Mitochondrial iPLA2 Activity Modulates the Release of Cytochrome c from Mitochondria and Influences the Permeability Transition*

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Martha E. Gadd1, Kimberly M. Broekemeier1, Elliott D. Crouser1, Jitendra Kumar1,**, Gustav Graff1, and Douglas R. Pfeiffer1,2

From the **Department of Molecular and Cellular Biochemistry, the 1Department of Internal Medicine, and the 1Institute for Mitochondrial Biology, Ohio State University, Columbus, Ohio 43210, the 1Department of Chemistry, Ohio Northern University, Ada, Ohio 45801, and 1Alcon Laboratories Inc., Fort Worth, Texas 76134

The mitochondrial Ca2+-independent phospholipase A2 is activated during energy-dependent Ca2+ accumulation under conditions where there is a sustained depression of the membrane potential. This activation is not dependent on induction of the mitochondrial permeability transition. Bromoeno lactone, which inhibits the phospholipase, is effective as an inhibitor of the transition, and this action can be overcome by low levels of exogenous free fatty acids. Apparently, activation of the Ca2+-independent phospholipase is a factor in the mechanisms by which depolarization and Ca2+ accumulation promote opening of the permeability transition pore. Sustained activity of the Ca2+-independent phospholipase A2 promotes rupture of the outer mitochondrial membrane and spontaneous release of cytochrome c on a time scale similar to that of apoptosis occurring in cells. However, more swelling of the matrix space must occur to provoke release of a given cytochrome c fraction when the enzyme is active, compared with when it is inhibited. Through its effects on the permeability transition and release of intermembrane space proteins, the mitochondrial Ca2+-independent phospholipase A2 may be an important factor governing cell death caused by necrosis or apoptosis.

Mitochondria from rat liver and rabbit heart have been shown to contain a Ca2+-independent phospholipase A2 (iPLA2) that has a molecular mass of ~80 kDa (1, 2). Like phospholipases of this type from other sources (3, 4), the mitochondrial enzyme is inactivated by bromoeno lactone (BEL), which acts through an activity-dependent mechanism, leading to a covalent modification within the active site (5). No physiological function of the iPLA2 in mitochondria has been established, but it has been shown that pretreatment with BEL attenuates the loss of phospholipids that accompanies ischemia/reperfusion injury and reduces the size of infarcts by ~50% (2). The relationship between mitochondrial energetic status and iPLA2 activity is an important factor to consider when contemplating potential physiological and pathophysiological roles of the enzyme. More specifically, activity is not seen in mitochondria that are respiring under state 4 conditions but is manifest upon the addition of uncoupler and is fully manifest following the development of inner membrane pores (1). The former property suggests that transient periods of deenergization might cause a transient activation of the iPLA2 in vivo, with a resulting accumulation of free fatty acids in mitochondria. Such an accumulation could be of interest in many regards, including opening of the permeability transition pore, which is favored by low levels of these compounds (6–8). Occurrence of the permeability transition leads to apoptosis in many cell types (9–11), so scenarios arise in which the iPLA2 contributes to the control of apoptosis by influencing the permeability transition and wherein the facilitative effects of depolarization on the transition (12, 13) might occur through activity of this enzyme.

As further regards the potential roles of the iPLA2, another factor to consider is its relatively high specific activity, compared with what might be expected from early reports. In media of high ionic strength, the activity level is sufficient to hydrolyze ~5 mol % of mitochondrial phospholipids/h, which could be sufficient to initiate and enforce the turnover of poorly functioning mitochondria (1). In addition, on the time scale of apoptosis (several h), this level of activity is expected to alter mitochondrial ultrastructure and might thereby change the poorly understood relationships between extents of swelling, membrane rupture, and the release of mitochondrial factors that control apoptosis.

The present study was undertaken as an initial test of potential mitochondrial iPLA2 functions along the lines of those described above and has identified three points of interest. It first of all shows that the iPLA2 is largely responsible for the accumulation of free fatty acids that occurs when mitochondria accumulate Ca2++. Second, it shows that on a relatively short time frame of several minutes, activity of the enzyme is indeed an important factor regulating the permeability transition and may, in part, constitute the link between mitochondrial energetic status and the ease with which the transition occurs. Finally, this study shows that on a longer time frame of hours, mitochondrial iPLA2 activity does indeed change the relationships between swelling and the release of an apoptogenic factor (cytochrome c), both in terms of the time course involved and the fraction released at a given extent of swelling. Aspects of these findings have been described in abstract form (14).

EXPERIMENTAL PROCEDURES

Preparation and Incubation of Mitochondria—Rat liver mitochondria were prepared by a standard procedure in which bovine serum albumin and EGTA were present in the homogenization medium but were absent from the medium used for washing (15). They were incu-
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bated in media containing 0.23 mM mannitol, 0.07 mM sucrose, 3 mM Hepes (Na⁺) (pH 7.4), plus further additions described in the figure legends. Alternatively, media were also employed in which 0.15 M KCl or 0.15 M KMes was used in place of mannitol/sucrose as further described under "Results." When succinate (Na⁺) was present as an oxidizable substrate, its concentration was 1.0, or 10 mM, and rotenone was present at 1 nmol/mg protein. In addition, the concentrations mannitol/sucrose, KCl, or KMes were reduced proportionally to maintain the osmotic pressure at 300 mosm when succinate was employed.

Small incubations were conducted in a cuvette to allow the continuous monitoring of mitochondrial swelling as apparent absorbance changes at 540 nm. An Aminco DW-2c spectrophotometer was employed for this purpose. Larger incubations were conducted in water-jacketed beakers that were open to the atmosphere, with stirring maintained to provide an availability of O₂ throughout the time course (16). When the experiments were conducted over long time frames (hours), all incubations were conducted simultaneously to prevent variations arising from aging of the preparation. During experiments in these vessels, swelling was monitored continuously using a Brinkman probe colorimeter that was interfaced to a computer. Alternatively, swelling was sometimes monitored discontinuously using the same instrument, by moving the probe from vessel to vessel and manually recording the reading. Changes in membrane potential were also of interest and were monitored simultaneously with swelling, using a TPP⁺ electrode as previously described (16).

Determination of PLA₂ Activity—Phospholipase activity was determined by monitoring the accumulation of free fatty acids (FFA) derived from the endogenous phospholipids (15). To extract these products, a 3-ml aliquot of the incubation was added to 4 ml of cold methanol to which 5.00 μg of heptadecanoic acid (17:0) had previously been added as an internal standard. The aqueous methanol solution was mixed before the addition of 8 ml of CHCl₃, and the resulting mixture was centrifuged to separate the organic and the aqueous phases. The aqueous phase (upper phase) was removed by aspiration, after which the lower organic phase was transferred to a 10-ml conical screw cap tube. This phase was brought to dryness under N₂, and the free fatty acids were converted to methyl esters (FAME) by reaction with diazomethane (17). During this procedure, the dried lipid phase was taken up in 1.1 ml of ether/methanol (10:1) to which 0.2 ml of the diazomethane solution was added. Reaction was allowed to continue for 15 min at room temperature. The samples were then brought to dryness and taken up in 1 ml of CHCl₃. Typically, they were thereafter stored overnight, at 4°C and under argon, prior to analysis by gas chromatography.

To begin the analysis, the stored samples were dried, and the lipids were taken up in 0.2 ml of hexane. These solutions were applied to silica gel minicolumns for the separation of FAME from other mitochondrial lipids (15). The columns were washed successively with 1.0 ml of hexane, 18 ml of CHCl₃, and finally 2.0 ml of hexane/ether (1:1). FAME are obtained in the hexane/ether wash. The hexane/ether solvent mixture was removed under N₂, and the FAME were taken up in 12 μl of hexane. They were then separated and quantitated using a gas chromatograph equipped with a capillary column and a computing integrator. Peak areas representing the original level of individual FFA were converted to units of nmol/mg mitochondrial protein. To convert the areas to units of mass, they were compared with the area of the internal standard peak, which represented the 5.00 μg of heptadecanoic acid that was added to the original extract.

Changes in FFA levels can usually be related to activity of a PLA₂ by considering the composition of compounds, because positional analysis data have shown which fatty acids are normally located at sn-1 and sn-2 positions of mitochondrial phospholipids (1, 18). However, activity of the mitochondrial iPLA₂ is apparently followed by that of a lysophospholipase, because FFA originally esterified at the two positions appear in approximately equal proportions (1). Furthermore, the mitochondrial functional parameters considered here depend more upon the total FFA present than upon the exact composition. Accordingly, the results of FFA analysis are reported as totals of all those detected and are in units of nmol/mg mitochondrial protein. Repetitive analysis of single samples has shown that the S.D. value for individual values is approximately ±4% (15).

Determination of Cytochrome c Release by HPLC—The preparation of samples for the determination of cytochrome c in mitochondrial stock suspensions or in samples arising from incubations has been described (19). Briefly, for stock suspensions, an aliquot containing 1.0 mg of mitochondrial protein was dispersed in 100 μl of 1% Lubrol WX to which 1 μl of 100 mM phenylmethylsulfonyl fluoride had previously been added. The dispersion was diluted to 1.0 ml with 100 mM KCl, and the resulting sample was centrifuged for 2 min in a microcentrifuge. For samples obtained from incubations, the initial volume was 1.0 ml, which also contained 1.0 mg of mitochondrial protein, and this was added to a microcentrifuge tube that already contained 1 μl of 100 mM phenylmethylsulfonyl fluoride. After centrifugation for 2 min in the microcentrifuge, 25 μM bovine serum albumin was added to the supernatants, which were saved for subsequent analysis. The pellets were immediately resuspended in 100 μl of incubation medium, dispersed with Lubrol WX, and then diluted to a total volume of 1.0 ml as described for the samples arising from mitochondrial stock suspensions.

For all samples, 100-μl aliquots representing 0.10 mg of mitochondrial protein were injected into the HPLC. A C₄ reverse phase column preceded by a guard column and a linear gradient ranging from 20 to 60% acetonitrile in water were employed. Both solvents A and B contained 100 mM KCl and 0.1% trifluoroacetic acid (v/v). The gradient was developed over a 15-min period, which was followed by a 5-min wash with the 60% solution and a subsequent 5-min wash with the 20% solution before injection of the next sample. The eluted cytochrome was detected at 393 nm to take advantage of an improved extinction coefficient at that wavelength, which results from the presence of trifluoroacetic acid (20). Cytochrome c release was expressed as a percentage of the total, as determined from the stock suspension samples. The sum of cytochrome c found in supernatants and pellets was furthermore compared with the total to verify that all of that protein was accounted for.

Other Methods—Rates of O₂ consumption were determined using a Clark-type electrode. Adenylate kinase and malate dehydrogenase activities were determined as described (21, 22), using the same samples that were generated for the determination of cytochrome c release (22). Initial rates were extracted from the progress curves by fitting an early portion to a linear model, using standard techniques. Incubations were conducted at 25 or 37°C, as specified in the figure legends, and at a mitochondrial protein concentration of 1.0 mg/ml. The concentration of protein in mitochondrial stock suspensions was determined by the Biuret reaction after dispersing the organelles in deoxycholate (Na⁺) (1% final concentration).

Work in progress indicates that rat liver mitochondria do indeed contain a lysophospholipase activity, which exceeds the activity of the iPLA₂. Nevertheless, it remains possible that low levels of lysophospholipids are present as a result of iPLA₂ activity and contribute to the consequences of activating that enzyme.
iPLA₂ Activity Produces the FFA Accumulation That Accompanies Ca²⁺ Uptake—It has long been known that energy-dependent Ca²⁺ accumulation provokes a limited hydrolysis of phospholipids and an accumulation of FFA (18). This phenomenon was originally attributed to the action of a mitochondrial secretory PLA₂, because such an activity had been identified in mitochondria and found to require high Ca²⁺ concentrations for activity (23, 24). However, the mitochondrial iPLA₂ is activated when deenergization occurs (1), and it is well known that Ca²⁺ accumulation can markedly depress membrane potential, depending upon conditions (25). Accordingly, we considered the possibility that it is actually the iPLA₂ that is responsible for the FFA accumulation that accompanies Ca²⁺ uptake.

Figs. 1 and 2 show the data that were obtained. During these experiments, swelling was monitored together with membrane potential (TPP⁺ accumulation) and FFA accumulation, so that development of the permeability transition and the extent of depolarization could be ascertained simultaneously. Confirming the earlier reports (1, 18), FFA do not accumulate in mitochondria that are coupled and oxidizing succinate (Fig. 1A), but accumulation is seen upon uncoupling (Fig. 1B), upon induction of the permeability transition (Fig. 1C), or when alamethicin is employed to form pores in the mitochondrial inner membrane (Fig. 1D). Swelling does not occur unless pores are formed by the permeability transition or the antibiotic, as is well known, and membrane potential behaves as would be expected (Fig. 1, A–D).

When mitochondria accumulate Ca²⁺ at 60 nmol/mg protein, a marked depolarization occurs, which is maintained and intensifies during subsequent incubation (Fig. 2, compare A and B). Some of the mitochondria belatedly undergo the permeability transition following Ca²⁺ accumulation, as indicated by modest swelling, and at the end of the experiment, 1.40 nmol/mg protein of FFA had been accumulated. This accumulation was in addition to the ~1.2 nmol/mg protein that was found as an endogenous content in this preparation. The inclusion of CsA eliminates the partial swelling occurring toward the end of the experiment and promotes some recovery of membrane potential following Ca²⁺ accumulation but has no effect on FFA accumulation (Fig. 2, compare A and D).
FIGURE 2. Relationships between Ca$^{2+}$ accumulation, membrane potential, and the accumulation of FFA in mitochondria. Mitochondria were incubated as described under "Experimental Procedures" and the legend to Fig. 1. Swelling (upper traces, solid line) and membrane potential (lower traces, dashed line) were monitored as also described. A, no further additions; B, CaCl$_2$ was added where shown at 60 nmol/mg protein; C, same as B except that 0.5 μM CsA was present from the beginning of the incubation; D, same as B except that 10 μM BEL was present from the beginning of the incubation; E, same as B except that 1 nmol/mg protein of ruthenium red was added before CaCl$_2$; F, same as B except that 2.0 mM inorganic phosphate (Na$_2$HPO$_4$) was present; G, same as B except that 1 nmol/mg protein of ruthenium red was added before CaCl$_2$; H, same as B except that SrCl$_2$ was added instead of CaCl$_2$. For all panels, at end of the incubations, ∆FFA was determined as described under "Experimental Procedures" and in the legend to Fig. 1.
BEL Inhibits the Mitochondrial Permeability Transition—To determine if FFA arising from iPLA₂ activity are truly a factor in promoting the permeability transition, we compared the effects of BEL with those of known inhibitors when the phenomenon is induced by Ca²⁺ accumulation. In the presence of ruthenium red or CsA (15), BEL inhibits the transition because the phenomenon is stimulated by FFA (6, 7), that the FFA of interest normally arise from iPLA₂ activity, and that the enzyme becomes active when the mitochondrial membrane potential is depressed upon the addition and accumulation of Ca²⁺.

iPLA₂ Activity Promotes the Spontaneous Release of Cytochrome c—To examine potential relationships between the mitochondrial iPLA₂ and apoptosis, apart from its effects on the permeability transition, we monitored the spontaneous release of cytochrome c and related parameters during extended incubations. The time frame of these experiments was selected to mimic that of cellular apoptosis (several hours) rather than the time frame of minutes, which is often employed in related studies using isolated mitochondria. An uncoupled condition was employed to promote activity, which was also favored by use of a physiological temperature and high ionic strength medium (KCl-based medium). In addition, CsA and EGTA were present to inhibit the permeability transition.

Under these conditions, mitochondria swell spontaneously, within ~45 min, to an extent that is similar to the swelling produced by alamethicin or the permeability transition (Fig. 4). This swelling is accompanied by an early release of cytochrome c equaling about 50% of the total, by the release of a similar fraction of adenylate kinase activity (intermembrane space marker), and of a slightly smaller fraction of malate dehydrogenase activity (matrix space marker). These findings suggest that the initial phase of relatively rapid swelling caused the outer membrane to rupture in about 50% of mitochondria and that the inner mem-

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5 Not pertinent to Fig. 3, but consistent with interpretations presented later in this work, exogenous FFA retain a capacity to promote the transition when Ca²⁺ accumulation has occurred before ruthenium red is added (data not shown).
brane was subsequently ruptured after a further period of ~30–60 min. Accordingly, we sought to identify the cause of this swelling and to minimize it, so that effects of iPLA₂ activity on the release of cytochrome c could be better observed.

Because the mitochondria were uncoupled and conditions were in place to inhibit the permeability transition when Fig. 4 was obtained, it seemed apparent that the early large amplitude swelling reflected an accumulation of KCl and that the driving force was neither colloid osmotic in nature nor derived from the ion pumping activities of mitochondria. Thus, solute concentration gradients were seen to be responsible. Furthermore, since the matrix space K⁺ concentration is similar to that of the external medium, it appeared that the Cl⁻ concentration gradient was the one of greatest interest. A scheme involving the mitochondrial inner membrane anion channel (IMAC) and the K⁺/H⁺ antiporter can account for the swelling, as further described under “Discussion,” which led us to substitute zwitterions for Cl⁻ (poor substrates for IMAC (26, 27)) as a way to diminish the initial swelling seen in Fig. 4. The sulfonic acid buffer ion Mes proved useful in this regard, so that further experiments were conducted in media containing 150 mM KMes.

In a KMes-based medium analogous to the KCl-based medium used to obtain Fig. 4, the initial swelling is greatly diminished, and fractional rupture of the outer and inner membranes is effectively curtailed (Fig. 5A). A second phase of swelling becomes apparent at about 2 h, and the release of cytochrome c begins. This is accompanied by an equal fractional release of adenylate kinase activity, showing that outer membrane rupture is responsible, and by a much smaller fractional release of malate dehydrogenase activity (inner membrane rupture). When BEL is present to inhibit the iPLA₂ (Fig. 5B), the period preceding the second swelling phase is longer, as is the time required to begin outer membrane rupture and release of cytochrome c.

Some significant aspects of Fig. 5 are better seen when the data are displayed in different ways and compared with the accumulation of FFA (Fig. 6). First of all, it is seen that 10 µM BEL does not completely prevent the accumulation of free fatty acids during extended incubations (Fig. 6A). The reason for this seems clear (see “Discussion”), but at present, the point is to recognize that swelling in the presence of BEL might be further reduced if FFA accumulation were more completely abolished. It is furthermore seen that the time course of cytochrome c release is shifted to the right by about 1.5 h when BEL is present and that about 30% of the cytochrome is never released under that condition (Fig. 6B).

Finally, and perhaps of greatest interest, BEL changes the relationship between fractional swelling and release of the cytochrome c, such that more is released per amount of swelling when the inhibitor is present (Fig. 6C). The differential can be quite large, such as near 60% swelling where it is 5–6-fold. This may have implications in the initiation of apoptosis, as further described below.

**DISCUSSION**

Uncoupling mitochondria through the action of carbonyl cyanide m-chlorophenylhydrazone (CCCP), the permeability transition pore, or the presence of alamethicin results in an accumulation of FFA due to action of the iPLA₂, as shown previously (1) and in Fig. 1. Before considering other aspects of the present data, it should be noted that the level of FFA accumulating on relatively short time frames (several min) shows some variability from preparation to preparation and with changes in experimental conditions that might alter the matrix content of water-soluble molecules and ions. Part of this variability appears to reflect an unidentified process that leads to declining levels of endogenous FFA during the early portion of incubations under energized conditions (e.g., Figs. 1A and 2A). Because this process varies in prominence when different preparations are compared, initial changes in the level of FFA can vary between preparations and do not reflect the rate of phospholipid degradation in a straightforward way. Effects of ionic strength on iPLA₂ activity (1) probably contribute to variability associated with altered matrix space solute composition brought about by changing conditions.

The above considerations notwithstanding, it is clear from Fig. 2 that the straightforward process of Ca²⁺ accumulation causes an iPLA₂-dependent accumulation of FFA. This accumulation does not require the permeability transition, because it can occur in the absence of significant swelling (Fig. 2, B and C) and because it occurs when CsA is present (Fig. 2C). It is dependent on iPLA₂ activity, because it is eliminated by BEL (Fig. 2, D and E). It is also eliminated when phosphate is present, which limits the period of diminished membrane potential associated with Ca²⁺ accumulation, and when no loss of membrane potential occurs upon Ca²⁺ addition due to inhibition of uptake by ruthenium red (Fig. 2, F and G, respectively). It is furthermore unrelated to the Ca²⁺ ion per se, because Sr²⁺ accumulation is similarly effective, both in terms of depressing membrane potential and promoting the accumulation of FFA (Fig. 2H). Finally, the accumulation of FFA is not easily attributed to
the changes in matrix pH produced by some of the specified conditions, because the enzyme displays a broad pH optimum located near pH 8 (1). Taken together, these data indicate that Ca\(^{2+}\) accumulation causes the accumulation of FFA, because it depresses membrane potential and thereby activates the iPLA\(_2\).

A relationship between pore opening and mitochondrial membrane potential has long been identified. More specifically, pore opening is antagonized and facilitated by high and low membrane potential, respectively (12, 28–30). Actions of sulfhydryl reagents (30–35) and compounds that modify arginine residues (36–38) led to the proposal that the pore contains a structural motif that senses the transmembrane potential and conveys this information to change the open-closed probability. It was also maintained that FFA facilitate pore opening because they alter the membrane surface potential by their presence in the bilayer, with the pore sensing this potential, together with the transmembrane potential, as a vector sum (6, 7). Here we see that part of the reason why a depressed membrane potential promotes an open pore is that it activates the iPLA\(_2\), which in turn leads to an increase in FFA and a change in the summed potentials that the pore encounters. Likewise, we see that activation of this enzyme is part of the reason why Ca\(^{2+}\) uptake promotes an open pore, in addition to the actions of Ca\(^{2+}\) that are exerted at internal binding sites. The FFA that accumulate upon activation of the iPLA\(_2\) can be very significant in controlling the pore, as seen in Fig. 3.

Occurrence of the mitochondrial permeability transition is the committed step in necrotic cell death, such as that which follows ischemia-reperfusion injury, and can initiate or reinforce cell death by apoptosis, depending upon how it is initiated (e.g. Refs. 39–44). It follows then that iPLA\(_2\) activity should promote both types of cell death through its effects on the pore open probability. Indeed, it has already been shown in a perfused heart model that pretreatment with BEL markedly reduces the size of infarcts produced by global ischemia (2). We are not aware of studies examining the effect of BEL on apoptosis via the mitochondrial pathway, although Bernardi and co-workers recently reported that apoptosis in MH1C1 cells brought about by ionophore A23187 reflects occurrence of the permeability transition promoted by accumulating arachidonic acid (54). They considered the arachidonic acid to arise through activity of a cytosolic Ca\(^{2+}\)-dependent PLA\(_2\), and although that might be the case, it is also true that A23187 plus Ca\(^{2+}\) uncouples mitochondria by allowing a futile cycle of Ca\(^{2+}\) uptake and release (45). Accordingly, the mitochondrial iPLA\(_2\) should have been active in their system, contributing to the phospholipid degradation and pore opening that they observed.

In a more general sense, the way that cytochrome \(c\) and other apoptotic factors are released from mitochondria is poorly understood, although a number of mitochondrial proteins that modulate the process have been identified (e.g. see Refs. 46–51). Basically, any proposed release mechanism must involve rupture of the outer membrane, the development of pores that are large enough to accommodate proteins of a substantial size (the reported size of apoptosis-inducing factor, for example, is 57 kDa (52)), or some type of mediated protein export process, for which there is no experimental support. Studies examining potential release mechanisms at the level of isolated mitochondria can be criticized because they are typically conducted on time frames of minutes, whereas apoptosis typically occurs on a time frame of hours. Thus, slowly acting mechanisms that might release significant amounts of proteins might go unnoticed in the isolated mitochondria system.

In view of time frame-related criticisms, one of our goals during the present investigation was to monitor spontaneous cytochrome \(c\) release from isolated mitochondria on long time frames that are pertinent to
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FIGURE 7. Scheme for the swelling of uncoupled mitochondria in KCl media. A shows that the IMAC was able to transport Cl⁻ but not the deprotonated form of MES. The result is an inside negative membrane potential that is formed in a Cl⁻–containing medium. In B, the membrane potential results in the formation of a pH gradient, inside acidic, through action of the uncoupler CCCP. The K⁺ for H⁺ antiporter then promotes an accumulation of K⁺ (C) to result in the overall process producing a net uptake of K⁺. Water entering in response to the increased matrix space osmotic pressure would thereafter cause swelling.

actual apoptosis and to determine if the iPLA₂ activity is sufficient to alter this time course in a significant way. Toward those ends, mitochondria were at first incubated a KCl-base medium, because high ionic strength is required to fully release the cytochrome from the inner membrane (19) and to promote activity of the enzyme (1). A temperature of 37 °C was employed, rather than the usual 25 °C, also to promote activity of the iPLA₂ and to better mimic physiological conditions. EGTA plus CsA were present to prevent the permeability transition, which is already well known to release cytochrome c, and to assure that any phospholipase activity was indeed Ca²⁺-independent. Finally, the uncoupler CCCP was present during the entire incubation to promote a consistent activity of iPLA₂.

The swelling that ensued under these conditions was somewhat unexpected and was problematic in terms of our goals. It can be explained by the mechanism illustrated in Fig. 7, in which IMAC provides for the transport of Cl⁻ into the matrix space, driven by the Cl⁻ concentration gradient, to generate an inside negative membrane potential (Fig. 7A). This electrical potential then drives an accumulation of H⁺ through the action of CCCP (Fig. 7B), and the resulting pH gradient promotes K⁺ accumulation via the K⁺/H⁺ antiporter (Fig. 7C). The net result is an ongoing accumulation of K⁺ and, therefore, an osmotic pressure-driven swelling. This swelling would appear modest on the usual time frame of experiments involving isolated mitochondria (10–20 min), but it disrupted the outer and inner membranes in 40–50% of the mitochondria during the first 1–2 h of incubation (Fig. 5). A second phase of outer membrane disruption beginning at ~4 h then released most of the remaining cytochrome c as the incubation continued. We found that BEL largely eliminates the second phase (data not shown), suggesting the involvement of iPLA₂ activity, but we decided not to further pursue this system in favor of attempts to minimize the initial phase of swelling and membrane disruption.

Consistent with the mechanism shown in Fig. 7 and reports describing the solute selectivity of IMAC (26), swelling seen during the first 2–3 h of incubation was reduced upon substituting the deprotonated form of MES for Cl⁻ in the medium, to the point that no disruption of mitochondrial membranes occurred during this period, and there was no release of cytochrome c. The presence of BEL extended the period of minimal swelling and the retention of membrane integrity to about 4 h. During the period between 4 and 8 h, significant swelling and membrane disruption did occur, but the final extent was diminished compared with that seen in the absence of BEL. The questions then arising are how iPLA₂ activity promotes the release of cytochrome c and whether this action might be important in the process of apoptosis.

Two types of processes come to mind that might explain the effect of activity on release of the cytochrome. In one, the iPLA₂ has access to outer membrane phospholipids, perhaps via contact sites, and reduces the volume that this membrane can contain as hydrolysis proceeds. The result would be rupture of the outer membrane with less expansion of the matrix volume and, in the extreme, without any expansion. This type of mechanism is not supported by Fig. 6C, which shows that the presence of BEL causes an increase in the fraction of cytochrome c released at intermediary fractions of swelling, the opposite of what that explanation would predict. The relationship depicted in Fig. 6C remains of interest, however, because it indicates that iPLA₂ can potentially tune the sensitivity of cells to apoptotic mechanisms that rely on expansion of the matrix space volume to disrupt the outer membrane and release cytochrome c.

The other process of possible interest is the permeability transition, which might occur and cause swelling after several hours, even in the presence of CsA and EGTA. Although CsA is very potent as an inhibitor of pore opening, its action is transient on the time scale of apoptosis (6). It is furthermore possible to open the pore in the absence of exogenous Ca²⁺ (e.g. see Ref. 53). Thus, the very high level of FFA that accumulates in 2 h when BEL is absent (equivalent to hydrolyzing about 8% of total mitochondrial phospholipids) may be sufficient to open the pore despite the precautions that were taken. If this is the case, then it is the residual activity (Fig. 6A) that eventually allows the release of cytochrome c when BEL is present (Fig. 6B). Regarding this residual activity, it should be noted that increasing the level of BEL employed did not reduce activity further (data not shown). We suspect that the residual activity reflects the mode of action of BEL, in which it becomes covalently bonded to a nucleophile group within the active site but not to the catalytic residue per se (5). It thus sterically interferes with substrate entering the active site, but this interference may not be complete. Thus, it seems worthwhile to determine if BEL analogs that contain bulkier constituents would be more effective at inhibiting the iPLA₂ and the release of cytochrome c and to investigate their actions on apoptosis initiated through the mitochondrial pathway.

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