP (images i–iii), at 10 s (iv–vi) and at 300 s (vii–ix) stimulation and 7 min after uncoupler addition (x–xii). (**B**) Comparison of [Ca^{2+}]$_c$ increases and mitochondrial depolarizations measured in each condition at the peak of the [Ca^{2+}]$_c$ rise and at 300 s stimulation. Data are normalized to prestimulation values. Calculated changes are the mean ± SEM of values from 60–80 cells from at least three different cultures. (**C**) Time courses of [Ca^{2+}]$_c$ and $\Delta\Psi_m$ changes in C2-treated (left and middle) and in naive (right) cells stimulated with 200 µM ATP for 6 min and subsequently washed into fresh buffer without C2 and ATP. (**D**) Comparison of $\Delta\Psi_m$ in naive and in C2-pretreated cells exposed to ATP for 10 min followed by 20 min incubation without C2 and ATP. The uncoupler-sensitive TMRE fluorescence intensities are normalized to the total fluorescence in each condition. Calculated changes are the mean ± SEM of values from the entire cell population in the imaging field (50–80 cells) from four or five different cultures.

It is important to point out that elevations of global [Ca^{2+}]$_c$ elicited by IP$_3$ in suspensions of permeabilized cells were in the submicromolar range (e.g. Figure 4D, i) whereas [Ca^{2+}]$_c$ responses evoked by addition of Ca^{2+} pulses that were used to induce PTP opening were in the range of 5–20 µM. This difference shows that IP$_3$-induced Ca^{2+} release was utilized extremely efficiently to fuel mitochondrial Ca^{2+} uptake. This result is consistent with the idea that IP$_3$ leads to activation of mitochondrial Ca^{2+} uptake via generation of a localized large increase in [Ca^{2+}]$_c$ near to the mitochondria (Rizzuto *et al.*, 1993, 1994, 1998; Hajnóczky *et al.*, 1995; Csordás *et al.*, 1999). To investigate further the relationship between the

mechanism of Ca^{2+} mobilization and mitochondrial Ca^{2+} signaling, [Ca^{2+}]$_c$ responses induced by IP$_3$ and Tg were monitored in the absence and presence, respectively, of RuRed (Figure 4B). Activation of the ER Ca^{2+} release sites by IP$_3$ caused a rapid and transient rise of [Ca^{2+}]$_c$ whereas inhibition of ER Ca^{2+} uptake sites by Tg led to a slow and substantial rise of [Ca^{2+}]. In response to IP$_3$ plus Tg a rapid rise of [Ca^{2+}]$_c$ was observed that was followed by a plateau phase.

The temporal pattern of mitochondrial Ca^{2+} uptake responses brought about by IP$_3$, Tg or IP$_3$ + Tg was estimated from the RuRed-sensitive component of [Ca^{2+}]$_c$ responses recorded at each condition. Enhancement of
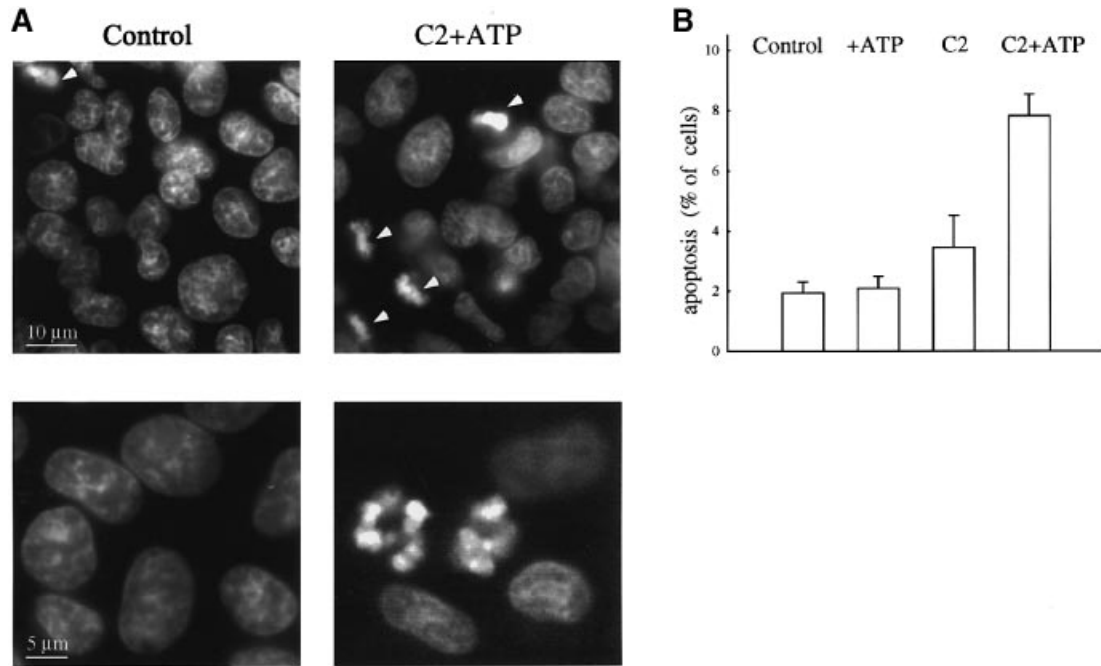
Fig. 6. Nuclear apoptosis in cells stimulated with IP$_3$-linked stimuli. Cell death in HepG2 cells was detected by a double staining Hoechst 33342/ propidium iodide technique. (**A**) Images show the Hoechst 33342 fluorescence in naive cells (left side) and in C2-treated cells stimulated with 200 μM ATP (right side). The cells were washed after exposure to apoptotic agents and ATP and incubated for an additional 90 min before the fluorescence measurement. Apoptotic nuclei are marked with arrows. Images in the lower row show nuclear staining at higher resolution. Propidium iodide was excluded from all cells on the fields. (**B**) Comparison of apoptotic responses in naive and C2-treated cells incubated with ATP or solvent. Data are the average of four separate experiments (mean ± SEM).

the IP$_3$-induced initial rise of [Ca^{2+}]$_c$ in the presence of RuRed suggests rapid transmission of the calcium signal into the mitochondria (Figure 4B, ii), whereas the gradually developing RuRed-sensitive [Ca^{2+}]$_c$ rise elicited by Tg suggests a slow loading of mitochondria with released Ca^{2+} (Figure 4B, iii). The massive effect of RuRed on the [Ca^{2+}]$_c$ rise evoked by IP$_3$ + Tg suggests that the largest initial and sustained mitochondrial Ca^{2+} uptake occurred under this condition (Figure 4B, i). Notably, Ca^{2+} loading of the mitochondria with repetitive Ca^{2+} pulses is more effective at evoking activation of PTP than continuous addition of the same Ca^{2+} dose (Ichas *et al.*, 1997). To test whether the brief mitochondrial Ca^{2+} load evoked by IP$_3$ by itself was sufficient to cause opening of PTP in C2-pretreated cells and to test the effect of the continuous Ca^{2+} load elicited by Tg, simultaneous fluorescence measurements of [Ca^{2+}]$_c$ and ΔΨ$_m$ were carried out in naive and C2-treated permeabilized cells. Figure 4C shows that IP$_3$-induced Ca^{2+} release appeared as a [Ca^{2+}]$_c$ transient without depolarization in naive cells (Figure 4C, i and iii), but it was followed by a collapse of the ΔΨ$_m$ (Figure 4C, i) and a large secondary, presumably, mitochondrial Ca^{2+} release response (Figure 4C, iii) in C2-treated cells. No similar mitochondrial response was evoked by Tg-induced Ca^{2+} release from ER in C2-treated cells (Figure 4C, ii and iv). In order to determine whether C2 exerts an effect on Ca^{2+} release from ER that might contribute to the effect of C2 on IP$_3$-induced [Ca^{2+}]$_c$ signal, mitochondrial Ca^{2+} uptake was blocked and the same experiments were repeated. Measurements of [Ca^{2+}]$_c$ that were carried out in the presence of uncoupler, to disconnect mitochondria from Ca^{2+} responses, showed that C2 did not affect IP$_3$- or Tg-induced Ca^{2+} release

from the ER Ca^{2+} store (Figure 4D). Taken together these experiments provide evidence that IP$_3$ receptor-mediated Ca^{2+} transients can trigger activation of PTP in cells exposed to proapoptotic stimuli and also suggest that the privileged Ca^{2+} transfer from IP$_3$ receptors to mitochondrial Ca^{2+} uptake sites plays a critical role in this process.

Activation of PTP by IP$_3$-linked calcium signals in intact HepG2 cells exposed to C2

In order to determine whether calcium signals evoked by IP$_3$-linked agonists are able to initiate the mitochondrial phase of apoptosis in C2-treated intact cells, simultaneous fluorescence measurements of ΔΨ$_m$ and [Ca^{2+}]$_c$ were carried out in HepG2 cells stimulated with ATP (Figure 5). Loading with tetramethylrhodamine ethyl ester (TMRE) and pretreatment with C2 (30 min) were carried out in serum-free buffer to avoid stimulation of the cells by serum-borne IP$_3$-linked agonists. Prior to ATP addition TMRE fluorescence intensities in naive and C2-pretreated cells were similar (not shown) suggesting that the brief preincubation with C2 did not cause mitochondrial depolarization. Addition of ATP evoked rapid and transient rises of [Ca^{2+}]$_c$ in naive and C2-treated cells as well (increase of the green component in Figure 5A, images iv and v) which often appeared as [Ca^{2+}]$_c$ oscillations (Figure 5C). A major decrease of TMRE fluorescence indicating mitochondrial depolarization synchronized to the onset of [Ca^{2+}]$_c$ spikes was not recorded in naive cells (essentially the same red components in images i and ii and iv and v of Figure 5A), but large depolarizations appeared gradually in C2-pretreated cells (decrease of the red component in image vii of Figure 5A, Figure 5B and C). If pretreatment of the cells with C2 was carried out
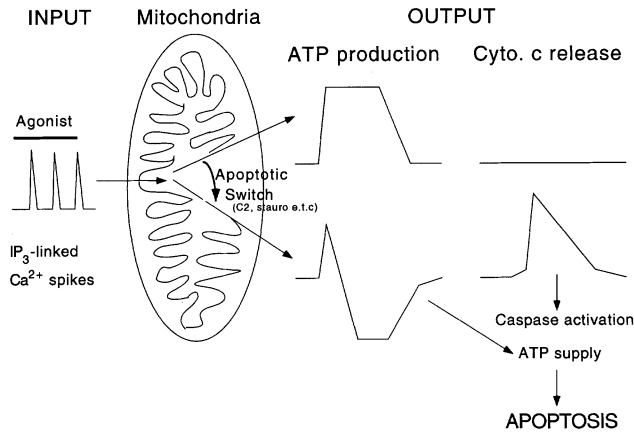
Fig. 7. Engagement of cells to the apoptotic pathway using IP$_3$-mediated mitochondrial calcium signals. Survival (upper row) and apoptotic (lower row) responses given by the mitochondria to Ca^{2+} spikes.

in the presence of CSA no depolarization followed ATP-induced [Ca^{2+}]$_c$ signal (Figure 5A, images iii, vi and ix, Figure 5B). Complete loss of TMRE fluorescence showing dissipation of $\Delta\Psi_m$ was achieved upon addition of uncoupler in naive, in C2-pretreated and in CSA + C2-pretreated cells (Figure 5A). Taken together, these data show that calcium signals evoked by IP$_3$-linked agonists led to large depolarizations in C2-pretreated but not in naive cells. Inhibition of depolarization by CSA suggests that the calcium signal-induced loss of $\Delta\Psi_m$ in C2-treated cells is due to the opening of PTP.

A remarkable feature of PTP activation upon mitochondrial Ca^{2+} uptake was the recovery of $\Delta\Psi_m$ after cessation of Ca^{2+} uptake (Figure 2). Providing that IP$_3$-linked [Ca^{2+}]$_c$ signals typically appear in the form of [Ca^{2+}] transients, we postulated that resealing of PTP and recovery of $\Delta\Psi_m$ may occur after decay of the [Ca^{2+}]$_c$ response in intact cells as well. In order to determine whether recovery of $\Delta\Psi_m$ occurs after the decay of [Ca^{2+}]$_c$ transients, fluorescence measurements of $\Delta\Psi_m$ were carried out using TMRE in naive and in C2-pretreated cells exposed to ATP followed by incubation without C2 and ATP (Figure 5C). These measurements showed recovery of $\Delta\Psi_m$ in C2-treated single cells (Figure 5C) and essentially the same $\Delta\Psi_m$ in naive and in C2-pretreated cells after washout of ATP and C2 (Figure 5D). This provided evidence that the decrease of $\Delta\Psi_m$ associated with the IP$_3$-linked calcium signal was reversible in cells exposed to apoptotic agents. Recovery of $\Delta\Psi_m$ suggests that the metabolic output of the mitochondria was also recovering and so mitochondria may provide ATP to meet the requirements of the apoptotic program. Another important implication is that the loss of $\Delta\Psi_m$ and presumably cytochrome c release evoked by IP$_3$-linked calcium spikes did not involve irreversible damage of the outer membrane.

Activation of the PTP complex is expected to yield complete dissipation of $\Delta\Psi_m$ at the level of individual mitochondria (Hüser et al., 1998). In our studies, depolarization associated with IP$_3$-linked [Ca^{2+}]$_c$ spikes manifested as a graded response at the level of single cells (Figure 5A and C). Also, depolarization was not detected in every cell of the cell population. These data suggest that only subsets of the mitochondria were involved in

depolarization and/or that individual mitochondria went through transient openings of PTP in a non-synchronized manner. Since the cells were challenged with maximal doses of ATP that evoked calcium signal in every cell without delay, the loss of $\Delta\Psi_m$ associated with [Ca^{2+}] spikes occurred in an essentially synchronized manner in individual cells. As such, depolarization was visible at the level of cell population average as well. In contrast to the experimental conditions applied here, the cells in the organisms are envisioned to undergo submaximal levels of IP$_3$-linked stimulation that cause [Ca^{2+}] spikes with cell-specific length of lag time and frequency of [Ca^{2+}] spiking. Though calcium spikes evoked by submaximal stimulation are fully competent to raise [Ca^{2+}]$_m$ (Hajnóczky et al., 1995), individual cells of the population will display Ca^{2+}-induced mitochondrial responses with variable delay. Providing that IP$_3$-linked calcium spiking is involved in initiation of the mitochondrial phase of apoptosis, one can easily fail to detect the depolarization preceding cytochrome c release in asynchronous multi-cellular systems.

Apoptosis in response to IP$_3$-linked calcium signals
To obtain a direct measure of the apoptotic activity in naive and in C2-pretreated HepG2 cells stimulated with ATP, apoptotic nuclei were visualized using Hoechst 33342 and necrotic or late apoptotic cells were visualized with propidium iodide. Prolonged incubations (3–8 h) with C2 led to a rise in the number of propidium iodide-positive cells, but if shorter incubations were carried out or C2 was washed out after stimulation with ATP all cells excluded propidium iodide. Condensed chromatin structure visualized using Hoechst 33342 was detected in a higher fraction of the cells exposed to C2 and subsequently to ATP than in naive cells or in cells exposed to C2 or ATP by itself (Figure 6, $P < 0.03$, $n = 4$). This result shows that stimulation of the cells with an IP$_3$-linked agonist led to apoptosis in C2-treated cells. The role of PTP in the signaling pathway is suggested by the fact that CSA (1 μM) inhibited the enhanced apoptotic response evoked by ATP in C2-treated cells (65.2 ± 14.7% inhibition of apoptosis measured in cells exposed to C2 and ATP, $P < 0.025$, $n = 4$). These data provide evidence that activation of the mitochondrial phase of apoptosis by IP$_3$-linked calcium signals leads to execution of the complete apoptotic program.

Control of life and death using IP$_3$-linked mitochondrial calcium signals
The schematic in Figure 7 shows the calcium signal-driven apoptotic pathway determined in the present study. A fundamental new feature of this mechanism is that engaging of cells to apoptosis is due to an altered cytochrome c and metabolic output of the mitochondria in response to IP$_3$-mediated [Ca^{2+}] signals. Survival and death responses given by the mitochondria are depicted in the upper and lower row, respectively. Privileged transfer of Ca^{2+} from IP$_3$ receptors to mitochondrial Ca^{2+} uptake sites yields large elevations of [Ca^{2+}]$_m$ during [Ca^{2+}]$_c$ signals driven by the IP$_3$ receptors (Rizzuto et al., 1993, 1994, 1998; Hajnóczky et al., 1995; Csordás et al., 1999). In health, mitochondrial Ca^{2+} signals mediated by IP$_3$ receptors are utilized to activate Ca^{2+}-sensitive

mitochondrial dehydrogenases and, in turn, to stimulate mitochondrial energy metabolism (McCormack *et al.*, 1990; Pralong *et al.*, 1994; Hajnóczky *et al.*, 1995; Robb-Gaspers *et al.*, 1998; Duchen, 1999). Increases in the mitochondrial ATP output serve to meet ATP requirements of the stimulated cell (Figure 7, upper row, left). In cells that are predestined to survive, PTP opening associated with cytochrome *c* release is not induced by IP$_3$-linked increases of $[Ca^{2+}]_m$ (Figure 7, upper row, right). By contrast, in cells exposed to certain proapoptotic stimuli, such as ceramide, the metabolic and cytochrome *c* release responses given by the mitochondria appear to be determined by activation of PTP. Although $[Ca^{2+}]_m$ has been known to be a major regulator of PTP, this study shows for the first time PTP activation and subsequent nuclear apoptosis to occur in response to IP$_3$-linked agonists. A major contribution of the Ca^{2+} spiking-induced opening of PTP to activation of the apoptotic machinery is cytochrome *c* release (Figure 7, lower row, right) since cytochrome *c* serves as a highly potent activator of the caspase enzymes. Opening of PTP also yields loss of mitochondrial ATP production (Figure 7, lower row, left) and brings about mitochondrial ATP consumption. One of the most important findings of our study is that the decay of Ca^{2+} spikes is followed by resealing of PTP. Recovery of the mitochondrial membrane potential suggests recovery of the metabolic function of the mitochondria as well (Figure 7, lower row, left). This may play a fundamental role in providing the energy-dependent steps of cellular breakdown with ATP during apoptosis. Inadequate ATP supply would cause the apoptotic process to turn into necrosis.

The agents (ceramide and staurosporine) that we used to evoke the 'apoptotic switch' in the mitochondria have been shown to activate the complete apoptotic program in a number of models on their own. Prolonged incubations with the proapoptotic agents also led to cell death in HepG2 cells. However, the short exposure to proapoptotic stimuli did not cause PTP opening and/or cytochrome *c* release and failed to yield enhanced apoptotic activity. Mitochondrial and nuclear apoptosis occurred only if rises of $[Ca^{2+}]_m$ were coincident with the short exposure to proapoptotic stimuli. This suggests that IP$_3$-linked Ca^{2+} signals can determine the fate of the cell at the beginning of exposure to apoptotic stimuli. It is also important to note that calcium signaling may also play a role in apoptosis evoked by prolonged incubation with apoptotic agents, since a number of serum-borne factors may yield activation of the IP$_3$-linked Ca^{2+} signaling pathway during cell culture. Interestingly, buffering of intracellular $[Ca^{2+}]$ at low levels (e.g. McConkey *et al.*, 1989; Dowd *et al.*, 1992; Story *et al.*, 1992; Robertson *et al.*, 1993; Kruman and Mattson, 1999) and inhibition of mitochondrial Ca^{2+} uptake sites (Zamzami *et al.*, 1995; Quillet-Mary *et al.*, 1997; Kruman and Mattson, 1999) have been described to inhibit apoptosis induced by apoptotic agents. This suggests that calcium signals triggered by components of the medium could have played a role in apoptotic responses described in previous studies. A wealth of data suggest that several distinct mechanisms can be utilized to evoke caspase-mediated cell death and so calcium signals do not represent an obligatory component of the death pathway, but our data demonstrate that IP$_3$-linked Ca^{2+} signals

provide an efficient messenger mechanism to execute apoptosis. Notably, the same signal appears to play a fundamental role in the physiological control of cellular energy metabolism and in the execution of cellular suicide under different conditions.

Due to the local Ca^{2+} regulation between IP$_3$ receptors and mitochondrial Ca^{2+} uptake sites, each short-lasting Ca^{2+} spike is able to activate mitochondrial Ca^{2+} uptake very efficiently. Although it is not feasible to measure the amount of Ca^{2+} delivered to the mitochondria during a single $[Ca^{2+}]$ spike, our experiments in permeabilized cells showed that the amount of Ca^{2+} mobilized by IP$_3$ represents a very small fraction of the amount of Ca^{2+} required to cause opening of PTP if Ca^{2+} was added to the bulk of the medium. Using inhibitors of the ER Ca^{2+} pumps to discharge the IP$_3$-sensitive Ca^{2+} store, Ca^{2+} release gives only a slow Ca^{2+} load of the mitochondria, which occurs without activation of the PTP complex. This provides further evidence arguing for a unique ability of IP$_3$ receptors to evoke rapid mitochondrial Ca^{2+} loads using small amounts of Ca^{2+}. Minimizing the amount of transported Ca^{2+} required to establish a biological response is a significant asset for the cells. Notably, most of the previous studies on the role of calcium in cell death were directed towards investigation of the actions of massive Ca^{2+} loads of the cells such as prolonged activation of Ca^{2+} entry and permanent Ca^{2+} depletion of intracellular Ca^{2+} stores. Under those conditions the effect of Ca^{2+} on the apoptotic machinery may be mediated by target mechanisms activated in the cytosol, e.g. calcineurin (Wang *et al.*, 1999) or in the ER lumen (Bian *et al.*, 1997). Our study shows for the first time that the physiological arrangements of local calcium signaling to the mitochondria may provide a means of activating the apoptotic death cascade.

Conclusions

We identified IP$_3$ receptor-mediated calcium spikes as a potent signal for the induction of mitochondrial apoptosis. Since calcium spikes are also intimately involved in the physiological control of mitochondrial metabolism, the same signal can be used to enhance cellular ATP supply and to direct the cell to the apoptotic track. Switch from the life program to the death program involves coincident detection of proapoptotic stimuli and calcium signals. Although prolonged exposure to proapoptotic stimuli or sustained increases of $[Ca^{2+}]_c$ would also move the cells to a death pathway (apoptotic or necrotic), detection of coincidence of short-living signals provides an attractive mechanism for the control of the fate of cells in living organisms. In particular, our data show that the impairment of mitochondrial metabolism associated with calcium spike-driven cytochrome *c* release is transient and so mitochondria can provide steps of the apoptotic machinery distal to cytochrome *c* release with ATP. The privileged role of IP$_3$ receptor-mediated calcium spikes in the control of mitochondrial functions is due to precipitous activation of Ca^{2+} release channels and to a local calcium signal transmission between IP$_3$ receptors and mitochondrial Ca^{2+} uptake sites during calcium spiking. In conclusion, our data establish the concept that IP$_3$ receptor-dependent mitochondrial calcium signaling provides an efficient

mechanism for control of the dual role of mitochondria in life and death.

Materials and methods

Cell culture
HepG2 cells (obtained from ATCC) were cultured in Dulbecco's Modified Essential Medium supplemented with 10% fetal bovine serum, 2 mM glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin in humidified air (CO_2 5%) at 37°C. For imaging experiments cells were plated onto poly-D-lysine-treated glass coverslips at a density of 20 000–25 000/cm^2 and were grown for 3–4 days. For cell suspension studies, cells were cultured for 4–6 days in 75 cm^2 flasks.

Fluorescence imaging measurements in intact HepG2 cells
Before use, the cells were preincubated in an extracellular medium (2% BSA/ECM) consisting of 121 mM NaCl, 5 mM NaHCO$_3$, 10 mM Na–HEPES, 4.7 mM KCl, 1.2 mM KH$_2$PO$_4$, 1.2 mM MgSO$_4$, 2 mM CaCl$_2$, 10 mM glucose and 2% bovine serum albumin (BSA), pH 7.4. To monitor [Ca^{2+}]$_c$ cells were loaded with 5 µM fluo3/AM or 5 µM fura2/AM for 20–30 min in the presence of 200 µM sulfinpyrazone at room temperature. Sulfinpyrazone was also present during the imaging measurements to minimize dye loss. To measure $\Delta\Psi_m$ the cells were loaded with 100 nM TMRE for 15 min at room temperature and 20 nM TMRE was present throughout the measurements. In order to visualize apoptotic and necrotic cells labeling of the cells with 10 µg/ml Hoechst 33342 for 5 min was carried out and 1 µg/ml propidium iodide was present during fluorescence imaging. Dye-loaded cells were washed two or three times with 2% BSA/ECM. Imaging measurements were performed in ECM containing 0.25% BSA (0.25% BSA/ECM) at 35°C.

Fluorescence images were collected using a laser scanning confocal microscope (Bio-Rad MRC 600) or using a cooled CCD imaging system as described previously (Csordás et al., 1999). Confocal imaging of [Ca^{2+}]$_c$ and $\Delta\Psi_m$ (using fluo3 and TMRE, Figure 5A) was carried out using 514 nm excitation (Ar laser) and a standard dual emission filter set. In fluorescence imaging experiments, images were acquired using an Olympus IX70 inverted microscope fitted with a 40× (UApo, NA 1.35) oil immersion objective and a cooled CCD camera (PXL, Photometrics) under computer control. The computer also controlled a scanning monochromator (DeltaRam, PTI) to select the excitation wavelength. Excitation at 340 and 380 nm was used for fura2, 340 nm was used for Hoechst 33342, 545 nm was used for TMRE and propidium iodide with multi-wavelength beamsplitter/emission filter combinations that allow simultaneous measurement of fura2 and TMRE fluorescence or Hoechst 33342 and propidium iodide fluorescence (Chroma Technology Corp., Brattleboro, VT).

Experiments were carried out with at least three different cell preparations and 20–80 cells were monitored in each experiment. Traces represent single-cell responses unless indicated otherwise. In order to determine apoptotic cell death at least 400 cells were counted, on 6–10 separate fields in each experiment and propidium iodide-negative cells with condensed, fragmented nuclei were counted as apoptotic.

Measurement of $\Delta\psi_m$ and [Ca^{2+}] in suspension of permeabilized HepG2 cells
Cells harvested using 0.25% trypsin–EDTA were washed in an ice cold Ca^{2+}-free extracellular buffer, containing 120 mM NaCl, 5 mM KCl, 1 mM KH$_2$PO$_4$, 0.2 mM MgCl$_2$, 0.1 mM EGTA, 20 mM HEPES–NaOH pH 7.4. Equal aliquots of cells (protein concentration 7 mg/ml or 10–12 × 10^6 cells) were resuspended and permeabilized with 30–40 µg/ml digitonin in 1.8 ml of an intracellular medium (ICM) composed of 120 mM KCl, 10 mM NaCl, 1 mM KH$_2$PO$_4$, 20 mM HEPES–Tris pH 7.2 supplemented with 1 µg/ml of each of antipain, leupeptin and pepstatin. ICM was passed through a Chelex column prior to addition of protease inhibitors to lower the ambient [Ca^{2+}]. All the measurements were carried out in the presence of 2 mM MgATP and ATP regenerating system composed of 5 mM phosphocreatine, 5 U/ml creatine kinase. Mitochondria were energized with 2 mM succinate (complex II substrate, experiments shown in Figures 1A–C and 2) or 1 mM/5 mM malate/glutamate (complex I substrate, experiments shown in Figures 1D, 3 and 4). In the experiments shown in Figures 1D, 3 and 4, 2.5 µg/ml oligomycin was also added to prevent reversed function of the mitochondrial H$^+$ ATPase. The final [Ca^{2+}] of the ICM was 250–350 nM. C2 and staurosporine were dissolved in dimethylsulfoxide

(DMSO) added to the incubations in small volumes of DMSO (<0.2%) and the same amount of DMSO was added to the control samples.

In order to carry out simultaneous measurements of [Ca^{2+}]$_c$ and $\Delta\Psi_m$, the permeabilized cells were supplemented with 0.5 µM fura2FF/FA and 800 nM JC1. The relatively low affinity of fura2FF for Ca^{2+} (K_d ~35 µM for fura2FF) was favorable for avoiding saturation of the dye during large increases of [Ca^{2+}]$_c$. Fluorescence was monitored in a multi-wavelength-excitation dual wavelength-emission fluorimeter (Delta RAM, PTI) using 340 and 380 nm excitation and 535 nm emission for fura2FF whereas 490 nm excitation/535 nm emission and 570 nm excitation/595 nm emission were used for JC1. Cytosolic [Ca^{2+}] is shown as the excitation ratio (340 nm/380 nm) of fura2FF/FA fluorescence whereas $\Delta\Psi_m$ is shown as the ratio of the fluorescence of J-aggregate (ex: 570 nm, em: 595 nm) and monomer (ex: 490 nm, em: 535 nm) forms of JC1 (Reers et al., 1991). Cytosolic [Ca^{2+}] measurements were also carried out using fura/FA (0.5 µM, ex: 340 and 380 nm, em: 500 nm). Experiments were carried out at 35°C and with simultaneous stirring.

In order to prepare cytosol fractions and determine caspase activity, at the end of the fluorescent measurement of $\Delta\Psi_m$ and [Ca^{2+}]$_c$, 1 mM EGTA was added to the samples, which were then centrifuged (14 000 g for 3 min and then the supernatant for 10 min). To measure cytochrome c release the fluorescent measurements of $\Delta\Psi_m$ were carried out as described above, except that the cells were washed after permeabilization to remove cytosolic cytochrome c and the extracts for Western blot analysis were prepared as described above. Protein concentration in the extracts was determined using the Bio-Rad protein assay with BSA as standard. Supernatants prepared for measurements of cytochrome c and caspase activity were kept at –80°C until use.

Calibration of fura2 signal was carried out at the end of each measurement adding 1.5 mM CaCl$_2$ and subsequently 10 mM EGTA–Tris pH 8.5. [Ca^{2+}]$_c$ was calculated by using a K_d of 224 nM. Increases of cytosolic [Ca^{2+}] obtained by addition of CaCl$_2$ were calculated using constants obtained from Bers et al. (1994).

Western blot analysis
The proteins (25 µg) were separated by SDS–PAGE (15% gel) under reducing conditions followed by transfer to polyvinylidene difluoride membrane (0.45 µm) (Millipore), at 150 mA for 210 min in transfer buffer (20 mM Tris–base, 150 mM glycine, 20% methanol). Non-specific binding was blocked by overnight incubation in blocking buffer (Pierce) at room temperature. This was followed by incubation with a monoclonal antibody to horse cytochrome c peptide (7H8.2C12, Research Diagnostics) at a concentration of 1 µg/ml for 7 h at 4°C. After washing the unbound antibody in phosphate-buffered saline (PBS pH 7.2) containing 0.1% Triton X-100, the blots were incubated with goat anti-mouse IgG conjugated to horseradish peroxidase (Pierce) at a dilution of 1:80 000 for 45 min at room temperature. Following washes with PBS containing 0.1% Triton X-100, the blots were developed using the enhanced chemiluminescence kit (Pierce).

Caspase assay
DEVD–AMC cleavage was measured essentially as described by Nicholson et al. (1995) and Hampton et al. (1997). Briefly, 180 µl of extract (protein concentration: 0.8–1.2 mg/ml) was preincubated for 10 min at 35°C and subsequently added to 1420 µl of assay buffer (10% w/v sucrose, 0.1% w/v CHAPS, 5 mM dithiothreitol, 100 mM HEPES–Tris pH 7.2). Assay mixture was incubated in the presence of 12.5 µM DEVD–AMC for 30 min at 35°C and the fluorescence of free amino-methylcoumarin (ex: 350 nm, em: 460 nm) was monitored using the fluorimeter described above.

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