BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS **253,** 185–191 (1998)
ARTICLE NO. RC989742

Potentiation of Apoptosis by Mitochondria in a Cell-Free System

Ph. Juin,[1] K. Tremblais, M. T. LeCabellec, M. Grégoire, K. Meflah, and F. M. Vallette[2]
Unité INSERM 419, IFR 26. 9 Quai Moncousu, 44035 Nantes Cedex 01, France

Received October 22, 1998

Using a cell-free system, we show that rat liver mitochondria, but not mitochondrial extracts, potentiated apoptosis triggered by cytosols derived from apoptotic cells. Apoptosis potentiated by mitochondria appeared to be inhibited by caspase 3 but not by caspase 1 inhibitors. A cytosolic caspase-3-like activity was increased by the addition of mitochondria to apoptotic cytosols; the latter activation was inhibited by the addition of bcl-2. Chelation of calcium by EGTA significantly and specifically inhibited the apoptosis potentiated by mitochondria as well as the increase of caspase-3-like activity. The incubation of mitochondria with apoptotic cytosols led to the release of cytochrome c, this latter phenomenon being inhibited by EGTA. Calcium or cytochrome c and dATP, however, did not reproduce the mitochondrial potentiation in the absence of the organelle. Thus, mitochondria can initiate and potentiate apoptosis through similar but not identical mechanisms. © 1998 Academic Press

Key Words: **apoptosis; calcium; caspases; cytochrome c; mitochondria.**

Apoptosis or programmed cell death is the physiological process which regulates both quantitatively and qualitatively cellular populations in higher eukaryotes (1). Apoptosis is characterized by typical morphological and biochemical changes, including blebbing of plasma and nuclear membranes, chromatin condensation, intracellular acidification, activation of proteases, and inter- and intra-nucleosomal degradation of DNA (2). These changes result in cell shrinkage and thereafter to the formation of apoptotic bodies. These particular cellular structures are eliminated without provoking physiological responses such as inflammation as it is usually observed in necrosis, the other form of cell death (2). Despite the importance of apoptosis in both normal and abnormal multicellular organism development, little is known about the molecular events involved in the initiation and/or the control of this process (1, 3). Over the past years, several molecular partners implicated in the so-called effector phase of apoptosis have been identified. They are proteases known as caspases and proto-oncogenes, members of the Bcl-2 family (4, 5). Caspases are cysteine proteases with a high hydrolyzing specificity at specific aspartate residues (6, 7) and are, probably together with other proteases, the main executors of the death programme (7). Caspases are synthezised as pro-enzymes that can be activated by auto-cleavage or by other caspases in an apparently ordered fashion (7). While some of these caspases (e.g. caspase 1) appear to be mainly activators of procytokine, other caspases called initiators (e.g. caspase 8) are responsible for the activation of "executioners" (e.g. caspase 3 or caspase 6) which are directly involved in the apopototic changes by the cleavages of key substrates (7). Bcl2 related proteins are either positive (bax, bak . . .) or negative (bcl-2, bcl-xl . . .) regulators of apoptosis (8-10). This property seems to be associated with the ability of members of this family to homo- or hetero-dimerize with themselves and/or to form ionic channels (8, 11). Several lines of evidence suggest that Bcl-2 family members regulate apoptosis upstream the "executive" phase which includes the caspases (7, 10). However, the existence, as well as the nature and the role in cell death of the interaction between caspases and bcl-2 related proteins has still to be firmly established (12).

The active participation of organelles in apoptosis has been suggested because the product of the proto-oncogene Bcl-2 is essentially associated to the outer membrane of mitochondria (5). In addition, it has been shown that the onset of apoptosis appears to be followed by an early dysregulation of the mitochondrial inner membrane potential ($\Delta\Psi$) both *in vivo* and *in*

[1] Present address: BCN Laboratory, Imperial Cancer Research Fund, P.O. Box 123, Lincoln's Inn Fields, London WC2A 3PX, UK.

[2] To whom all correspondence should be addressed at: Unité INSERM 419, 9 Quai Moncousu, 44035 Nantes Cedex 01, France. Fax: 33-2-40084082. E-mail: fval@nantes.inserm.fr.

Abbreviations: ACE, apoptotic cytosolic extract; CCE, control cytosolic extract; GSH, reduced form of gluthation; NaB, sodium butyrate; PMSF, phenylmethylsulfonyl fluoride; EGTA, ethylene glycol-bis-(β-aminoethyl ether); PTP, permeability transition pore.

0006-291X/98 $25.00

vitro (5). It has recently been postulated that a crucial and important step in the execution of apoptosis could be the Bcl-2 regulated opening of the permeability transition pore (PTP) (5). The PTP is an elusive structure that could be constituted by the coupling of a channel of the outer membrane, the voltage dependent anionic channel (VDAC) with the megachannel of the inner membrane (13). This pore has been associated with the exchange of small molecules such as Ca^{++}, reactive oxygen species (ROS), or ATP, between mitochondria and the cytosol (13). Several works have demonstrated that cytoplasmic extracts from Xenopus oocytes induced morphological and biochemical apoptotic features in cell free systems only in the presence of mitochondria enriched fractions and that the mitochondrial release of cytochrome c and/or of an as yet uncharacterized intermembrane space protease might be important steps during the final phase of apoptosis (14-18).

MATERIALS AND METHODS

The chemicals used in this study were similar to those used in Juin et al. (14).

Purification of mitochondria and nuclei. Purified mitochondria were prepared from normal rat liver as described in (15) except that a percoll gradient was added in the procedures. Functionality and integrity of these mitochondria were routinely checked by their ability to import nuclear encoded mitochondria proteins as described in (15). Rat liver nuclei were prepared according to (16).

Induction of apoptosis. Cells were grown to confluence then apoptosis was induced either by a sodium butyrate (NaB) (5 mM, 72h) or by a staurosporine (stau) (1μM, 24h) treatment. After either treatment, most of the cells detached from the culture dishes and displayed characteristic morphological apoptotic features such as chromatin condensation of nuclei and blebbing of plasma membrane (17). The apoptotic bodies were purified by differential centrifugation as previously described in (17). The cytosol extracts (CEs) from both control (CCE) and apoptotic (ACE) and the cell free reaction were obtained according to the procedure described in Juin et al (14). At the end of the incubation, 9 μl of the mix was removed and incubated with 1μl of a 25 μM Hoechst 33342 solution for 10 min at room temperature before examination by fluorescence microscopy (Olympus BX 60). Nuclei were scored as apoptotic if marginalization and condensation of the chromatin was observed as described in (14). For the quantification of apoptotic nuclei, 50 nuclei were evaluated in different fields in a minimum of 3 different independent experiments.

Measurement of protease activities. The fluorogenic caspase (Ac-DEVD-AMC, Ac-VEID-AMC, ac-IETD-AMC) or chymotrypsin substrates bz-VGR-AMC (Bachem, France) were incubated at a concentration of 10 μM in the presence of the different reaction mixtures for 3 or 4 h at 37°C as described in (14).

Association of in vitro translated bcl-2 with mitochondria. Bcl-2 was transcribed/translated with the TNT system (Promega, France) and associated with mitochondria as previously described (15). After the completion of the association, mitochondria were pelleted and resuspended in isotonic ACEs.

Electrophoresis and Western blotting. Electrophoresis and Western blot analysis were performed using standard methods except that antibodies bound to Immobilon-P (Millipore, France) were detected by enhanced chemiluminescence (Amersham, France).

RESULTS

Potentiation of Apoptosis by Normal Mitochondria in a Cell-Free System

2×10^5 rat liver nuclei and 10 to 100 μg of CEs were incubated with or without 50 μg mitochondria for 2h at 37°C. Few aberrant morphological changes were observed in nuclei incubated in the presence of A15A5 CCE (Figure 1A). The addition of mitochondria or mitochondrial extracts with 100μg CCE did not produce any signs of apoptosis (data not shown). On the other hand, typical nuclear apoptotic events such as collapsing of the chromatin against the nuclear periphery and its condensation were observed only when nuclei were incubated with 25 μg ACE derived from NaB treated A15A5 to reach a maximum effect (i.e 80 % apoptotic nuclei) with 100 μg ACE (Figure 1B and C). When 50 μg of mitochondria was added to 25 μg ACE, the nuclei displayed intense chromatin condensation together with the shrinking of the nuclei (Figure 1D) and internucleosomal degradation of the DNA (Figure 1E).

Effect of Caspase Inhibitors on the Mitochondrial Potentiation of Apoptosis

We tested the influence of several protease inhibitors such as trypsin or chymotrypsin inhibitors (TLCK and TPCK at 100 μM), calpain inhibitors I and II (10 to 100 μM), E 64 (100 μM) or 100 μM H-APF-OH, an inhibitor of the Nuclear Scaffold Protease (18) on the cell free apoptosis in the presence or not of mitochondria. We found that these inhibitors had different effects on the cell free apoptosis but that these effects were always independent of the presence or the absence of mitochondria (Juin and Vallette, unpublished data).

As caspases are required for apoptosis both *in vivo* and *in vitro,* we have investigated their role in mammalian cell free apoptosis. We used YVAD-CHO, an inhibitor of caspase 1, and DEVD-CHO, an inhibitor of caspase 3-like proteases, in the reaction mixtures. We used either 100 μg ACE/CCE or 25 μg ACE plus 50 μg purified rat liver mitochondria in order to obtain a comparable range of induced morphological apoptosis. As illustrated in Figure 2, the percentage of apoptotic nuclei was decreased dramatically upon the addition of DEVD-CHO both in the presence of 100 μg ACE and 25 μg ACE plus 50 μg mitochondria. However, the addition of YVAD-CHO (100 μM) was effective only when the cell-free assay was performed in the absence of mitochondria. Under these conditions, both in absence and in the presence of mitochondria, the addition of the free radical scavenger GSH prevented the onset of nuclear apoptosis (Figure 2).

Caspase-3 like activities in cytosols were measured in ACE or CCE in the presence or in the absence of mitochondria using the chromogenic peptide ac-DEVD-AMC (see Materials and Methods). Mitochondria

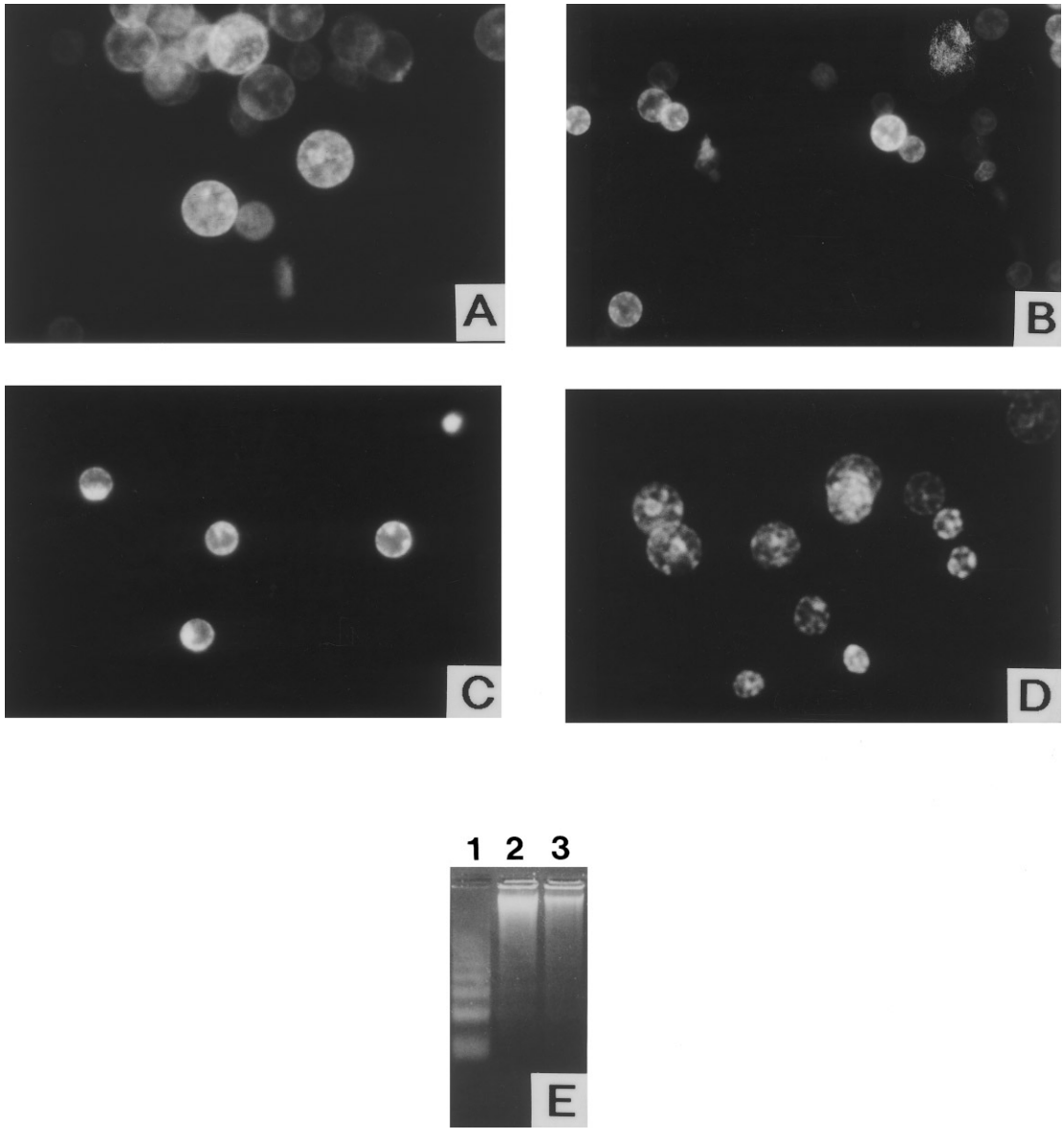

FIG. 1. Apoptotic changes in rat liver nuclei exposed to various concentrations of cytosolic extracts in the presence or in the absence of mitochondria: 2×10^5 nuclei were incubated in the cell free buffer for 2h at 37°C with (A) 100 μg of CCE (magnification × 500), (B) 25 μg ACE (magnification × 350) (C) 100 μg ACE (magnification × 350), (D) 25 μg ACE plus 50 μg mitochondria (magnification × 500), (E) DNA prepared from D (lane 1), C (lane 2), and A (lane 3), were loaded on a 1.5 % agarose gel to analyze internucleosomal cleavages.

and/or nuclei were pelleted by centrifugation at 9,000g for 10 minutes at 4°C and the DEVDase activity was measured both in the supernatants and in the pellets. We found no DEVDase activity in mitochondrial and/or nuclei pellets (data not shown). The addition of mitochondria to 25 μg ACE provoked a 3-fold increase in caspase 3-like activity in the supernatant resulting in an activity comparable to that found in 100 μg ACE (Figure 3A). The DEVDase activity in the presence of mitochondria was completely inhibited by 100μM DEVD-CHO but was unaffected by 100 μM YVAD-CHO. Consistent with DNA and morphological analyses, in the absence of mitochondria, both YVAD-CHO and DEVD-CHO blocked the DEVDase activity (Figure 3A). It should be noted that the addition of GSH had no effect on the activation of the caspase-3 like activity (data not shown). A chymotrypsin-like activity (i.e. cleavage of ac-VGR-AMC) was not significantly affected by the addition of mitochondria under similar conditions (data not shown), ruling out a general increase of proteolytic activity induced by the addition of the organelle. To examine further the nature of the DEVDase activity, we measured the cleavage of ac-VEID-AMC, a substrate preferentially cleaved by

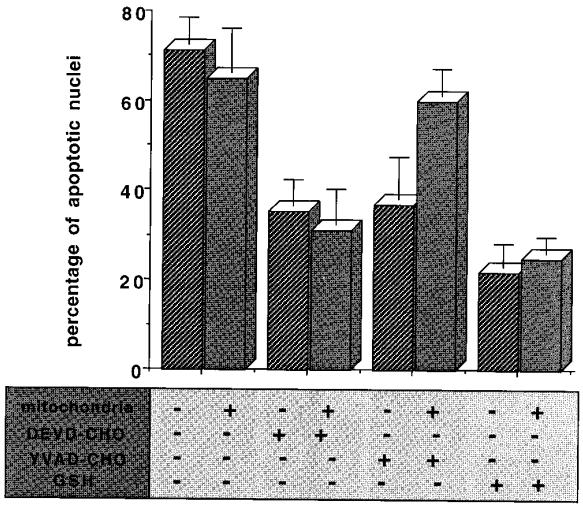

FIG. 2. Proportion of apoptotic nuclei observed in the presence of inhibitors of caspase-3 or caspase-1. Rat liver nuclei were incubated in the cell free buffer for 2h at 37°C, with CCE and ACE supplemented or not with mitochondria with 100μM caspase 1 inhibitor (YVAD-CHO), caspase 3 inhibitor (DEVD-CHO) or GSH. Apoptotic nuclei were scored as described in Materials and Methods; a minimum of 50 nuclei from different fields were counted from 3 independent experiments (means ± SD). In the presence of 50 μg mitochondria 25 μg ACE are added and in the absence of mitochondria, 100 μg ACE are added to induce similar proportion of apoptotic nuclei.

caspase-6, or ac-IETD-AMC, a caspase-8 subtrate (19) in CCE or ACE suplemented or not with 50 μg of mitochondria. As shown in Figure 3B no or little caspase activation was observed in CCEs incubated with or without mitochondria. In ACE both ac-VEID-AMC and ac-DEVD-AMC were equally cleaved suggesting that caspase-3 and caspase-6 were both active in these fractions. On the other hand, in the presence of mitochondria, the DEVDase activity was increased considerably more than the VEIDase activity suggesting a specific increase of a caspase-3 like activity under these conditions (Figure 3B). It should be noted that no or little caspase-1 activity was found in ACE in the presence or not of mitochondria.

Effect of bcl-2 on the Mitochondrial Potentiation of Apoptosis

We have examined the effect of bcl-2 on the mitochondrial activation of caspase-3 like activity. No or little bcl-2 related proteins were found associated with rat liver mitochondria but *in vitro* translated bcl-2 readily bound to these mitochondria under our experimental conditions (Tremblais *et al*, in preparation). As illustrated in Figure 4, the addition of bcl-2 (approximately 5 pmoles) had no effect on the DEVD cleaving activity present in ACEs derived either from NaB or stau treated A15A5 cells. On the other hand, with both ACEs, no activation of DEVDase was observed with bcl-2 associated with mitochondrial membranes. This effect was found with both stau and NaB ACEs. The latter result suggests that the mitochondrial potentiation observed under our experimental conditions was inhibited by bcl-2.

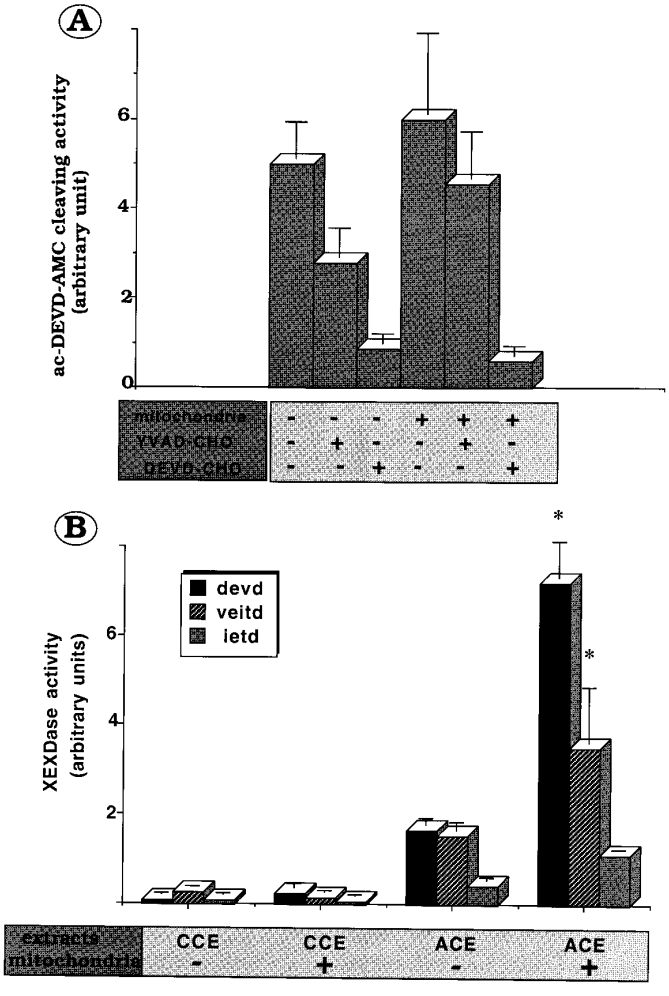

FIG. 3. Mitochondrial induction of a caspase 3 activity in ACE. (A) Inhibition of ac-DEVD-AMC cleaving activity in ACE by YVAD-CHO or DEVD-CHO in the presence or in the absence of mitochondria. The DEVD cleaving activity was measured as described in Materials and Methods. ACEs exhibiting similar DEVDase activity (i.e 100 μg ACE or 25 μg ACE + 50 μg mitochondria) were incubated for 2 h in the presence or in absence of mitochondria with or without 100μM YVAD-CHO or DEVD-CHO. Results shown were obtained from data obtained from 3 different experiments (means ± SD). (B) Caspases activities in control A5A15 cells (CCE) or in NaB treated cells (ACE) in this absence or in the presence of 50 μg mitochondria. The caspase activities were measured as described in Materials and Methods, using ac-DEVD-AMC as a substrate for caspase 3 (■), ac-VEID-AMC as a substrate for caspase 6 (▨) and ac-IETD-AMC as substrate for caspase 8 (▤). Results shown were obtained from data obtained from 3 different experiments (means ± SD). Difference between the level of DEVDase and VEIDase activities (*) was found significative in ACE + mitochondria, using an unpaired t-test ($p = 0.0122$), and not significative in ACE in absence of mitochondria.

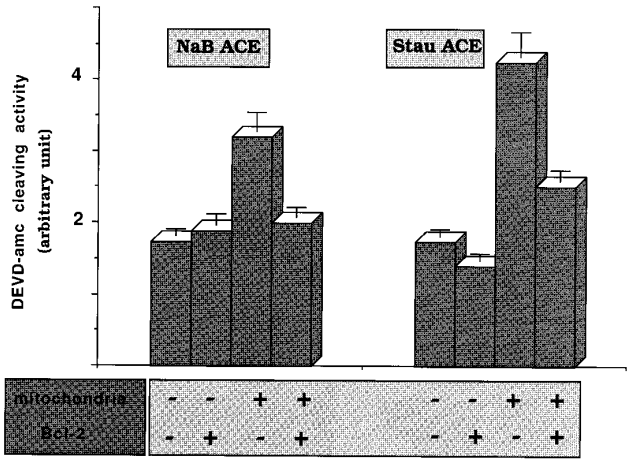

FIG. 4. Effect of bcl-2 on the mitochondrial potentiation of the ac-DEVD-amc cleaving activity. In vitro translated human bcl-2 or yeast cytochrome b2 (a mock protein) were added to either 100 μg ACEs (from NaB or stau treated rat glioma A15A5) or 25 μg ACEs plus 50 μg rat mitochondria and incubated for 2h at 30°C. The DEVD cleaving activity was measured as described in Materials and Methods. Results shown were obtained from data obtained with the 2 different treatments from a maximum of 3 different experiments (means ± SD).

Effect of a Calcium Chelator on the Mitochondrial Potentiated Apoptosis and on Caspase 3 Activity

As illustrated in Figure 5A, the addition of EGTA (10 mM) to ACE derived from NaB treated A15A5 (25 μg and 100μg) had no effect on the proportion of apoptotic nuclei. When rat liver nuclei were incubated with mitochondria (50 μg) plus ACE (25 μg), EGTA decreased by 50 % the amount of apoptotic nuclei (Figure 5A). A similar result was obtained with ACE derived from stau treated A15A5 (data not shown). In ACE, the DEVDase activity was not inhibited by the addition of EGTA (10 mM) (Figure 5B). In the presence of mitochondria, the caspase 3 like activity was partially inhibited by the addition of the calcium chelator at the same concentration (Figure 5B). Conversely, the addition of calcium (from 100 μM to 500 μM) to ACE in the absence of mitochondria had no effect on DEVDase activity (14). Thus, an increase in cytosolic calcium due to its release from mitochondria cannot account for the increase of the DEVDase activity observed in the presence of the organelle. The addition of ruthenium red to mitochondria had no effect on the caspase activity implying that the uptake of calcium by the mitochondria was not implicated in this mitochondrial potentiation (data not shown).

Effect of Calcium and of Cyclosporine A on the Release of Cytochrome c

Cytochrome c has been shown to be released from mitochondria in the cytosols of apoptotic cells at a very early stage and to induce an increase in caspase 3-like activity (20-24). Percoll-purified rat liver mitochondria were incubated with CCE (25 μg) or ACE (25 μg) as described above. At the end of the incubation, the mitochondria were collected by centrifugation (9,000g at 4°C for 10 minutes) and the presence of cytochrome c and the β subunit of F1-ATPase analyzed. As shown in Figure 6A, the incubation of mitochondria with CCE did not provoke any loss of cytochrome c or of the β subunit of F1-ATPase (Figure 6A). However, incubation with ACE resulted in a rapid release of cytochrome c into the supernatant (data not shown). This loss was

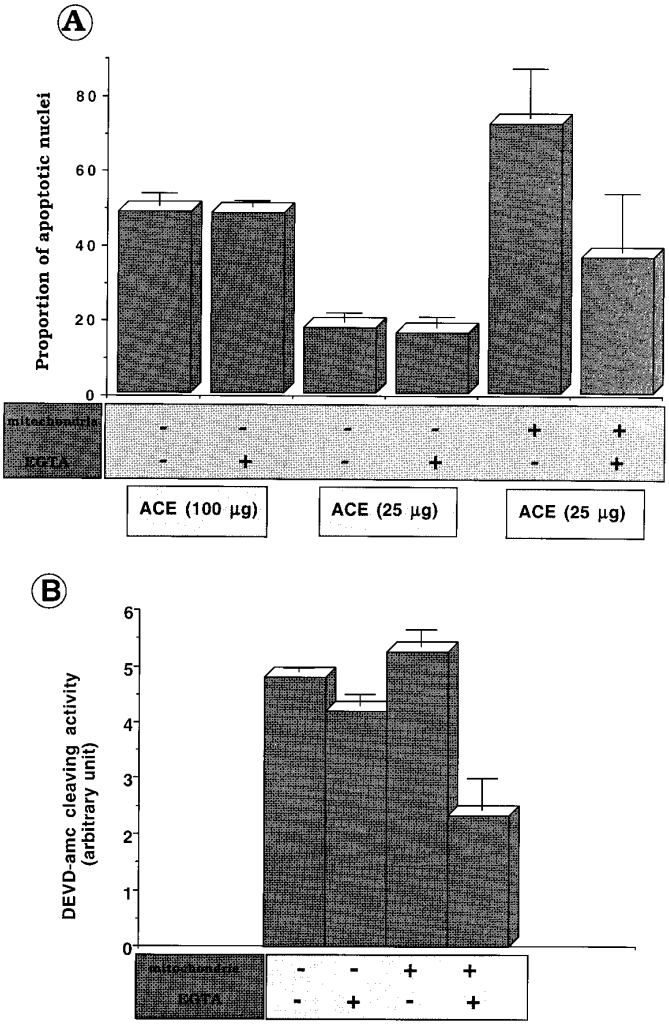

FIG. 5. Differential effect of EGTA on the cell-free apoptosis in the presence or in the absence of mitochondria. Rat liver nuclei were incubated as described in Figure 1 in the cell-free buffer for 2h at 37°C, with ACEs supplemented or not with mitochondria. At the end of the incubation, the incubation mixture was centrifuged 9,000 g for 10 mins at 4°C. Apotic nuclei were counted in the pellets and the ac-DEVD-amc cleaving activity measured in the supernatant. Results shown are calculated from data obtained from 5 different experiments (means ± SD). (A) Influence of EGTA (10mM) on the proportion of apoptotic nuclei observed in the presence or the absence of mitochondria (50 μg) and with 25 or 100 μg ACE. (B) Effect of EGTA on the ac-DEVD-amc cleaving activity under the same conditions.

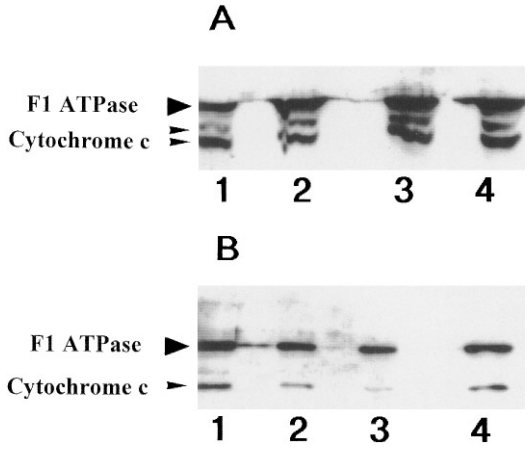

FIG. 6. Western blot analysis of the release of cytochrome c in the presence of EGTA or cyclosporine A. Rat liver mitochondria were incubated with CCE and ACE at 37°C for 2h. At the end of the incubation, the incubation mixture was centrifuged at 9,000 g for 10 minutes at 4°C. The pellets was resuspended in denaturing loading buffer and analyzed on a 15% SDS-PAGE. Western blot analyses were performed using an antibody raised against pigeon cytochrome c or the β subunit of the rat F1-ATPase. (A) Lane **1:** 50 μg mitochondria; Lane **2:** 25 μg CCE incubated with 50 μg mitochondria plus Cyclosporine A. lane **3:** 25 μg CCE incubated with 50 μg mitochondria; lane **4:** 25 μg CCE incubated with 50 μg mitochondria plus EGTA Note the presence of dimers of cytochrome c. (B) Lane **1:** 50 μg mitochondria; Lane **2:** 25 μg ACE incubated with 50 μg mitochondria plus Cyclosporine A. lane **3:** 25 μg ACE incubated with 50 μg mitochondria; lane **4:** 25 μg ACE incubated with 50 μg mitochondria plus EGTA. Data shown are representative of 3 independent experiments.

a specific response to ACE as the level of the β subunit of F1ATPase remained unchanged (Figure 6B). We also studied the effect of the calcium chelator EGTA (10mM) on the release of cytochrome c in this cell free system. The addition of EGTA to the reaction mixture partially inhibited the release of cytochrome c (Figure 6B). Furthermore, we have tested the involvement of the PTP on this release by studying the effect of cyclosporine A (1 μM), an inhibitor of this pore, in the cell-free system. An similar effect to that of EGTA, was obtained by the addition of cyclosporine A to ACE although the release of cytochrome c was not completely abolished (Figure 6B).

DISCUSSION

Apoptosis Is Potentiated by Normal Mitochondria

The addition of mitochondria alone or in the presence of control cytosols did not induce apoptotic changes in liver nuclei in the cell free assay (Figure 1A). On the other hand, when mitochondria were added with low concentrations of apoptotic cytosols, more pronounced apoptotic features were observed (Figure 1B, C). We have observed this potentiation using apoptotic cytosols derived from cells of different origins (the rat glioblastoma A15A5, the rat colorectal carcinoma cell line ProB and the human leukemia cell line HL60). With the A15A5 and ProB cells, we also used different treatments (stau and NaB) which both produced similar results although they act differently *in vitro* on the mitochondrial metabolism (25, 26). The apoptosis obtained in the presence of mitochondria and ACEs suggest than the organelle acts as a catalyst of the reaction on cytosolic factor(s) present in the apoptotic cells.

Mitochondria Potentiated Apoptosis Is Linked to an Increase of Caspase-3 Activity

Proteolysis is a central element in the executioner phase of apoptosis (27). We used inhibitors of caspase-1 and caspase-3 like proteases as it is generally assumed that the amount of caspase activities in cytosols is related to their apoptogenic effects on nuclei (7). We found that apoptosis was inhibited by the tetrapeptide inhibitor DEVD-CHO both in the presence and in the absence of mitochondria (Figure 2). On the other hand, apoptosis was delayed by an inhibitor of caspase-1 (YVAD-CHO) only in the absence of mitochondria. The latter results suggested that mitochondrial induced apoptosis is dependent upon one step of the apoptotic programme, namely a DEVD cleaving activity. This contention was strengthened by the fact that a caspase-3 like activity increased in the apoptotic cytosols supplemented with mitochondria (Figure 3). This activity is likely to be due to caspase 3 itself as the ratio between DEVDase and VEIDase activities is similar to that observed with pure caspase 3 (data not shown). Our data show that this potentiation is not sensitive to 100 μM ac-YVAD-CHO, a caspase-1 inhibitor. Kluck et al. (21) have recently reported a similar phenomenum with Xenopus cell extracts, in which 100 μM YVAD had only a little effect on cell free apoptosis. Interestingly, the latter cell-free system requires a mitochondrial enriched fraction (16). As no or little caspase-1 like activity was found in ACEs in the presence or in the absence of mitochondria, a likely explanation for this lack of inhibition in the presence of mitochondria is that the caspase 3 activity induced by the addition of mitochondria is not as sensitive to ac-YVAD-CHO as that present in ACEs in absence of the organelle. In agreement with this hypothesis, Martins et al. (28) recently shown that the DEVDase activity contained in apoptotic HL60 cells was less sensitive to ac-YVAD-CHO than the VEIDase activity.

Mitochondria could provide the DEVDase activity by releasing intermembrane space proteases. However, mitochondrial extracts obtained either by an osmotic shock, by several freezing/thawing cylcles or by sonication, appeared to be devoided of a DEVD cleaving inducing activity (data not shown). Our results suggest that incubation of cytosols with intact mitochondria is more likely to result into an activation of a caspase-3 like cytosolic fraction.

Mitochondrial Potentiated Apoptosis Is Partially Inhibited by the Addition of EGTA

This calcium chelator blocked the mitochondrial induced caspase 3 activation and the release of cytochrome c from the mitochondria.

Nuclear apoptotic morphological events are affected by several classical elements such as GSH or the addition of bcl-2. The nuclear apoptosis and the DEVDase activation are affected by EGTA only in the presence of mitochondria. This latter result suggests that calcium ions are involved in mitochondrial apoptosis upstream of the increase in the DEVD-cleaving activity. As the addition of ruthenium red at a concentration of 10 μM does not affect this phenomenum (data not shown), the recapture of cytosolic Ca^{++} ions by the mitochondria can be ruled out. The addition of Ca^{++} up to 1 mM in ACE did not increase any morphological or biochemical apoptotic features in the cell free system (21). This result suggests that the mere release of calcium ions by mitochondria in the cytosol is not responsible of the increase of the DEVDase activity. Several reports have shown that mitochondrial cytochrome c was accumulates in the cytosol of apoptotic cells where it activates a caspase-3 like activity. The release of cytochrome c from mitochondria occurs through an unknown mechanism (20, 21, 24). We have studied the release of cytochrome c by analyzing the cytochrome c remaining in mitochondrial pellets after incubation with ACEs or CCEs as these apoptotic cytosols used in this study already exhibited high levels of endogenous cytochrome c. As illustrated in Figure 6, in our cell free, mitochondria released cytochrome c only upon incubation with ACEs. The addition of EGTA partially inhibited this release which suggests an involvement of the calcium at this important step of the apoptotic programme. As calcium ions are potent regulators of the Permeability Transition pore (13), we used an other inhibitor of the PTP, the cyclopsorin A and found that it inhibited also the cytochrome c release (Figure 6). The PTP appears to control the release of cytochrome c in our system and the Ca^{++} could be a key element in cytochrome c release by regulating the opening of the PTP during apoptosis. In our experimental conditions, no increase in apoptotic properties of cytosols are observed upon supplementation with bovine cytochrome c and dATP (14). This suggests that additional factors either from mitochondrial or cytosolic origins are involved in the activation process observed in this study.

It remains thus to be established whether the mitochondrial potentiation of ACE is due to the induction of a pathway independent from that triggering apoptosis in CCE (20-24).

ACKNOWLEDGMENTS

We thank Pr Joel Lunardi (URA1130 CNRS) for the gift of antibodies against the β subunit of the rat F1-ATPase. The plasmid encoding for bcl-2 was a gift from Dr G. Evan (ICRF, London). This work was supported by grants from the Institut National de la Santé et de la Recherche Médicale (INSERM U419), the Association pour la Recherche contre le Cancer (ARC), The Ligue Nationale contre le Cancer (LNC), and the Fondation pour la Recherche Medicale (FRM). P.J. and K.T. are recipients of fellowships from ARC.

REFERENCES

1. Jacobson, M. D., Weil, M., and Raff, M. C. (1997) *Cell* **88,** 347–354.
2. Kerr, J., Wyllie, A., and Currie, A. (1972) *Br. J. of Cancer* **26,** 239–257.
3. Rudin, C. M., and Thompson, C. B. (1997) *Annu. Rev. Med.* **48,** 267–281.
4. Reed, J. C. (1994) *J. Cell Biol.* **124,** 1–6.
5. Kroemer, G. (1997) *Cell Death Diff.* **4,** 443–456.
6. Martin, S. J., and Green, D. R. (1995) *Cell* **82,** 349–352.
7. Salvesen, G. S., and Dixit, V. M. (1997) *Cell* **91,** 443–446.
8. Reed, J. C. (1997) *Nature* **387,** 773–776.
9. Vaux, D. L., and Stasser, A. (1996) *Proc. Natl. Acad. Sci. (USA)* **93,** 2239–2244.
10. Kroemer, G. (1997) *Nature Med.* **3,** 614–620.
11. Schendel, S. L., Montal, M., and Reed, J. C. (1998) *Cell Death Diff.* **5,** 372–380.
12. Goldstein, P. (1997) *Science* **275,** 1081–1083.
13. Zoratti, M., and Szabo, I. (1995) *Biochem. Biophys. Acta* **1241,** 139–176.
14. Juin, P., Pelletier, M., Oliver, L., Tremblais, K., Grégoire, M., Meflah, K., and Vallette, F. M. (1998) *J. of Biol. Chem.* **273,** 17559–17564.
15. Vallette, F. M., Juin, P., Pelleschi, M., and Henry, J. P. (1994) *J. of Biol. Chem.* **269,** 13367–13374.
16. Newmeyer, D. D., Farshon, D. M., and Reed, J. C. (1994) *Cell* **79,** 353–364.
17. Boisteau, O., Gautier, F., Cordel, S., Henry, F., Harb, J., Douillard, J. Y., Vallette, F. M., Meflah, K., and Grégoire, M. (1997) *Apoptosis* **2,** 403–412.
18. McConkey, D. J. (1996) *J. of Biol. Chem.* **271,** 22398–22406.
19. Talanian, R. V., Quinian, C., Trautz, S., Hackett, M. C., Mankovich, J. A., Banach, D., Ghayur, K. D., and Wond, W. W. (1997) *J. of Biol. Chem.* **269,** 13367–13374.
20. Kluck, R. M., Bossy-Wetzel, E., Green, D. R., and Newmeyer, D. D. (1997) *Science* **275,** 1132–1136.
21. Kluck, R. M., Martin, S. J., Hoffman, B. M., Zhou, J. S., Green, D. R., and Newmeyer, D. D. (1997) *EMBO J.* **16,** 4639–4649.
22. Liu, X., Kim Naekyung, C., Yang, J., Jemmerson, R., and Wang, X. (1996) *Cell* **86,** 147–157.
23. Reed, J. C. (1997) *Cell* **91,** 559–562.
24. Yang, J., Liu, X., Bhalla, K., Kim, C. N., Ibrado, A. M., Cai, J., Peng, T.-I., Jones, D. P., and Wang, X. (1997) *Science* **275,** 1129–1132.
25. Jacobson, M., Weil, M., and Raff, M. (1996) *J. Cell Biol.* **133,** 1041–1051.
26. Heerdt, B. G., and Augnelicht, L. H. (1991) *J. of Biol. Chem.* **266,** 19120–19126.
27. Sukharev, S. A., Pleshakova, O. V., and Sadovnikov, V. B. (1997) *Cell Death Diff.* **4,** 457–462.
28. Martins, L. M., Kotte, T., Mesner, P. W., Basi, G. S., Sinha, S., Frigon, N., Tatr, E., Tung, J. S., Bryant, K., Takahashi, A., Svingen, P. A., Madden, B. J., McCormick, D. J., Earnshaw, W. C., and Kaufmann, S. H. (1997) *J. Biol. Chem.* **272,** 7421–7430.