Pore Formation and Uncoupling Initiate a Ca^{2+}-Independent Degradation of Mitochondrial Phospholipids†

Kimberly M. Broekemeier,*,‡,§ James R. Iben,‡ Emily G. LeVan,‡ Elliott D. Crouser,|| and Douglas R. Pfeiffer§

Department of Chemistry, Ohio Northern University, Ada, Ohio 45018, and Departments of Internal Medicine and of Molecular and Cellular Biochemistry, The Ohio State University, Columbus, Ohio 43210-1218

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ABSTRACT: Mitochondria contain a type IIA secretory phospholipase A_2 that has been thought to hydrolyze phospholipids following Ca^{2+} accumulation and induction of the permeability transition. These enzymes normally require millimolar Ca^{2+} for optimal activity; however, no dependence of the mitochondrial activity on Ca^{2+} can be demonstrated upon equilibrating the matrix space with extramitochondrial Ca^{2+} buffers. Ca^{2+}-independent activity is seen following protonophore-mediated uncoupling, when uncoupling arises through alamethicin-mediated pore formation, or upon opening the permeability transition pore. Under the latter conditions, activity continues in the presence of excess EGTA but is somewhat enhanced by exogenous Ca^{2+}. The Ca^{2+}-independent activity is best seen in media of high ionic strength and displays a broad pH optimum located between pH 8 and pH 8.5. It is strongly inhibited by bromoenol lactone but not by arachidonyl trifluoromethyl ketone, dithiothreitol, and other inhibitors of particular phospholipase A_2 classes. Immunoanalysis of mitochondria and mitochondrial subfractions shows that a membrane-bound protein is present that is recognized by antibody against an authentic $iPLA_2$ that was first found in $P388D_1$ cells. It is concluded that mitochondria contain a distinct Ca^{2+}-independent phospholipase A_2 that is regulated by bioenergetic parameters. It is proposed that this enzyme, rather than the Ca^{2+}-dependent type IIA phospholipase A_2, initiates the removal of poorly functioning mitochondria by processes involving autolysis.

The mitochondrial permeability transition (MPT)[1] is the committed step in necrotic cell death, is associated with apoptotic cell death, and is implicated as a component of the system that generates cytoplasmic Ca^{2+} waves (*1*). Several investigators have proposed that the MPT, as its most basic function, leads to the removal and replacement of poorly functioning mitochondria in cells. According to this hypothesis, mitochondria that are damaged by reactive oxygen species or exposure to chemical toxins, that contain mutated DNA, or that are functioning poorly for other reasons undergo the MPT more readily than normal mitochondria. Following permeabilization, the affected fraction is removed by autolysis (*2*), autophagy (*3*), because the import of new proteins/structural components no longer occurs (*4*), or by some combination of these processes and considerations. This concept has been referred to as "mitochondrial apoptosis" or "mitopoptosis" and encompasses the known roles of the MPT in cell death, by specifying a different consequence of the phenomenon according to the fraction of the mitochondrial population that is involved. When the fraction is small, only those mitochondria undergoing the transition are affected because the release of proapoptotic factors is too limited to support cellular apoptosis, and surviving mitochondria can easily compensate for the loss of biochemical capacity at the whole cell level. However, as the permeable fraction grows, cellular apoptosis can be initiated or, in the extreme, necrotic cell death occurs if most of the mitochondria have been permeabilized (*2*−*4*).

Autophagy is clearly a process that is potentially able to remove mitochondria that undergo the MPT in vivo (*5*), and the energy requirements of mitochondrial protein import are well established (*6*). In contrast, the extent of autolysis that might be expected to follow the MPT is not as clear. Phospholipid hydrolysis is of obvious interest in this regard, and recognition of a close relationship between mitochondrial phospholipases and the MPT dates back to the 1970s (*7*, *8*). The early studies showed that unsaturated free fatty acids (FFA) and lysophospholipids accumulate as the MPT spreads throughout a population of mitochondria in vitro (*8*, *9*). Both

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* Address correspondence to this author at the Department of Chemistry, Ohio Northern University, Ada, OH 45018. Phone: 419-772-2435. Fax: 419-772-2330. E-mail: k-broekemeier@onu.edu.

‡ Department of Chemistry, Ohio Northern University.

§ Department of Molecular and Cellular Biochemistry, The Ohio State University.

|| Department of Internal Medicine, The Ohio State University.

[1] Abbreviations: $AACOF_3$, arachidonyl trifluoromethyl ketone; BEL, bromoenol lactone; *p*-BPB, 4-bromophenacyl bromide; CCP, carbonyl cyanide *m*-chlorophenylhydrazone; $cPLA_2$, cytoplasmic phospholipase A_2; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); DTT, dithiothreitol; FAME, fatty acid methyl esters; FFA, free fatty acids; $iPLA_2$, Ca^{2+}-independent phospholipase A_2; MPT, mitochondrial permeability transition; NEM, *N*-ethylmaleimide; PE, phosphatidylethanolamine; PLA_1, phospholipase A_1; PLA_2, phospholipase A_2; PTP, permeability transition pore; RuRed, ruthenium red; $sPLA_2$, secretory phospholipase A_2; TFP, trifluoperazine.

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lipid classes are products of PLA_2 activity, and neither accumulates in energized mitochondria that are not Ca^{2+} loaded. These considerations led to interpretations wherein a Ca^{2+}-regulated PLA_2 is activated when Ca^{2+} was accumulated to initiate the MPT, and it was thought that the lipid degradation products cause the increased permeability of the inner membrane (*10*). It is now clear that the MPT does not reflect the formation of a membrane lipid phase defect (*11*, *12*) but is due to opening of a proteinaceous inner membrane pore, the permeability transition pore (PTP). It is also clear that phospholipid degradation products, alone or in combination with Ca^{2+}, are not competent to open the PTP (*10*); however, see refs *13* and *14*. Nevertheless, FFA are potent facilitators of PTP opening and increase the effectiveness of traditional inducing agents, with a half-maximal effect obtained near 2 nmol/mg of protein (*14*−*16*). The level of FFA arising from endogenous phospholipids is small compared to what might be expected if the MPT truly functions to initiate the autolysis of mitochondria.

The enzyme thought to be responsible for phospholipid degradation associated with the MPT has been isolated from liver mitochondria (*17*, *18*), and the corresponding cDNA has been cloned and sequenced (*19*). On the basis of the amino acid sequence and the location of cysteine residues, it has been classified as a type IIA phospholipase A_2 (*19*). The most prominent enzyme of this type is secreted by platelets (*20*). Type IIA enzymes are low molecular mass proteins (∼14 kDa) that belong to the general category of secretory phospholipases A_2 ($sPLA_2$). Under most conditions, type IIA PLA_2 require millimolar Ca^{2+} and display an alkaline pH optimum (*21*, *22*). There is no acyl chain preference, but an apparent selectivity for phosphatidylethanolamine (PE) is often observed. The latter property is dependent on the phase state of the phospholipids, such that it is not clear if type IIA PLA_2 are PE selective in vivo (*23*).

In concurrence with the sequence data, the enzyme from rat liver mitochondria displays these expected biochemical characteristics, including an apparent preference for PE, a lack of acyl group selectivity, an alkaline pH optimum, and a requirement for high Ca^{2+} concentrations (*24*). While these characteristics support the notion that the enzyme is in fact a type II PLA_2 (*25*), they raise questions regarding the potential of the enzyme to rapidly generate FFA for regulation of the PTP and to participate significantly in mitochondrial autolysis following the MPT. Other uncertainties have arisen from Southern blot analysis, which indicates that there is only a single gene in mammalian cells that encodes for a type IIA PLA_2 (*26*). The typical secretory signal sequence is present, and there is no evidence of alternative splicing. Accordingly, it is also not clear how this enzyme is directed to mitochondria for participation in physiological processes.

To further clarify the significance of phospholipid hydrolysis accompanying the MPT, and to address some of the uncertainties noted above, we reexamined and expanded upon previous studies relating to the regulation of PLA_2 activity in isolated mitochondria. Unexpectedly, the results show that a Ca^{2+}-independent activity is present in addition to the Ca^{2+}-dependent activity described before. This newly discovered activity is most apparent in media of high ionic strength. It is observed following the MPT, upon permeabilizing mitochondria with alamethicin, or when uncoupling is achieved by the use of protonophores and ionophores. It seems probable that it is the Ca^{2+}-independent activity, rather than the one described previously, which acts to regulate the MPT and participates in mitochondrial autolysis. Aspects of these findings have been presented in a preliminary form (*27*, *28*).

EXPERIMENTAL PROCEDURES

Reagents. Bromoenol lactone (BEL) was purchased from Cayman Chemicals, Ann Arbor, MI. Arachidonyl trifluoromethyl ketone ($AACOF_3$) was obtained from BIOMOL Research Laboratories, Inc., Plymouth Meeting, PA. 4-Bromophenacyl bromide (*p*-BPB) and 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) were from Sigma. $iPLA_2$ antibody was purchased from Cayman Chemicals. Horseradish peroxidase linked anti-rabbit antibody was purchased from Amersham-Pharmacia. The ECL Western blotting detection kit and Hyperfilm were also purchased from Amersham-Pharmacia. Color-coded molecular weight markers were obtained from Bio-Rad. Electrophoresis grade reagents were purchased from Bio-Rad. Other reagents were obtained from commercial sources and were the best available grade. Mannitol/sucrose solutions were deionized by passage over Chelex 100. Freshly distilled solvents and acid-cleaned glassware were utilized during lipid analysis.

Preparation and Fractionation of Mitochondria. Liver mitochondria were prepared from male Sprague-Dawley rats (∼250 g). The final pellets were alternatively suspended in a mannitol−sucrose medium or in a medium containing KCl. Mitochondria that were suspended in the mannitol−sucrose medium were obtained by a standard procedure (*29*). EGTA (0.5 mM) and bovine serum albumin (2 mg/mL) were present in the homogenizing medium but were omitted from the washing medium which contained 230 mM mannitol, 70 mM sucrose, and 3 mM Hepes (Na^+), pH 7.4 (MSH medium). The final pellet was suspended at ∼60 mg of protein/mL in MSH and maintained on ice. When the final pellet was to be suspended in the KCl medium, homogenization and the initial low speed centrifugation were carried out as described above. However, after the mitochondria were sedimented for the first time, the MSH was replaced by a medium containing 150 mM KCl, and 3 mM Hepes (Na^+), pH 7.4 (KCl medium). This medium was then utilized for the subsequent washing steps and for maintaining the final preparation.

Percoll gradient purified mitochondria were prepared as described before (*12*). Briefly, 1.0 mL of the mitochondrial preparation, containing 30−40 mg of protein, was layered onto 23 mL of 30% Percoll, which contained mannitol, sucrose, and Hepes (Na^+) as in MSH. Centrifugation was subsequently conducted at 50000*g*, for 30 min, using a Beckman Ti60 rotor (0−4 °C). Mitochondria were obtained at the bottom of the tube, whereas contaminants were observed near the top. The mitochondrial layer was recovered, diluted to ∼30 mL with MSH, and centrifuged at 10000*g* for 10 min (0−4 °C). The pellet was resuspended and washed three additional times, employing the same conditions. The final pellet was then suspended in the MSH or KCl medium, at 30−40 mg of protein/mL, and maintained on ice.

To obtain subtractions for Western blot analysis, Percoll gradient purified mitochondria were diluted to ∼15 mg of protein/mL with MSH, and an aliquot was saved for

immunoanalysis and marker enzyme determinations. The remainder was disrupted by sonication on ice, using a Bronson sonifier (6 × 30 s intervals, power setting = 7). A portion of the sonicate was also saved, and the remainder was centrifuged at 100000*g* for 1 h, and at 4 °C, to obtain the mitochondrial membranes and soluble components as separate fractions. After the supernatant was removed, the pellet was resuspended in a volume of MSH equal to the original volume. The samples were then stored at −20 °C and were analyzed within 3 days of preparation.

For all preparations, the protein concentration was determined by the biuret reaction in the presence of 1% deoxycholate (Na^+). Bovine serum albumin in the medium used for the final suspension was employed as a standard.

Incubation of Mitochondria. Incubations were conducted at 25 °C, in 15 mL glass tubes, with moderate stirring maintained throughout. The protein concentration was 1.0 mg/mL. Media contained 10 mM succinate (Na^+), rotenone at 2 nmol/mg of protein, and 3 mM Hepes (Na^+), pH 7.4. An osmotic pressure near 300 mOsM was maintained by the presence of 207 mM mannitol and 63 mM sucrose or by the presence of 135 mM KCl. Other additions and conditions of interest are described in the figure legends.

Determination of PLA_2 Activity. Phospholipase activity was determined by monitoring the accumulation of FFA (*29*). To extract these products, a 3 mL aliquot of the incubation was added to 4 mL of cold methanol to which 5 μg of heptadecanoic acid (17:0) had previously been added as an internal standard. The aqueous methanol solution was mixed before addition of 8 mL of $CHCl_3$, 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 N_2, and the free fatty acids were converted to methyl esters (FAME) by reaction with diazomethane (*30*). 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. The 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 $CHCl_3$. Typically, they were then stored overnight, at 4 °C and under argon.

Prior to analysis by GLC, 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 (*29*). The columns were washed successively with 1.0 mL of hexane, 1.8 mL of $CHCl_3$, 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 N_2, and the FAME were taken up in 12 μL of hexane. They were then separated and quantitated using a GLC equipped with a capillary column and a computing integrator. Peak areas representing the original level of individual FFA were converted to units of nanomoles per milligram of mitochondrial protein. To convert the areas to units of mass, they were compared to the area of the internal standard peak, which represented the 5 μg of heptadecanoic acid that was added to the original extract.

FFA levels were related to PLA_2 activity by recalling that positional analysis of the fatty acid composition in liver mitochondrial phospholipids shows that polyunsaturated fatty acids are located predominantly at the *sn*-2 position of the glycerol moiety, while the saturated fatty acids are predominantly located at the *sn*-1 position (*8*). Accordingly, we assumed that all polyunsaturated FFA (predominantly 18:2, 20:4, and 22:6) arose from the activity of a PLA_2. Similar amounts of saturated compounds (predominately 16:0 and 18:0) also accumulate under most of the conditions employed. We assumed that these arise by a lysophospholipase acting after PLA_2 or through a PLA_1 acting independently. Accordingly, the saturated compounds were excluded when activity was calculated. Oleic acid (18:1) is the major monounsaturated fatty acid in mitochondrial phospholipids. It is found primarily in PE (39 mol % of total phospholipid) and in cardiolipin (8.1 mol % of phospholipid) (*8*). In PE it is distributed between the *sn*-1 position and the *sn*-2 position at a ratio of 2:3. In cardiolipin it is found nearly exclusively at the *sn*-2 position (*8*). Fifty percent of the accumulating oleic acid was assumed to arise through activity of PLA_2.

The fatty acid analysis procedure is highly reproducible overall because of the inclusion of an internal standard. Standard deviations of replicated determinations were ±3−4% (n = 10), depending on whether total, saturated, or unsaturated fatty acids are considered. Accordingly, the symbol size employed in the figures approximates the error bars for those determinations. Animal to animal variation was larger in terms of specific activities under a given condition (±∼30%), such that all data shown in a single figure were obtained from the same preparation of mitochondria. Experiments were repeated three times, with the data shown being representative.

Western Blot Analysis. Intact, disrupted, and fractions of mitochondria were subjected to SDS−polyacrylamide gel electrophoresis using an 8.5% gel and a Bio-Rad mini-gel apparatus. Samples were obtained by diluting the stored materials 1:1 with sample buffer, which contained 10% glycerol (v/v), 5% β-mercaptoethanol (v/v), and 2.5% SDS (w/v) in 0.25 M Tris-HCl (pH 6.8). Equal volumes of these (15 μL), originally derived from the same amount of mitochondria, were loaded, and the proteins were separated using constant voltage (100 V) and an 80 min run time. The proteins were then transferred completely to a poly(vinylidene difluoride) (PVDF) membrane, as indicated by the removal of all prestained molecular weight markers from the gel.

Before the presentation of antibodies, nonspecific sites were blocked with Tris-buffered saline (TBS) containing 150 mM NaCl and 100 mM Tris-HCl (pH 7.5), plus 0.05% Tween (v/v) (TTBS) and 10% (w/v) defatted milk. The $iPLA_2$ antibody was reconstituted according to the supplier's instructions and diluted 1:1000 with TBS containing 1% defatted milk. After the PVDF membrane was exposed to the diluted antibody for 1 h, it was washed 3 × 15 min with TBS containing 1% defatted milk and then exposed to the secondary antibody. The horseradish peroxidase-linked anti-rabbit secondary antibody had been diluted 1:20000 with TBS containing 1% defatted milk. Exposure was for 1 h, after which the membrane was washed 3 × 5 min with TBS. Proteins of interest were visualized by enhanced chemiluminescence, using a kit obtained from Amersham and ECL Hyperfilm from the same supplier.

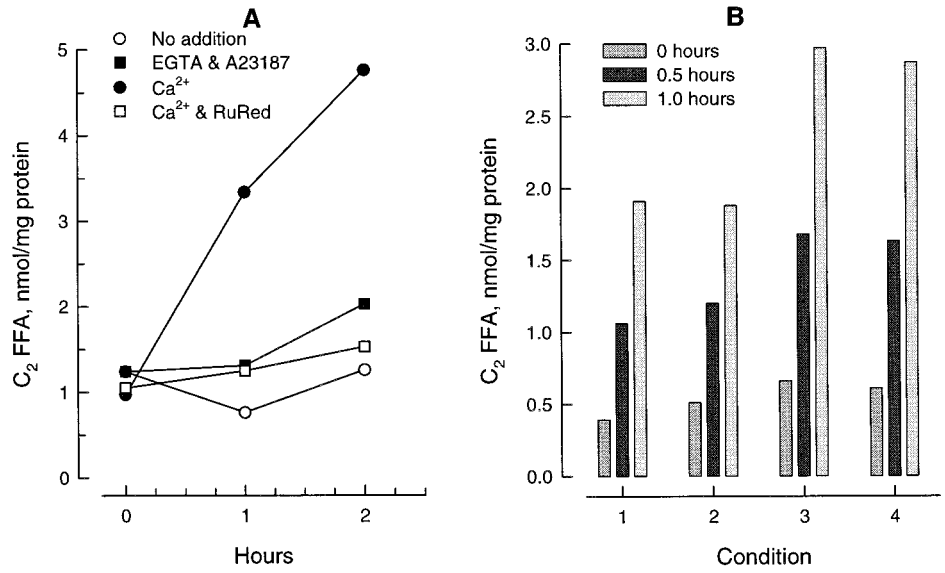


FIGURE 1: Ca^{2+} accumulation activates mitochondrial PLA_2 activity. Mitochondria were prepared and incubated in KCl-based media as described in Experimental Procedures. Panel A: (○) no additions after mitochondria; (■) 0.5 mM EGTA and 1 μM A23187 were added after 3 and 4 min of incubation, respectively; (●) 80 μM $CaCl_2$ was added at 4 min; (□) addition of 80 μM $CaCl_2$ at 4 min was preceded by ruthenium red (RuRed), which was added at 3 min and at 1 nmol/mg of protein. Panel B: individual bars show the accumulation of PLA_2 reaction products at 0, 0.5, or 1.0 h, as further detailed in the figure. Four incubation conditions were employed, and for all of these, A23187 (1 μM), valinomycin (0.1 μM), and CCP (3 nmol/mg of protein) were present. For conditions 1 and 2, the pH was 7.4. For condition 1, 100 μM EGTA was present, whereas for condition 2, 100 μM $CaCl_2$ was present. For conditions 3 and 4, the pH was 8.6. For condition 3, 100 μM EGTA was present, whereas for condition 4, 100 μM $CaCl_2$ was present.

The samples analyzed by Western blot were also evaluated by marker enzyme analysis. Malate dehydrogenase and monoamine oxidase activities were determined as described previously (*12*). Malate dehydrogenase is a soluble protein located in the mitochondrial matrix space, whereas monoamine oxidase is associated with the outer membrane.

RESULTS

Figure 1A confirms an earlier report that first showed that Ca^{2+} accumulation activates a PLA_2 in rat liver mitochondria (*8*). The FFA products of this activity accumulate progressively in mitochondria that have accumulated Ca^{2+} but not when endogenous Ca^{2+} alone is available (typically 4−8 nmol/mg of protein). Ca^{2+} must be accumulated into the matrix space as shown by the effect of ruthenium red, which inhibits the mitochondrial Ca^{2+} uniporter and prevents the activation of PLA_2 that is produced by exogenous Ca^{2+} (Figure 1A).

The report confirmed by Figure 1A, and most other studies investigating the properties of phospholipases in mitochondria, employed low ionic strength media in which mannitol and/or sucrose were (was) used to establish the osmotic pressure. The present data were obtained in a KCl-based medium, although all other conditions were the same. When comparing the present and previous data, we noticed that activity under the current conditions is ~3-fold greater than was seen before. We also noticed that activity was stimulated by a small, but consistent factor when ionophore A23187 and EGTA were employed to deplete the mitochondria of endogenous Ca^{2+} (Figure 1A). For a PLA_2 that truly requires Ca^{2+} this would not be expected.

To investigate the effect of Ca^{2+} concentration on enzyme activity, we conducted experiments similar to those shown in Figure 1A, wherein the external medium contained an EGTA−Ca^{2+} buffer system in addition to the other components. A23187, valinomycin, and a protonophore were present simultaneously to equilibrate Ca^{2+}, H^+, and K^+ gradients across the inner membrane. We utilized this approach, in preference to loading mitochondria with differing levels of Ca^{2+}, to minimize changes in matrix pH that would otherwise accompany changes in the matrix-free Ca^{2+} concentration and affect activity. Unexpectedly, no external Ca^{2+} concentration could be established that was low enough to substantially limit the activity of mitochondrial PLA_2. An example of these data is shown in Figure 1B, which demonstrates that PLA_2 activity is the same when mitochondria are equilibrated with 100 μM external EGTA or 100 μM Ca^{2+} and that this is true when the prevailing pH is 7.4 or 8.6.

Under the conditions of Figure 1A mitochondria remain coupled during the incubations, whereas they were uncoupled under the conditions of Figure 1B. To determine if this difference relates to the apparent difference in Ca^{2+} requirement indicated by the two panels, the effect of uncoupling per se was investigated. This factor was furthermore examined in media of low and high ionic strength, given that the comparison of Figure 1A with our earlier report suggests that this parameter affects activity. Figure 2 shows that uncoupling mitochondria in the presence of excess EGTA markedly activates the PLA_2 and further documents the effect of medium composition on the rate that is attained. While uncoupler was effective under either condition, the activation was modest in the low ionic strength medium (Figure 2A). Mitochondria that were prepared in MSH and then incubated in the KCl medium showed a higher rate (Figure 2B), whereas the highest rate was attained when mitochondria were both prepared and incubated in KCl (Figure 2C).

The use of EGTA together with uncoupler was directed at chelating the endogenous and adventitious Ca^{2+}, since it seemed possible that even these low amounts might satisfy

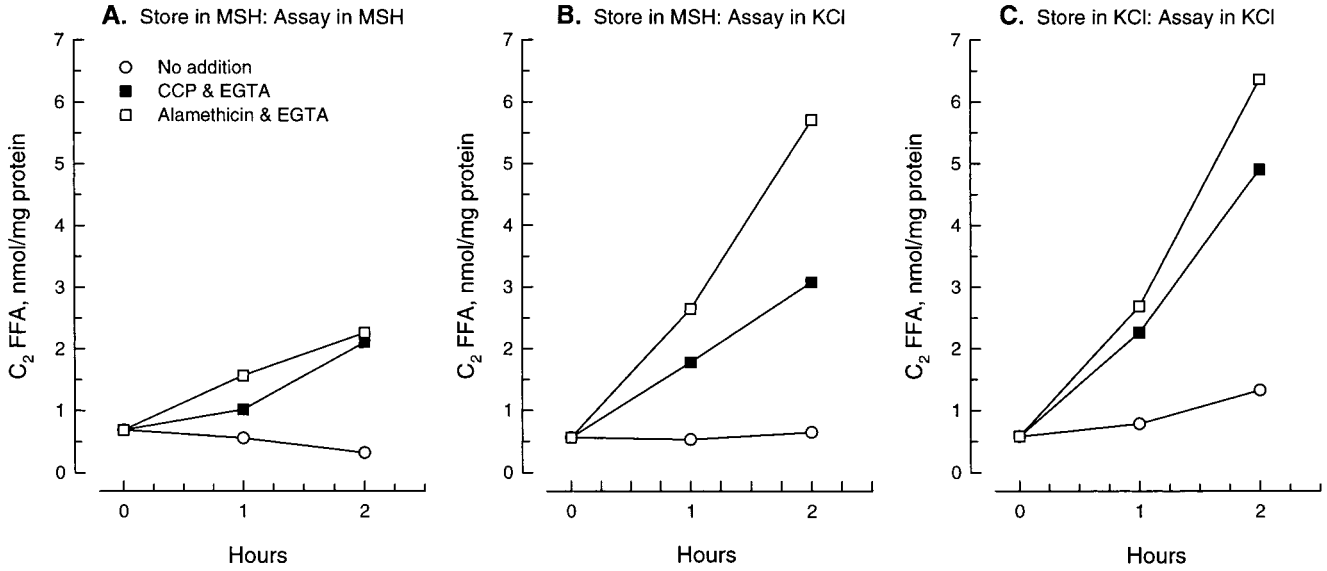


FIGURE 2: Uncoupling activates mitochondrial PLA_2 activity. Mitochondria were prepared and incubated in MSH-based media (panel A), prepared in MSH and incubated in KCl (panel B), or prepared and incubated in KCl (panel C) as described in Experimental Procedures. Key for all panels: (○) no additions after mitochondria; (■) 0.5 mM EGTA and 3 nmol of CCP/mg of protein were added at 3 and 4 min, respectively; (□) 1 μg of alamethicin/mg of protein and 0.5 mM EGTA were added at 3 and 4 min, respectively.

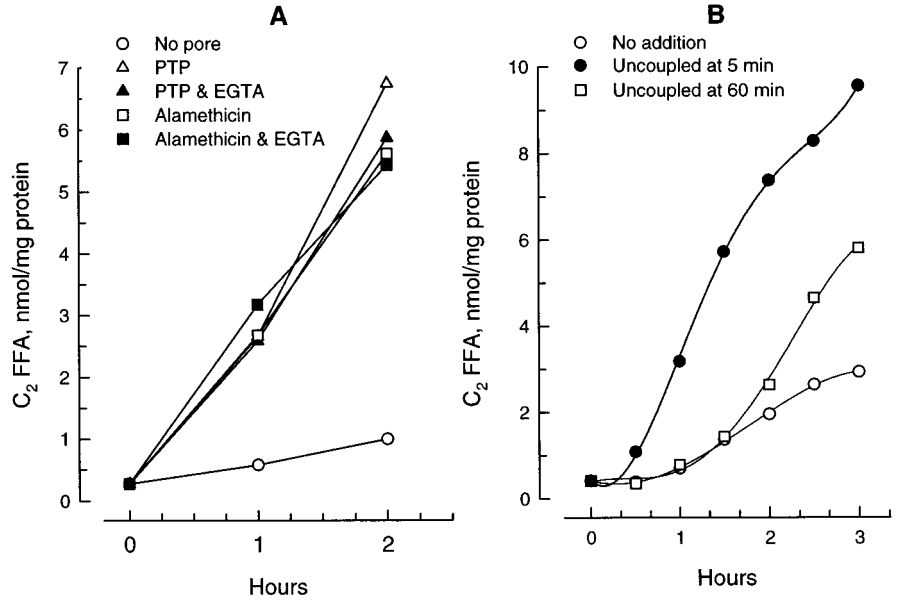


FIGURE 3: Additional factors effecting activity. Mitochondria were prepared in MSH and incubated in KCl media. Panel A: (○) 0.5 mM EGTA and 1 μM A23187 were added at 3 and 4 min, respectively; (△) the PTP was induced at 4 min by uncoupling Ca^{2+}-loaded mitochondria (80 nmol/mg of protein) in the presence of ruthenium red, as further described elsewhere (*53*); (▲) same as (△) except that 0.5 mM EGTA was added after the PTP was opened; (□) Ca^{2+}-loaded mitochondria (80 nmol/mg of protein) were permeabilized with alamethicin, which was added at 4 min and at 1 μg/mg of protein; (■) same as (□) except that 0.5 mM EGTA was added after the action of alamethicin was complete. Panel B: (○) no additions to the medium; (●) 3 nmol of CCP/mg of protein was added at 5 min; (□) 3 nmol of CCP/mg of protein was added at 60 min.

an enzyme requirement in a way that is influenced by uncoupler. However, it is known that the mitochondrial Ca^{2+} uniporter is poorly reversible under this condition (*31*) and that a fraction of the endogenous Ca^{2+} remains in mitochondria under conditions similar to those employed (*32*). Accordingly, Figure 2 also shows the effect of alamethicin plus EGTA on the activity of PLA_2. As with the PTP, alamethicin forms pores in the mitochondrial inner membrane, and these allow internal and external solutes of molecular weight less than ~1700 to equilibrate their distribution (*33*, *34*). Thus mitochondria are fully uncoupled by alamethicin, and when it is present, EGTA should be fully equilibrated will all pools of Ca^{2+}. PLA_2 activity determined in the presence of alamethicin and EGTA is even higher than that seen in the presence of uncoupler plus EGTA, while the advantage of a high ionic strength is maintained (Figure 2C).

The action of alamethicin in stimulating PLA_2 activity is not unique, in that opening the PTP has an equivalent effect (Figure 3A). In addition, there is no apparent timing requirement to obtain an active enzyme in that uncoupling by a protonophore is equally effective when it occurs after 5 min of incubation or after 60 min (Figure 3B). Thus, it is clear that the activity does not require Ca^{2+}, that it is seen

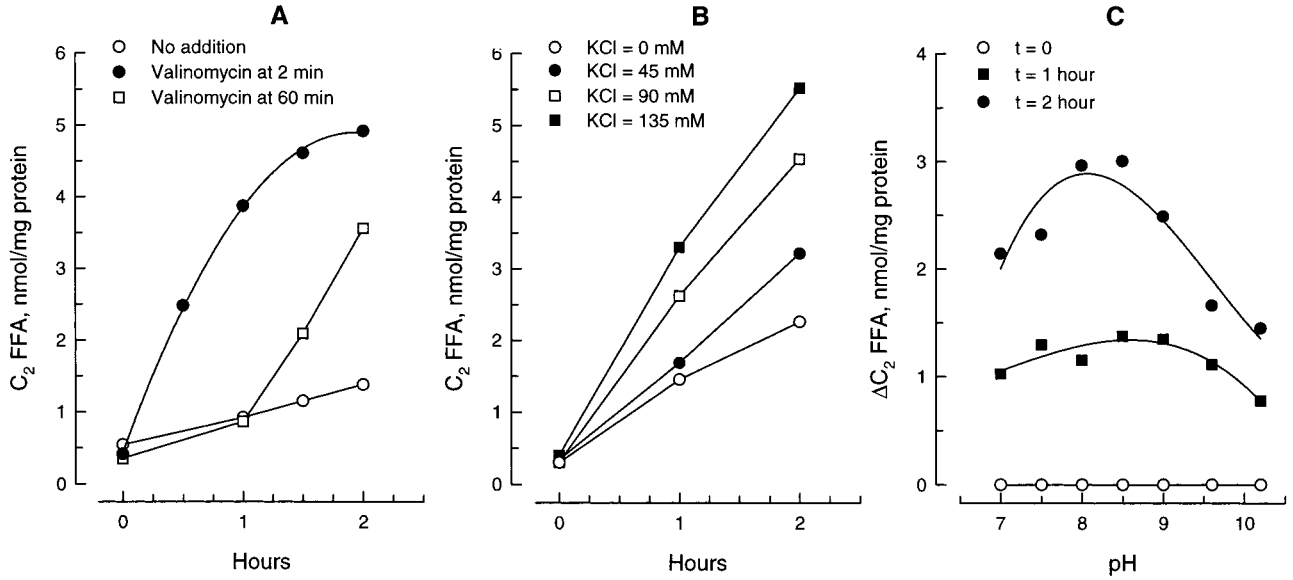


FIGURE 4: Effects of K^+ and pH on activity. Panel A: Mitochondria were prepared and incubated in MSH-based media. Key: (○) no additions after mitochondria; (●) 0.1 nmol of valinomycin/mg of protein was added at 2 min; (□) 0.1 nmol of valinomycin/mg of protein was added at 60 min. Panel B: Mitochondria were prepared in MSH medium and were incubated in media containing the indicated concentrations of KCl. These media were made by mixing MSH- and KCl-based media to give the KCl concentration desired while maintaining an osmotic pressure of 300 mOsM. EGTA (0.5 mM) was also present. PLA_2 was activated with alamethicin, which was added at 2 min and at 1 μg/mg of protein. Panel C: Mitochondria were prepared in MSH-based medium. They were incubated in a modified KCl-based medium which contained 5 mM each of Mes, Hepes, and Ches to allow buffering at the pH values shown. CCP (3 nmol/mg of protein) and 0.1 nmol of valinomycin/mg of protein were added at the beginning to equilibrate transmembrane gradients of H^+ and K^+ and to uncouple the mitochondria. (○, ■, and ●) Product levels observed at 0, 1.0, and 2.0 h, respectively.

in uncoupled mitochondria, and that it is more active in a KCl medium compared to a medium of low ionic strength.

Insight as to why activity is affected by the medium conditions used to isolate and store mitochondria, and to assay activity, is provided by Figure 4. Mitochondria maintained and incubated in MSH media lose a substantial fraction of endogenous K^+ (*14*; data not shown). Simply providing valinomycin to allow such mitochondria to reaccumulate K^+ activates PLA_2, although the full extent of this effect is not maintained (Figure 4A). Part of the response to valinomycin may reflect an increased matrix K^+ level, since mitochondria that are treated with alamethicin show a dependence of activity on K^+ concentration (Figure 4B). However, the response likely also reflects changing bioenergetic parameters in that activity displays an alkaline pH optimum (Figure 4C). Accordingly, the increased matrix pH that is expected to accompany the valinomycin-mediated K^+ accumulation should contribute to higher activity. Increased matrix pH is expected to lower membrane potential, and this consideration may also favor high activity, given that uncoupling stimulates activity (Figure 2).

Regarding activation by Ca^{2+}, panels A and B of Figure 5 reiterate that Ca^{2+} accumulation and alamethicin-mediated uncoupling are similarly effective in activating PLA_2, regardless of whether low or high ionic strength conditions are employed. In addition, it is seen that Ca^{2+} added together with alamethicin is more effective than either agent utilized alone (Figure 5A,B). The latter finding is particularly apparent at low ionic strength, and when data are expressed as a percent increase produced by Ca^{2+} in alamethicin-treated mitochondria, compared to the activity seen with alamethicin alone (Figure 5C).

The actions of inhibitors were examined, and Western blot analysis was employed to determine the type of PLA_2 that is responsible for Ca^{2+}-independent activity. Figure 6 shows that BEL is the most potent inhibitor among the compounds that were tested. While BEL inhibits a number of lipid metabolizing enzymes, such as phosphotidate phosphohydrolase, it is relatively specific for Ca^{2+}-independent PLA_2 ($iPLA_2$) when compared to $sPLA_2$ and cytoplasmic PLA_2 ($cPLA_2$) (*35*, *36*). Dithiothreitol (DTT) is a classic inhibitor of $sPLA_2$ and acts by reducing disulfide linkages that are critical for maintaining activity (*37*). It is not very potent against the mitochondrial activity (Figure 6B). On the how hand, *p*-BPB is also effective as an inhibitor of s-type enzymes (*37*), and this compound partially inhibited the mitochondrial activity (Figure 6). $AACOCF_3$ is often employed as an inhibitor of $cPLA_2$ (*38*) but can have inhibitory activity toward $iPLA_2$ under some conditions (*39*). In our hands, it stimulated the mitochondrial activity, while this did not occur with a long-chain acyl alcohol [16:0(OH)] (Figure 6). The latter finding is of interest because both compounds should perturb the membrane acyl group region in a similar manner. The sulfhydryl reagents *N*-ethylmaleimide (NEM) and dithionitrobenzoic acid (DTNB) were partially effective, and this was also true for trifluoperazine (TFP). TFP has been used as an inhibitor of the MTP and the phospholipid degradation that accompanies the phenomenon (*14*, *29*).

Thus, the actions of BEL and DTT support the possibility that the Ca^{2+}-independent activity reflects the presence of an $iPLA_2$ in mitochondria, but the actions of some inhibitors might be seen as reasons to doubt that interpretation. Such doubts are relieved, however, by the Western blot analysis shown in Figure 7. A primary antibody directed against a synthetic peptide that corresponds to a partial sequence of the $iPLA_2$ from $P388D_1$ cells recognizes a protein in these mitochondria. $P388D_1$ is macrophage-like cell line, and the $iPLA_2$ from that source was the first one discovered (*36*,

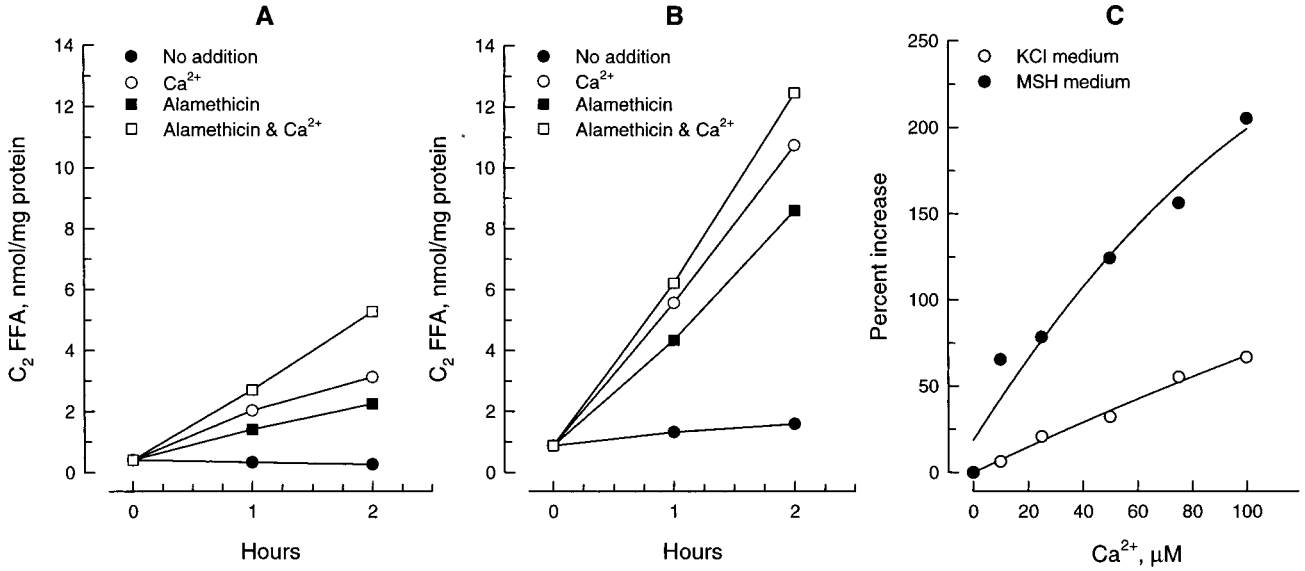
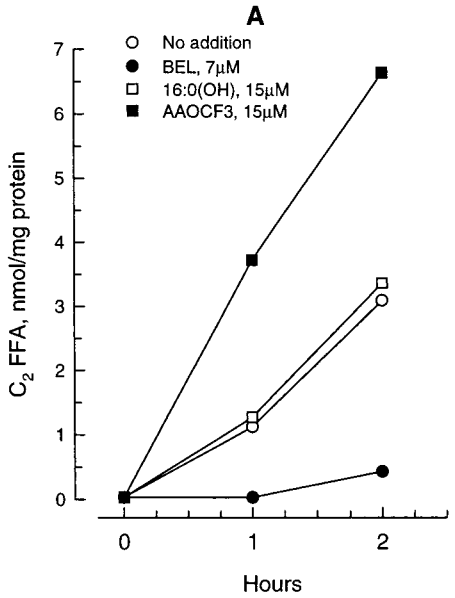


FIGURE 5: Relationship between the Ca^{2+}-dependent and Ca^{2+}-independent activities. A single mitochondrial preparation was divided in half following the low-speed centrifugation, and the mitochondria were sedimented as usual. Thereafter, the preparation was completed using MSH medium for one half and KCl medium for the other half. Subsequent incubations were conducted in MSH- or KCl-based media in panels A and B, respectively. Key for both panels: (●) no additions after mitochondria; (○) 80 μM $CaCl_2$ was added at 2 min; (■) 1 μg of alamethicin/mg of protein was added at 3 min; (□) 80 μM $CaCl_2$ and 1 μg of alamethicin/mg of protein were added at 2 and 3 min, respectively. Panel C: Values were obtained from experiments such as those shown in panels A and B, with both $CaCl_2$ and alamethicin employed, except that the $CaCl_2$ was varied as shown. Key: (○) KCl-based medium was employed; (●) MSH-based medium was employed.



B

Inhibitor	Activity, %	Known Target
No addition	100	N.A.
BEL, 7μM	12	$iPLA_2$
$AACOF_3$, 15μM	215	cyto PLA_2
16:0(OH), 15μM	109	membrane
NEM, 1mM	40	sulfhydryl
DTT, 1mM	87	$sPLA_2$
DTNB, 1mM	76	histidine
p-BPB, 0.4 mM	25	$sPLA_2$
TFP, 40 μM	40	MPT

FIGURE 6: Action of inhibitors on the Ca^{2+}-independent activity. Mitochondria were purified by Percoll density gradient centrifugation and were thereafter stored and incubated in KCl-based media. Panel A: PLA_2 was activated by alamethicin, which was added at 4 min and at 1 μg/mg of protein. The medium contained potential inhibitors as indicated in the figure. Panel B summarizes the effectiveness of all inhibitors tested. In all cases the inhibitor was added 1 min after the addition of mitochondria.

40). It is classified as a type VIA PLA_2 and has a molecular weight of ≥80000, which generally corresponds to that of the major band seen in Figure 7. The band is present in mitochondria prepared by the standard procedure (lane 9), after Percoll gradient purification (lanes 6 and 7), and when mitochondria have been disrupted by sonication (lane 5). In the membrane and soluble fractions obtained from disrupted mitochondria the band is primarily seen in the membrane fraction (lanes 3 and 4), but a faint band at the same molecular weight appears in the soluble fraction (lanes 1 and 2). Marker enzyme analysis showed that the soluble fraction contained the malate dehydrogenase activity and still contained ~10% of the monoamine oxidase activity. The former finding shows that sonication had released the soluble proteins. The latter indicates that a minor component of the membrane fraction remained in the supernatant following centrifugation (data not shown). Thus, the putative mitochondrial $iPLA_2$ is predominately membrane associated, but we cannot exclude the possibility that a small fraction is soluble.

In addition to the major band, faint bands are seen having molecular weights near 42000 and 30000 (Figure 7). The larger of these is a soluble component and has been detected by others utilizing this antibody (*41*, *42*). The other is seen

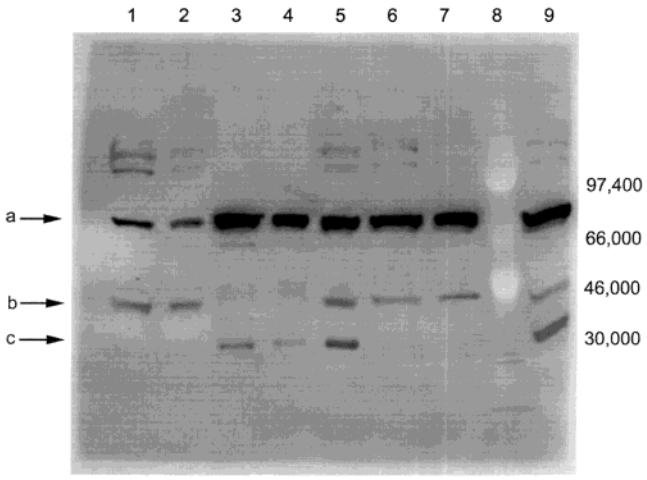


FIGURE 7: A membrane-associated mitochondrial protein is recognized by antibody against a known $iPLA_2$. Western blot analysis was carried out as described in Experimental Procedures using samples prepared as also described therein. In reverse order, the lane designations are as follows: lane 9, intact mitochondria; lane 8, molecular weight markers; lanes 6 and 7, intact Percoll purified mitochondria; lane 5, sonicated Percoll purified mitochondria; lanes 3 and 4, mitochondrial membranes; lanes 1 and 2, mitochondrial soluble fraction. Lanes 9, 6, 5, 4, and 2 contained equal equivalents of original mitochondria, equal to 40 μg of mitochondrial protein. Lanes 7, 3, and 1 contained two times that amount of mitochondrial equivalents.

only after mitochondria have been sonicated. It is not clear if these are relevant proteins or degradation products.

DISCUSSION

Following our abbreviated descriptions (*27*, *28*), this is the first demonstration of a Ca^{2+}-independent PLA_2 activity in mitochondria. Questions that arise in view of the finding include the following: (1) is this truly a mitochondrial activity or might it be associated with contaminating structures; (2) what is the relationship of the activity to the mitochondrial $sPLA_2$ that was identified previously; and (3) what is the relationship of the present activity to $iPLA_2$s from other sources? Regarding the first question, the present data strongly indicate that the activity is indeed mitochondrial and that it is most likely associated with the inner membrane. Data supporting these interpretations include the marked effects produced by agents and conditions that alter the bioenergetic status of mitochondria. Thus, a chemical uncoupler (CCP) that collapses the protonmotive force by transporting protons into the matrix space is an activator (Figures 2 and 5), as is alamethicin, which uncouples by forming large pores in the inner membrane (Figures 2, 3, and 5). Opening the PTP and adding alamethicin are equally effective (Figure 3), and activity is furthermore stimulated by valinomycin (Figure 4). The latter agent is an electrogenic K^+ ionophore that alters bioenergetic parameters (particularly membrane potential and pH gradient) upon transporting K^+ into the matrix space of the energized organelle. In general, these agents and conditions would not have marked effects on the low level contaminants that are found in standard mitochondrial preparations (microsomes, lysosomes, peroxisomes) and, therefore, would not be expected to influence the activity of phospholipases that they may contain.

To further examine the location of the Ca^{2+}-independent PLA_2 activity, we compared the properties of standard mitochondrial preparations to those that were further purified by Percoll density gradient centrifugation. The latter procedure is very effective at reducing the already low levels of contaminants found in standard preparations (e.g., ref *12*). No significant differences in specific activities were observed when the two preparations were compared, and there was no indication that activity is differently regulated following density gradient purification (data not shown). Thus, on the basis of measurements of activity and factors that regulate activity it seems clear that the PLA_2 of interest is truly mitochondrial. The immunoanalysis data shown in Figure 7 also support this interpretation, as further described below.

Regarding the relationship between the Ca^{2+}-independent enzyme and the known mitochondrial $sPLA_2$, indications from activity measurements alone are equivocal. The fact that Ca^{2+} increases the activity seen in alamethicin-treated mitochondria (Figure 5) could indicate that the two activities arise from distinct proteins. However, the Ca^{2+} requirement of the $sPLA_2$ varies markedly with substrate composition and may reflect, in part, effects of Ca^{2+} on physical properties of the lipid substrate, including phase state, packing density, and membrane surface charge (*43*−*45*). Accordingly, on the basis of Figures 1−5 alone, it is also possible that the known $sPLA_2$ can function in a Ca^{2+}-independent manner, when acting on its normal substrate, at high ionic strength, and that this has heretofore escaped detection.

The latter possibility is discounted, however, when the inhibitor sensitivity profile of the Ca^{2+}-independent activity (Figure 6) and the immunoanalysis data (Figure 7) are also considered. The inhibitor BEL is routinely used in a diagnostic manner to distinguish between processes mediated by $iPLA_2$ and $sPLA_2$ or $cPLA_2$, and the sensitivity of the mitochondrial activity to BEL is among the highest reported (*36*). In contrast, the actions of other inhibitors examined do not make a strong case for the activity arising from either an $sPLA_2$ or a $cPLA_2$, although the inhibitory activity of *p*-BPB is deserving of further comment. *p*-BPB is a histidine reagent that inhibits $sPLA_2$ by reacting with an active site histidine (*37*). It is generally ineffective against $iPLA_2$ or $cPLA_2$, which utilize an active site serine. Nevertheless, *p*-BPB might certainly react with a histidine removed from the active site in such enzymes and alter activity by changing the tertiary structure. Thus, partial inhibition of the mitochondrial activity by *p*-BPB does not necessarily conflict with the strong inhibition produced by BEL, which indicates that the mitochondrial activity is an authentic $iPLA_2$.

Regarding the immunoanalysis data, they show that antibody against a known $iPLA_2$ recognizes one or more mitochondrial proteins and that the dominant example displays a molecular weight in the same range as that of the known enzyme (Figure 7). As is true with PLA_2 activity per se, the prevalence of this protein is unaffected by Percoll gradient purification, indicating that it also is an authentic component of mitochondria. At $\geq$80000, the indicated molecular weight far exceeds that of the mitochondrial $sPLA_2$ ($\sim$14000), supporting the notion that most of the activity seen here is from the putative $iPLA_2$ and not from the $sPLA_2$ operating in a Ca^{2+}-independent manner.

The immunoanalysis data furthermore indicate that the $iPLA_2$ is primarily membrane bound, presumably to the inner membrane, given the controlling effects of mitochondrial energetic status on activity discussed above. The human gene

encoding the $iPLA_2$ (tType VIA) that is recognized by the antibody employed here gives rise to five putative splice variants, with two, or possibly three, of these possessing activity (*46*). The two known active proteins are designated type VIA-1 and -2, with the latter protein possessing a 54 amino acid insertion that is not present in the former. Rats appear to produce analogous active proteins (*41*). Type VIA-1 is thought to be predominantly a soluble protein (*36*). When type VIA-2 is overexpressed in COS-7 cells, it is found to be membrane associated. Recently, a membrane-bound $iPLA_2$ (type VIB) was described by Gross and co-workers (*47*). This protein shares sequence homology with type VIA only at the active site, a nucleotide binding site, and another nine amino acid sequence. It has a signal sequence directing it to peroxisomes. Given that the $iPLA_2$ of interest here is primarily membrane bound and is recognized by an antibody to type VIA, it may be the VIA-2 $iPLA_2$ that is found in mitochondria. However, following the recommendations of Dennis and co-workers (*48*), isolation, sequencing, and cloning will be required before this interpretation is verified and before the activity can be classified with certainty.

It is interesting to consider the possible functions that might be carried out by the $iPLA_2$ and the $sPLA_2$ in vivo. $iPLA_2$ from other sources are thought to participate in phospholipid remodeling via the Lands cycle, and there are indications that they might also participate in cell signaling, apoptosis, and other processes (reviewed in ref *36*). Phospholipid remodeling is an unlikely function for the enzyme in liver mitochondria, given that the acyl-coenzyme A−1-acyllysophospholipid acyltransferase activity that is required to complete the Lands cycle is not present (*12*). A role in controlling the level of phospholipids in mitochondria is a possibility, although there is no obvious relationship between that role and the factors that regulate activity.

Indeed, it is useful to reiterate the regulatory features of the $iPLA_2$ while considering potential functions. Given the intramitochondrial location and membrane association, the enzyme in situ has ready access to phospholipid substrate, experiences the preferred high ionic strength environment, is at or near the optimum pH, and has no requirement for high Ca^{2+} levels. Nevertheless, it is inactive unless the mitochondria are deenergized, and this is a novel property among the presently known properties of PLA_2 enzymes. The way in which that deenergization produces activation cannot be specified at present but might reflect a structural element that senses membrane potential directly, an association with respiratory chain complexes and a sensitivity to their redox status, an effect of redox status on disulfide bonds within the enzyme per se, or possibly regulation through changing nucleotide ratios, given that an $iPLA_2$ from $P388D_1$ cells contains a nucleotide binding site (*49*, *50*). Whatever the linkage may be, the function of this enzyme should relate directly to the bioenergetic status of mitochondria. An attractive hypothesis in our view is that the $iPLA_2$ initiates the autolysis/autophagy of poorly functioning mitochondria, as was initially described in the introduction. Under this scenario, poorly functioning mitochondria would equate with diminished energization, and the $iPLA_2$ would become more active as a consequence. Rising levels of FFA would favor an open PTP (*33*), as would the state of diminished energization per se (*51*), and upon occurrence of the MPT the $iPLA_2$ activity would be fully expressed. Activity is sufficient to hydrolyze a large fraction of total mitochondrial phospholipids on a time scale of hours. In conjunction with proteases and nucleases, it might fully remove a disabled mitochondrion (*2*), or it might lead to fusion with a lysosome and autophagy (*3*). Participation of the apparent $iPLA_2$ is favored in comparison to the $sPLA_2$ because it does not imply a steep rise in the mitochondrial Ca^{2+} level prior to attaining activity. It furthermore does not require Ca^{2+} disregulation at the cellular level to maintain activity once the MPT has occurred. Thus, the $iPLA_2$ would be fully functional in deenergized mitochondria during cellular apoptosis, for example.

The function of the $sPLA_2$ continues to be obscure, given the dependence of activity on high Ca^{2+} levels (Figure 5; *29*, *44*, *45*). Such levels are expected during necrosis, so the activity should be expressed during that process, although this presumably would have no intended function. Under physiological conditions, mitochondria located near to Ca^{2+} conducting channels experience high local Ca^{2+} concentrations and accumulate above average levels of the cation (*52*). The $sPLA_2$ activity might be realized in those mitochondria, and the arachidonic acid produced might lead to the production of lipid mediators and their further participation in cell signaling. These interpretations and possibilities are under further investigation.

NOTE ADDED IN PROOF

The presence of an $iPLA_2$ in rabbit heart mitochondria has been discovered independently and reported by Williams and Gottlieb (*54*).

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REFERENCES

1. Bernardi, P., Basso, E., Colonna, R., Costantini, P., Di Lisa, F., Eriksson, O., Fontaine, E., Forte, M., Ichas, F., Massari, S., Nicolli, A., Petronilli, V., and Scorrano, L. (1998) *Biochim. Biophys. Acta 1365*, 200−206.
2. Broekemeier, K. M., Klocek, C. K., and Pfeiffer, D. R. (1998) *Biochemistry 37*, 13059−13065.
3. Lemasters, J. J., Nieminen, A.-L., Qian, T., Trost, L. C., Elmore, S. P., Nishimura, Y., Crowl, R. M., Cascio, W. E., Bradham, C. A., Brenner, D. A., and Herman, B. (1998) *Biochim. Biophys. Acta 1366*, 177−196.
4. Skulachev, V. P. (1998) *Biochim. Biophys. Acta 1363*, 100−124.
5. Klionsky, D. J., and Emr, S. D. (2000) *Science 290*, 1717−1721.
6. Pfanner, N., and Neupert, W. (1990) *Annu. Rev. Biochem. 59*, 331−353.
7. Pfeiffer, D. R., Kaufman, R. F., and Lardy, H. A. (1978) *J. Biol. Chem. 253*, 4165−4171.
8. Pfeiffer, D. R., Schmid, P. C., Beatrice, M. C., and Schmid, H. H. O. (1979) *J. Biol. Chem. 254*, 11485−11494.
9. Schmid, P. C., Pfeiffer, D. R., and Schmid, H. H. O. (1981) *J. Lipid Res. 22*, 882−886.
10. Gunter, T. E., and Pfeiffer, D. R. (1990) *Am. J. Physiol. 258*, C755−C786.
11. Broekemeier, K. M., Dempsey, M. E., and Pfeiffer, D. R. (1989) *J. Biol. Chem. 264*, 7826−7830.
12. Broekemeier, K. M., Schmid, P. C., Dempsey, M. E., and Pfeiffer, D. R. (1991) *J. Biol. Chem. 266*, 20700−20708.
13. Scorrano, L., Penzo, D., Petronilli, V., Pagano, F., and Bernardi, P. (2002) *J. Biol. Chem. 276*, 12035−12040.
14. Broekemeier, K. M., and Pfeiffer, D. R. (1995) *Biochemistry 34*, 16440−16449.

15. Bernardi, P., Broekemeier, K. M., and Pfeiffer, D. R. (1994) *J. Bioenerg. Biomembr. 26*, 509−517.
16. Hamidinia, S. A., Erdahl, W. L., Chapman, C. J., Taylor, R. W., and Pfeiffer, D. R. (2002) *J. Biol. Chem.* (in preparation).
17. De Winter, J. M., Vianen, G. M., and Van den Bosch, H. (1982) *Biochim. Biophys. Acta 712*, 332−341.
18. Natori, Y., Karasawa, K., Arai, H., Tamori-Natori, Y., and Nojima, S. (1983) *J. Biochem. (Tokyo) 93*, 637.
19. Van Schaik, R. H. N., Verhoeven, N. M., Neijs, F. W., Aarsman, A. J., and Van den Bosch, H. (1993) *Biochim. Biophys. Acta 1169*, 1−11.
20. Hayakawa, M., Kudo, I., Tomita, M., Nojima, S., and Inoue, K. (1988) *J. Biochem. (Tokyo) 104*, 772.
21. Dennis, E. A. (1994) *J. Biol. Chem. 269*, 13057−13060.
22. Murakami, M., Kudo, I., and Inoue, K. (1995) *J. Lipid Mediat. Cell Signal. 12*, 119−130.
23. Lenting, H. B. M., Neys, F. W., and Van den Bosch, H. (1987) *Biochim. Biophys. Acta 917*, 178−185.
24. Waite, M., and Sisson, P. (1971) *Biochemistry 10*, 2377−2383.
25. Bjornstad, P. (1966) *J. Lipid Res. 7*, 612−620.
26. Komada, M., Kudo, I., and Inoue, K. (1990) *Biochem. Biophys. Res. Commun. 168*, 1059−1065.
27. Broekemeier, K. M., and Pfeiffer, D. R. (1999) *Biophys. J. 76*, A229−A229.
28. Pfeiffer, D. R., Gunter, T. E., Eliseev, R., Broekemeier, K. M., and Gunter, K. K. (2002) *IUBMB Life 52*, 205−212.
29. Broekemeier, K. M., Schmid, P. C., Schmid, H. H. O., and Pfeiffer, D. R. (1985) *J. Biol. Chem. 260*, 105−113.
30. Schlenk, H., and Gellerman, J. (1960) *Anal. Chem. 32*, 1412−1414.
31. Litsky, M. L., and Pfeiffer, D. R. (1997) *Biochemistry 36*, 7071−7078.
32. Gunter, T. E., Chace, J. H., Puskin, J. S., and Gunter, K. K. (1983) *Biochemistry 22*, 6341−6351.
33. Pfeiffer, D. R., Gudz, T. I., Novgorodov, S. A., and Erdahl, W. L. (1995) *J. Biol. Chem. 270*, 4923−4932.
34. Sansom, M. S. P. (1993) *Eur. Biophys. J. 22*, 105−124.
35. Hazen, S. L., Zupan, L. A., Weiss, R. H., Getman, D. P., and Gross, R. W. (1991) *J. Biol. Chem. 266*, 7227−7232.
36. Winstead, M. V., Balsinde, J., and Dennis, E. A. (2000) *Biochim. Biophys. Acta 1488*, 28−39.
37. Kudo, I., Murakami, M., Hara, S., and Inoue, K. (1993) *Biochim. Biophys. Acta 1170*, 217−231.
38. Street, I. P., Lin, H.-K., Laliberté, F., Ghomashchi, F., Wang, Z., Perrier, H., Tremblay, N. M., Huang, Z., Weech, P. K., and Gelb, M. H. (2001) *Biochemistry 32*, 5935−5940.
39. Ackermann, E. J., Conde-Frieboes, K., and Dennis, E. A. (1995) *J. Biol. Chem. 270*, 445−450.
40. Ackermann, E. J., Kendall, J. M., and Dennis, E. A. (1994) *J. Biol. Chem. 269*, 9227−9233.
41. Forsell, P. K. A. L., Kennedy, B. P., and Claesson, H. E. (1999) *Eur. J. Biochem. 262*, 575−585.
42. Liu, S. J., and McHowat, J. (1998) *Am. J. Physiol. Heart Circ. Physiol. 44*, H1462−H1472.
43. De Winter, J. M., Korpancova, J., and Van den Bosch, H. (1984) *Arch. Biochem. Biophys. 234*, 243−252.
44. Lenting, H. B. M., Neys, F. W., and Van den Bosch, H. (1988) *Biochim. Biophys. Acta 961*, 129−138.
45. Zurini, M., Hugentobler, G., and Gazzotti, P. (1981) *Eur. J. Biochem. 119*, 517−521.
46. Larsson, P. K. A., Claesson, H. E., and Kennedy, B. P. (1998) *J. Biol. Chem. 273*, 207−214.
47. Mancuso, D. J., Jenkins, C. M., and Gross, R. W. (2000) *J. Biol. Chem. 275*, 9937−9945.
48. Six, D. A., and Dennis, E. A. (2000) *Biochim. Biophys. Acta 1488*, 1−19.
49. Lio, Y. C., and Dennis, E. A. (1998) *Biochim. Biophys. Acta 1392*, 320−332.
50. Ma, Z., Wang, X., Nowatzke, W., Ramanadham, S., and Turk, J. (1999) *J. Biol. Chem. 274*, 9607−9616.
51. Petronilli, V., Cola, C., and Bernardi, P. (1993) *J. Biol. Chem. 268*, 1011−1016.
52. Rizzuto, R., Bastianutto, C., Brini, M., Murgia, M., and Pozzan, T. (1994) *J. Cell Biol. 126*, 1183−1194.
53. Igbavboa, U., and Pfeiffer, D. R. (1988) *J. Biol. Chem. 263*, 1405−1412.
54. Williams, S. D., and Gottlieb, R. A. (2002) *Biochem. J. 362*, 23−32.

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