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Ca^{2+}-independent Cytosolic Phospholipase A_2 from Macrophage-like $P388D_1$ Cells

ISOLATION AND CHARACTERIZATION*

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A novel form of an ATP-regulated, oligomeric, Ca^{2+}-independent phospholipase A_2 ($iPLA_2$) has been purified from the cytosol of the murine macrophage-like cell line $P388D_1$. The purification procedure included ammonium sulfate precipitation and sequential column chromatography on octyl-Sepharose, ATP-agarose, Mono Q fast protein liquid chromatography (FPLC), and hydroxyapatite FPLC. The resulting enzyme preparation was purified over 400,000-fold with a final specific activity of approximately 5 μmol/min/mg using a mixed micelle assay system of Triton X-100 and dipalmitoyl phosphatidylcholine (PC). The purified enzyme was Ca^{2+}-independent and did not show a preference for either *sn-2* arachidonic acid or *sn-1* alkyl-ether containing phospholipids when utilizing mixed micelles as substrate. It was found to hydrolyze dipalmitoyl-PC ~4-fold faster than 1-palmitoyl-2-arachidonyl-PC and ~15-fold faster than 1-*O*-hexadecyl-2-arachidonyl-PC. Triton X-100 increased the $P388D_1$ $iPLA_2$ activity with optimal activity found at a Triton/phospholipid molar ratio of 4:1. The purified enzyme was activated 2–6-fold by ATP as well as other di- and triphosphate nucleosides. This activation was sensitive to the concentration of Triton X-100 present in the assay. SDS-polyacrylamide gel electrophoresis carried out on the purified enzyme yielded a single major band at a molecular weight of about 80,000. However, radiation inactivation experiments, carried out on the cell homogenate, demonstrated a target size of 337 ± 25 kDa, indicating that the catalytically active $iPLA_2$ exists as a large oligomeric complex, either through self-aggregation or association of the enzyme with other proteins.

As part of our continuing study of phospholipid metabolism, its regulation, and subsequent involvement in the release of important biological mediators, we have attempted to identify and characterize the many different phospholipases that exist in the macrophage-like cell line $P388D_1$. Early on, we identified at least four different phospholipase A (1) and two very active lysophospholipase (2) activities in this single type of cell. Two of the phospholipase A activities are optimal at alkaline pH. They include a membrane-associated Ca^{2+}-dependent phospholipase A_2 (PLA_2)[1] and a Ca^{2+}-independent PLA_2 ($iPLA_2$). The other two phospholipases are active at acidic pH, and at least one has PLA_1 activity (1). In addition, through polymerase chain reaction cloning,[2] we have demonstrated that the recently discovered 85-kDa, low [Ca^{2+}] requiring cytosolic PLA_2 ($cPLA_2$) (3, 4) is also present in these cells. Of the enzymes listed above, the membrane-associated PLA_2 (5–7) and the two lysophospholipases (2, 8, 9), have been purified and characterized. In the present study, we report the purification and characterization of a cytosolic $iPLA_2$ from these cells.

$iPLA_2$s have been shown to exist in a variety of tissues (10, 11) and species (10, 12), and they have been implicated in a number of important cellular processes (13–15). However, unlike their Ca^{2+}-dependent counterparts, very little is known about these enzymes and only a few $iPLA_2$s have been purified to date (16–19). Of those purified, the most extensively studied is the plasmalogen-specific $iPLA_2$ from canine myocardium (18, 20, 21).

The data reported herein on the $P388D_1$ $iPLA_2$, which is obtained from a single cell source, demonstrate that it and the myocardial $iPLA_2$ are unique among known PLA_2s in that they are both modulated by ATP, and they both form high molecular weight complexes of roughly 400,000. Furthermore, we show using radiation inactivation techniques that an active form of the $P388D_1$ $iPLA_2$ is oligomeric. These interesting characteristics suggest that the $P388D_1$ $iPLA_2$ may play a key regulatory role in lipid metabolism as well as a presumed role in arachidonic acid release for eicosanoid production.

While these characteristics suggest regulatory similarities of the $P388D_1$ and the myocardial $iPLA_2$s, the $P388D_1$ $iPLA_2$ is distinct from the myocardial $iPLA_2$ in that it remains sensitive to ATP activation throughout the purification, and the formation of its high molecular weight complex is necessary to maintain full catalytic activity. It is not known at this time whether these differences reflect unique regulatory properties of two distinct enzymes or if they reflect purifications of similar enzymes that are present in two distinct states: the canine myocardial $iPLA_2$ as a labile, homogenous, catalytic protein and the murine $P388D_1$ $iPLA_2$ as a stable, functional, regulatory/catalytic complex. In either case, the characteristics of $P388D_1$ $iPLA_2$, including phospholipid preference, positional specificity, pH optimum, and detergent sensitivity, are different from those reported for the purified myocardial $iPLA_2$. This suggests that the $P388D_1$ $iPLA_2$ has unique properties; a preliminary report of these results has been presented (22).

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[1] The abbreviations used are: PLA_2, phospholipase A_2; $iPLA_2$, calcium-independent PLA_2; $cPLA_2$, cytosolic PLA_2; PLA_1, phospholipase A_1; ATP(PCP), adenosine 5′-(β,γ-methylene)triphosphate; PC, phosphatidylcholine; PE, phosphatidylethanolamine; FPLC, fast protein liquid chromatography; PAGE, polyacrylamide gel electrophoresis; DPPC, dipalmitoyl phosphatidylcholine; DTT, dithiothreitol; LS-1, low speed supernatant; LS-P, ammonium sulfate precipitate.

[2] S. J. Barbour and E. A. Dennis, unpublished results.

EXPERIMENTAL PROCEDURES

Materials

1-Palmitoyl-2-[1-^{14}C]palmitoyl-*sn*-glycero-3-phosphorylcholine and other radiolabeled phospholipids were purchased from DuPont NEN. The unlabeled phospholipids were purchased from Avanti Polar Lipids, except for 1-*O*-hexadecyl-2-arachidonyl-*sn*-glycero-3-phosphorylcholine, which was purchased from Biomol (Plymouth Meeting, PA). The octyl-Sepharose CL-B, Mono Q HR 5/5 FPLC, and Superose 6 HR 10/30 were from Pharmacia LKB Biotechnology Inc. Adenosine 5'-triphosphate agarose (attachment at N-6, catalogue No. A-9264) and rabbit muscle phosphofructokinase were purchased from Sigma. Dithiothreitol (DTT) was ultragrade from Calbiochem. The 8–16% Tris-glycine gradient gels, SDS-PAGE molecular weight markers, and the hydroxyapatite column were purchased from Novex (San Diego, CA). All other reagents were analytical grade or better.

Phospholipase Assays

Phospholipase A_2 Assay—The standard iPLA$_2$ assay contained 400 μM Triton X-100, 100 μM dipalmitoyl phosphatidylcholine (PC), 5 mM EDTA, and 100 mM Hepes (pH 7.5) in a final volume of 500 μl. In addition, 0.8 mM ATP was present in all assays unless otherwise indicated and described in the legends. Assays were initiated by the addition of enzyme to the substrate mixture and were incubated at 40 °C for 15–60 min with agitation.

Because the Triton X-100 was used throughout the purification to stabilize the enzyme, its subsequent addition in the assay was unavoidable. Therefore, the substrate was prepared such that a final concentration of 400 μM Triton X-100 was maintained in the assay. This was typically achieved by preparing the substrate as described below with 300 μM Triton X-100 and asssaying with 50 μl of the column fractions (containing 1 mM Triton X-100).

The substrate was prepared by evaporating the required amounts of dipalmitoyl-PC and 1-palmitoyl-2-[1-^{14}C]palmitoyl-*sn*-glycero-3-phosphorylcholine (200,000 cpm per assay) to dryness under a stream of N_2. The dried phospholipids were resuspended with 450 μl of a buffer containing appropriate amounts of Hepes, EDTA, and Triton X-100. Mixed micelles were formed by a combination of heating (above 40 °C), vortexing, and water bath sonication until the solution clarified. In assays that used vesicle substrates, the phospholipids were resuspended in assay buffer without Triton X-100, and vesicles were formed by sonication at room temperature in a MSE probe sonicator until the solution clarified (about 5 min).

The reaction was stopped by adding 2.5 ml of the Dole reagent (24), (2-propanol:heptane:0.5 M H_2SO_4, 400:100:20, v/v/v) and vortexing. The product mixture was subsequently processed according to the modified (23) Dole extraction system (24) as previously described (5). Data are presented as the rate of hydrolysis of substrate (adjusted for the rate of hydrolysis in a control lacking enzyme).

In a few experiments, the TLC extraction method was utilized instead of the Dole extraction method. In these experiments, the standard assay was quenched by the addition of 1.0 ml of chloroform:methanol: acetic acid (2:4:1, v/v/v), followed by the addition of 0.5 ml of chloroform and 0.5 ml of water. The reaction was subsequently processed as previously described (5). Briefly, the organic layer containing the reactants and the products was dried, resuspended in 30 μl of chloroform: methanol (2:1, v/v), and spotted onto a Silica Gel G plate (Analtech). The lipids were separated by a solvent composed of chloroform:methanol: acetic acid:water (25:15:4:2, v/v/v/v). The lipids were visualized with I_2 vapor, and the zones corresponding to fatty acid, lyso-PC and, dipalmitoyl-PC were scraped and counted.

Lysophospholipase Assay—The lysophospholipase assay contained 100 μM 1-palmitoyl-2-hydroxy-*sn*-glycerol-3-phosphorylcholine (200,000 cpm of [1-^{14}C]palmitoyl-2-hydroxy-*sn*-glycerol-3-phosphorylcholine), 400 μM Triton X-100, 5 mM EDTA, 0.8 mM ATP, and 100 mM Hepes (pH 7.5). The substrate was prepared as described under "PLA$_2$ assay" and assayed according to Zhang and Dennis (2) using the modified Dole extraction method.

Protein Purification

Preparation of Homogenate—P388D$_1$ cells obtained from the American Type Culture Collection were grown to confluency and harvested as described by Ross *et al.* (1). The cells were washed once in serum-free media and adjusted to a concentration of 3.3×10^7 cells/ml with homogenization buffer (10 mM Hepes (pH 7.5), 1 mM EDTA, 1 mM DTT, and 0.34 M sucrose). The cells were lysed using nitrogen cavitation at 650 p.s.i., in a Parr cell disruption bomb (Parr Instruments). This whole cell lysate was immediately centrifuged at 1,000 × *g*, 5 °C, for 30 min, to remove unbroken cells and debris. The resulting low speed supernatant (LS-1) was utilized for the purification and was routinely stored at −20 °C for up to 6 months without any significant loss of activity. All of the following steps in the purification were carried out at 4–8 °C.

Ammonium Sulfate Precipitation—Typically, the LS-1 derived from 6 × 10^9 cells (190 ml) was thawed, and sufficient solid ammonium sulfate was added to bring its concentration to 40% of saturation. This solution was stirred slowly overnight and centrifuged the following day (12,000 × *g*, for 30 min). The resulting supernatant was discarded, and the pellet (LS-P) was resuspended in 190 ml of buffer A (10 mM Hepes (pH 7.5), 1 mM EDTA, 1 mM DTT, 1 mM ATP, and 1 mM Triton X-100).

Octyl-Sepharose Column—The LS-P was loaded directly onto an octyl-Sepharose column (5.0 × 10.0 cm), at a flow rate of 6 ml/min. Prior to addition of the protein, the column was equilibrated with at least 10 column volumes of buffer B (buffer A + 350 mM NaCl), to ensure the equilibration of the Triton X-100. After the LS-P was loaded, the column was washed with buffer B at 10 ml/min until the A_{280} rose slightly, typically 3–4 column volumes, and eluted with buffer C (10 mM Hepes (pH 7.5), 1 mM EDTA, 1 mM DTT, and 1 mM Triton X-100). The eluent was collected in 15-ml fractions. The active fractions from the octyl-Sepharose column were pooled (approximately 250 ml) and concentrated (to about 15 ml) using an Omega stirred cell (from Filtron) with a 10,000 molecular weight cut-off membrane. The concentrate was resuspended with 200 ml of buffer C and concentrated again (to about 25 ml). This procedure was carried out to remove ATP from the sample which would otherwise interfere with the affinity column used in the following step.

ATP-Agarose—After the second concentration step, the sample was immediately applied to an ATP-agarose column (1.0 × 6.0 cm), which was previously equilibrated with buffer C at a flow rate of 1 ml/min. After loading, the column was washed with buffer C (10 ml), followed by buffer D (buffer C + 10% glycerol), until the A_{280} leveled off. The iPLA$_2$ activity was eluted from the column at a reduced flow rate of 0.5 ml/min with buffer D containing 1 mM ATP and was collected in 5-ml fractions.

Mono Q FPLC—The active fractions eluted from the ATP-agarose column were pooled and passed over a Mono Q HR 5/5 FPLC column (flow rate 1 ml/min, 2-ml fractions) that had been pre-equilibrated with at least 60 ml of buffer containing 5 mM Tris-HCl (pH 7.5), 1 mM EDTA, 1 mM DTT, 1 mM ATP, 1 mM Triton X-100, and 10% glycerol. The enzyme was eluted with the same buffer, and the active fractions in the first peak were pooled.

Hydroxyapatite FPLC—The pooled fractions from the Mono Q column were loaded onto a hydroxyapatite FPLC column (7.5 mm × 8.0 cm, 0.3 ml/min) which was previously equilibrated with buffer consisting of 10 mM KH_2PO_4 (adjusted to pH 7.5 with NaOH), 1 mM EDTA, 1 mM DTT, 1 mM Triton X-100, and 10% glycerol. The column was washed with 2 volumes of equilibration buffer and eluted with a linear gradient of 10–300 mM KH_2PO_4 (pH 7.5) containing 1 mM EDTA, 1 mM DTT, 1 mM Triton X-100, and 10% glycerol.

SDS-Polyacrylamide Gel Electrophoresis—SDS-PAGE was routinely carried out following the method of Laemmli (25), and the bands were visualized by silver staining according to Hancock and co-workers (26). Molecular weight markers were from Novex.

Protein Determination—Protein concentrations were determined using the Bio-Rad Protein Assay Kit for steps 1–3. Because of the low levels, Quantigold (Diversified Biotech) was used for steps 4–6. Bovine serum albumin from Bio-Rad was used as the standard.

Radiation Inactivation

P388D$_1$ crude cell homogenate (LS-1) was prepared as described above. Aliquots (500 μl, approximately 2 mg of protein) of LS-1 were placed in glass ampules (Kimble LAB-12012), frozen at −70 °C, and sealed using an oxygen-gas flame. The samples were maintained at −70 °C except during irradiation, which was carried out at −135 °C. Irradiation of the samples was performed using high energy electrons as previously described (27). After irradiation, the vials were opened and purged with nitrogen prior to thawing. The iPLA$_2$ activity was determined using the standard assay conditions.

Target analysis of the radiation inactivation data was performed as previously described (27). Target sizes were determined from the slope of the inactivation curves using a least squares fit. The target size is reported ± the standard deviation, which represents the variation between experiments.

Phosphofructokinase Affinity Elution

The affinity elution of phosphofructokinase from the ATP-agarose column (attachment N-6) was carried out following the method of Hazen

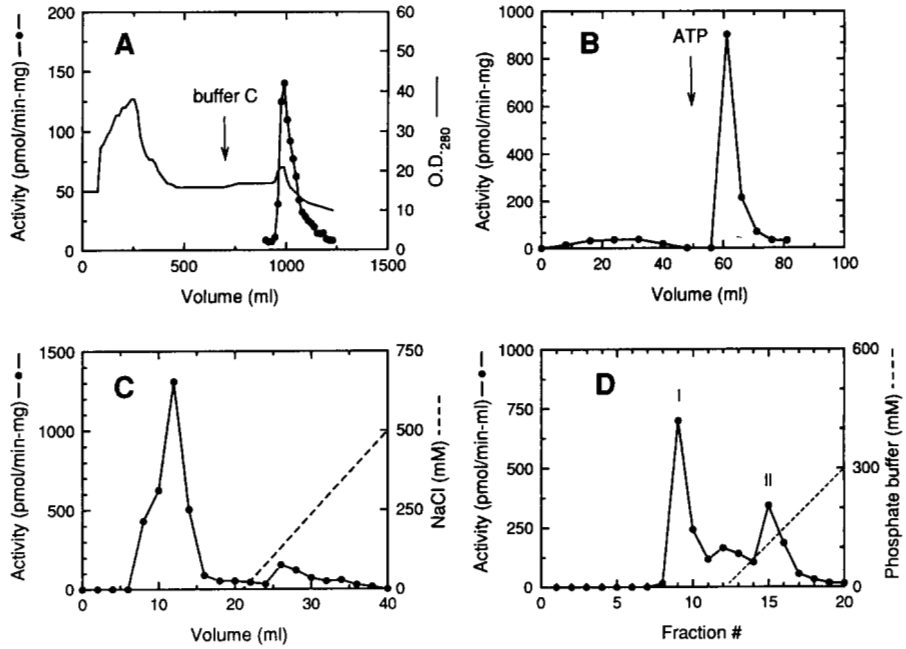

FIG. 1. **Typical column chromatography profiles of the Ca^{2+}-independent PLA_2 from $P388D_1$ cells.** *A*, octyl-Sepharose CL-B; *B*, ATP-agarose; *C*, Mono Q-FPLC; *D*, hydroxyapatite FPLC. The first active peak from this column is referred to as peak I and was taken as the purified Ca^{2+}-independent PLA_2. The optical density of the protein was not recorded in column profiles shown in *B–D* due to absorption interference by the Triton X-100.

and Gross (21). Approximately 0.6 mg of rabbit muscle phosphofructokinase (previously exchanged into buffer E which contains 10 mM imidazole (pH 8.3), 25% glycerol, and 1 mM DTT) or 20 ml of LS-P (resuspended in buffer E) were loaded onto the column (0.8 ml) at a flow rate of 2 min/ml. The column was then washed with 60 ml of buffer E followed by sequential washes of buffer E with the following additions; 50 μM fructose 6-phosphate (12 ml), no addition (5 ml), 50 μM ADP (12 ml), no addition (5 ml), a mixture of 50 μM fructose 6-phosphate and 50 μM ADP (12 ml), a mixture of 10 mM fructose 6-phosphate and 100 μM ADP (12 ml), and 1 mM ATP (12 ml). The elution profile of the phosphofructokinase was followed by SDS-PAGE, and the $iPLA_2$ activity was followed utilizing standard assay conditions.

Western Blotting

Western analysis was carried out using conventional methods. Proteins were separated on 10% SDS-PAGE and transferred to an Immobilon-P membrane (Millipore), and immunoblots were carried out according to Hazen and Gross (21). The phosphofructokinase antibody utilized was a generous gift of Richard Gross, Washington University School of Medicine.

RESULTS

Protein Purification—Initial attempts to purify the $iPLA_2$ from $P388D_1$ cells were frustrated by the low abundance and instability of the enzyme. The instability problem was overcome by including Triton X-100 in the column buffers. We found that Triton X-100 stabilized the $iPLA_2$ activity and increased recoveries of the enzyme during each chromatography step. Additionally, Triton X-100 increased the observed $iPLA_2$ activity in a concentration-dependent manner when included in the assay (see below).

Typical column profiles are shown in Fig. 1, *A–D*, and data from the purification of a single enzyme preparation are shown in Table I. The first chromatography step utilized an octyl-Sepharose column where roughly 95% of the contaminating protein eluted in the load and wash fractions. The majority of the $iPLA_2$ activity was retained by the column and was eluted in a low salt buffer (buffer C) (Fig. 1*A*). Typical recoveries from this column ranged from 50–90%. The $iPLA_2$ fractions from the octyl-Sepharose column were pooled, contaminating ATP was removed, and the protein was applied to an ATP-agarose affinity column. A sharp peak of $iPLA_2$ activity eluted from this column in the presence of ATP, yielding an 800-fold purification step (Fig. 1*B*). We routinely found that at least 10–20% of the $iPLA_2$ activity passed through the ATP column without binding. These fractions also failed to bind the ATP affinity column when reapplied, indicating that this is a distinct pool of $iPLA_2$ activity or $iPLA_2$ with tightly bound ATP.

The $iPLA_2$ activity that was eluted by ATP from the affinity column was chromatographed over a Mono Q FPLC column. A broad peak of $iPLA_2$ emerged in the wash fractions (Fig. 1*C*). This step achieved a 4-fold purification with recovery of roughly 70–90% of the loaded $iPLA_2$ activity. Finally, the Mono Q fractions were applied to a hydroxyapatite FPLC column. Two peaks of $iPLA_2$ activity consistently eluted from this column. Peak I eluted in the isocratic wash of 10 mM phosphate, represented ~50–80% of the loaded activity, and was apparently homogenous. Peak II eluted between 10 and 300 mM phosphate along with other proteins (Figs. 1*D* and 2), and comprised ~10–20% of the loaded activity. Therefore, the $iPLA_2$ in peak II was discarded, and the purified $iPLA_2$ in peak I was characterized further. This $iPLA_2$ was purified over 400,000-fold with a final specific activity of roughly 5 μmol/min/mg. It was fairly stable and could be stored at −20 °C for at least 2 weeks.

Assessment of purity was carried out using SDS-PAGE under reducing conditions. In three separate purifications, peak I from the hydroxyapatite column displayed a single major band near M_r = 80,000 when standard silver staining techniques were utilized. SDS-PAGE carried out on the individual fractions from the hydroxyapatite column (Fig. 2) demonstrated that the intensity of this band correlated with the elution profile of the $iPLA_2$ activity from this column (Fig. 1*D*). In some preparations, we observed a few other minor bands, but they did not correlate with $iPLA_2$ activity. Attempts were made to elute the purified $iPLA_2$ from a native gel in order to determine if the M_r = 80,000 band represented the $iPLA_2$, but these were unsuccessful.

Dependence of $iPLA_2$ Activity on Triton X-100 and ATP—Early in our studies, we discovered that both Triton X-100 and ATP stimulated the $iPLA_2$ activity from $P388D_1$ cells. In addition, the extent of ATP activation was sensitive to the concentration of Triton X-100 present in the assay. The ammonium sulfate precipitate (LS-P) was assayed with 100 μM phospholipid vesicles alone and then in the presence of increasing amounts of Triton X-100, forming mixed micelles with molar ratios ranging from 2:1–12:1. These assays were carried out in

TABLE I

Typical purification of the $iPLA_2$ from the macrophage-like cell line P388D$_1$

The data are taken from a single enzyme preparation starting with 6×10^9 cells and carried through each purification step. For steps 5 and 6, only a portion of the material was utilized, and the table entries were adjusted accordingly.

Step	Total protein	Total activity	Yield	Specific activity	Purification
	mg	*nmol/min*	*%*	*nmol/min/mg*	*-fold*
1. LS-1	1,130	13.6	100	0.012	1
2. LS-P	547	15.2	112	0.028	2
3. Octyl-Sepharose	26	10.3	76	0.40	33
4. ATP-agarose	0.02	6.4	47	320	26,000
5. Mono Q-FPLC	0.0036	4.8	35	1,300	110,000
6. Hydroxyapatite-FPLC	0.0002	1.1	8	5,500	460,000

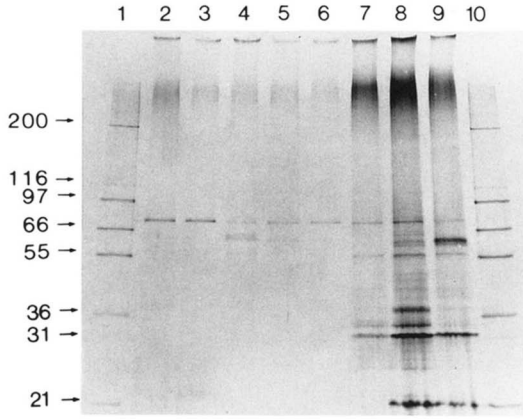

FIG. 2. **SDS-PAGE of hydroxyapatite column fractions.** 1.0-ml aliquots from the hydroxyapatite column fractions (Fig. 1*D*), were exchanged into water using a Centricon 10 (Amicon), lyophilized, and resuspended in 15 μl of reducing sample buffer. The samples were loaded on a 8–16% gradient gel, separated by SDS-PAGE under reducing conditions, and visualized by silver stain. *Lanes 1* and *10* are molecular weight markers, and *lanes 2–9* contain hydroxyapatite fractions 9–16.

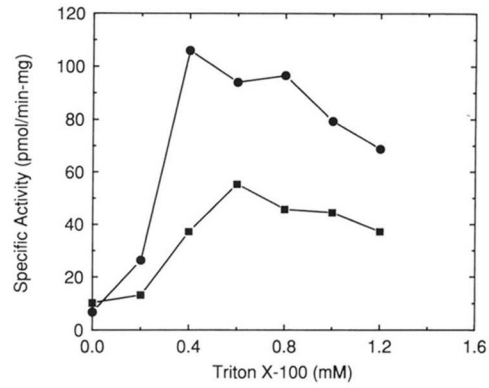

FIG. 3. **Effects of Triton X-100 and ATP on Ca^{2+}-independent PLA_2.** LS-P was prepared as described under "Experimental Procedures," and the pellet was resuspended in a buffer consisting of 10 mM Hepes (pH 7.5), 1 mM EDTA, and 1 mM DTT. LS-P was assayed with 100 μM DPPC vesicles (the data point lacking Triton X-100) and Triton X-100/DPPC mixed micelles in the presence (●) or absence (■) of 0.80 mM ATP.

the presence or absence of added ATP (Fig. 3).

In both cases, the LS-P displayed an initial increase in activity with increasing amounts of Triton X-100 followed by a decrease. In the absence of added ATP, maximal activity was found at a Triton X-100 concentration of ~0.6 mM (molar ratio 6:1) and was about 5-fold greater than that observed with vesicles. In the presence of added ATP, the $iPLA_2$ activity displayed a second increase above that observed with Triton X-100 alone. This additional increase varied at different Triton X-100 concentrations. In this experiment, there was a 2.8-fold activation due to ATP at a Triton X-100 concentration of 0.4 mM. At higher concentrations of Triton X-100, the ATP activation fell to about 1.8-fold. Interestingly, in the absence of Triton X-100 (vesicle assay), no ATP activation could be observed.

Maximal $iPLA_2$ activity was observed with the combination of 0.4 mM Triton X-100 and 0.8 mM ATP and was roughly 10-fold greater than the $iPLA_2$ activity observed with vesicles of DPPC. Experiments carried out with either whole cell lysate, LS-1, or partially purified $iPLA_2$ from the ATP-agarose column resulted in similar trends.

Nucleotide Activation—In seven separate experiments, we found that the purified $iPLA_2$ (peak I, hydroxyapatite column) was activated 2–6-fold by the addition of 0.8 mM ATP under standard assay conditions. To investigate the specificity of this activation, the purified enzyme was assayed in the presence of various nucleotides, shown in Table II. The $iPLA_2$ showed little specificity for nucleoside triphosphates; roughly equal activations were observed with ATP, GTP, CTP, and UTP. $iPLA_2$ activity was also activated by a nonhydrolyzable analogue of ATP (ATP(PCP)), indicating that a classic phosphorylation reaction was not required. In addition, ADP activated the enzyme while

TABLE II

Nucleotide activation of the purified $iPLA_2$

$iPLA_2$ activity was determined in triplicate using the standard assay conditions with 2.2 ng of the purified $iPLA_2$

Nucleotide (0.8 mM)	Activity	% control
	μmol/min/mg	
None	7.0	100
AMP	8.6	120
ADP	19	270
ATP	19	270
GTP	18	260
CTP	19	270
UTP	14	200
ATP(PCP)	19	270

AMP did not, suggesting that a di- or triphosphate moiety is required for activation.

In some enzyme preparations, there was no detectable ATP activation of the purified enzyme. This was apparently caused by some ATP eluting with the enzyme from the hydroxyapatite column. This problem was overcome by either rechromatographing the enzyme on the hydroxyapatite column or concentrating the sample once and diluting the concentrate back to the original volume in buffer devoid of ATP. Under these circumstances, a minimum 2-fold activation was achieved.

Molecular Size of Active $iPLA_2$—Although SDS-PAGE experiments carried out on the purified enzyme suggested a molecular weight of 80,000, several other experiments utilizing both crude and purified $iPLA_2$ yielded molecular weight estimates of 300,000–400,000. In one experiment, the $iPLA_2$ from the cell homogenate was first clarified by centrifugation at $100,000 \times g$ and then filtered through a 300,000 molecular weight cut-off membrane. 80% of the total $iPLA_2$ activity (assayed in the presence of ATP), was retained upon filtration,

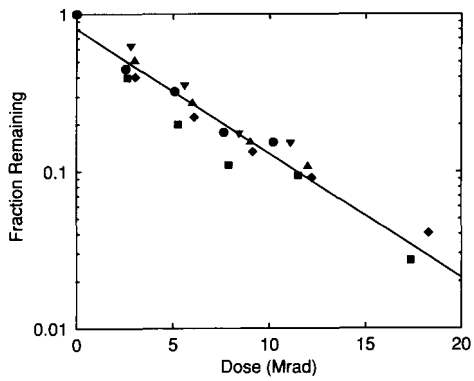

FIG. 4. **Radiation inactivation.** LS-1 was irradiated as described under "Experimental Procedures" over a range of 1–23 megarads. The results of five separate experiments are shown. The activity of the irradiated samples was measured and compared to that of unirradiated controls, which represent 100% activity. The remaining enzyme activities were plotted against the radiation dose received. The assay conditions were varied as described. ▼, 4:1 mixed micelles, 100 μl of protein/assay; ▲ and ●, 6:1 mixed micelles, 100 μl of protein/assay; ♦ and ■, 6:1 mixed micelles, 200 μl of protein/assay. Each point represents the average of either duplicates or quadruplicates.

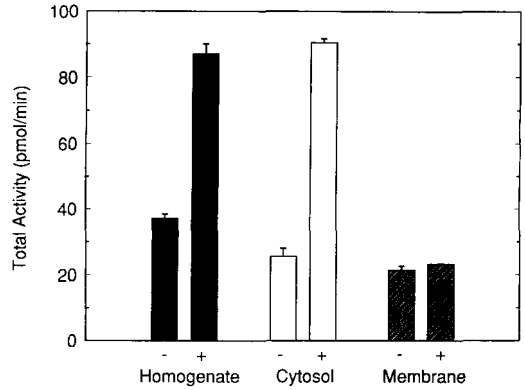

FIG. 5. **Subcellular distribution of the Ca^{2+}-independent PLA_2 activity in P388D_1 cells.** LS-1 was subjected to ultracentrifugation (100,000 × *g*, 1 h, 0–5 °C) to separate the cytosol and membrane fractions. The resulting pellet was resuspended to its original volume with homogenization buffer. Assays were carried out in the absence (−) or presence (+) of 0.80 mM ATP using standard assay conditions.

while only 17% passed through the membrane. The retentate was activated 4-fold by ATP, while the filtrate was not. In a second experiment, the molecular weight of the purified $iPLA_2$ was estimated under nondenaturing conditions, by using a Superose 6 FPLC gel filtration column. The enzyme activity eluted in a very broad peak, centered at M_r = 400,000, and it maintained its ATP activation.

These results suggest that the $iPLA_2$ is present as a high molecular weight complex. This could be due to an aggregation of the $iPLA_2$ protein alone or it could also be due to aggregation of the $iPLA_2$ with other proteins. In either case, it is not clear whether the aggregation plays any role in the catalytic activity of the $iPLA_2$. To help clarify this point, radiation inactivation experiments were carried out on LS-1. Radiation inactivation determines the molecular size of the catalytically active unit (28). LS-1 was irradiated over a range of 1–23 megarads and assayed under standard assay conditions in the presence of 0.8 mM ATP. The average of five separate experiments gave a target size of 337 ± 25 kDa (Fig. 4). This implies that a 340-kDa complex is required for the ATP-activated $iPLA_2$ activity. This complex could be formed through either self-aggregation or association of the enzyme with other proteins.

Cellular Distribution of the $iPLA_2$ in the Cytosol and Membrane Fractions—To determine if the $iPLA_2$ activity is associated with the soluble or membrane fractions of P388D_1 cells, the low speed crude homogenate was centrifuged at 100,000 × *g*. The resulting supernatant (the cytosolic enriched fraction) and pellet (the membrane/organelle enriched fraction) were assayed using standard assay conditions in the presence or absence of ATP (Fig. 5). 80% of the total activity (measured in the presence of ATP) was found in the cytosolic fraction, while the other 20% was associated with the membranes and organelles. The cytosolic activity was activated by ATP, approximately 3.2-fold, while the membrane-associated activity was not. These data indicate that the purified $iPLA_2$ in peak I from the hydroxyapatite column is a cytosolic protein.

Also shown in Fig. 5 is the total activity of the starting material (homogenate). The total activity detected in the homogenate was less than the combined cytosolic and membrane-associated $iPLA_2$ activities. This is probably due to the dilution of the radiolabeled phospholipid in the assay by the endogenous cellular phospholipid present in both the homogenate and membrane fractions, but reduced in the cytosolic fraction.

The presence of a non-ATP-sensitive membrane-associated $iPLA_2$ correlates with the other observations that a fraction of the P388D_1 $iPLA_2$ did not bind to the ATP affinity column (Fig. 1*B*) and that a fraction of the P388D_1 homogenate passed through a M_r = 300,000 membrane and was insensitive to ATP. Collectively, these results suggest the presence of at least two Ca^{2+}-independent PLA_2 activities in P388D_1 cells.

Substrate Specificity—When the purified $iPLA_2$ was assayed under standard assay conditions using mixed micelles of 100 μM phospholipid and 400 μM Triton X-100, the enzyme showed no preference for either *sn-2* arachidonic acid- or *sn-1* alkyl-ether-containing phospholipids. It hydrolyzed dipalmitoyl-PC roughly 4-fold faster than 1-palmitoyl-2-arachidonyl-PC and roughly 15-fold faster than 1-*O*-hexadecyl-2-arachidonyl-PC (Table III). The enzyme also showed little preference for either choline- or ethanolamine-containing phospholipids, since it hydrolyzed both arachidonyl-PC and arachidonyl-PE at roughly the same rates.

Preliminary studies were also carried out with partially purified $iPLA_2$ utilizing vesicle substrates as well as mixed micelles. With vesicles, the apparent substrate preference was reversed with 1-*O*-hexadecyl-2-arachidonyl-PC and 1-palmitoyl-2-arachidonyl-PC > dipalmitoyl-PC, even though the specific activity of the P388D_1 $iPLA_2$ using 1-*O*-hexadecyl-2-arachidonyl-PC vesicles was only 20% the specific activity using dipalmitoyl-PC mixed micelles. Because it has been well established with the extracellular PLA_2s that the nature of the substrate interface can greatly affect the enzyme activity observed, caution should be used in reaching a conclusion about the true substrate preference for any such enzymes.

To determine if the purified enzyme was specific for the hydrolysis of the *sn-2* position of phospholipids, we checked for both lysophospholipase activity and phospholipase A_1 (PLA_1) activity. As shown in Table III, the enzyme demonstrated a small activity toward [1-^{14}C]palmitoyl-lyso-PC; however, it was 20-fold lower than the $iPLA_2$ activity. The positional specificity of the $iPLA_2$ was determined by following the release of radiolabeled products from both 1-palmitoyl-2-[1-^{14}C]palmitoyl-*sn*-glycero-3-phosphorylcholine and 1-[1-^{14}C]palmitoyl-2-[1-^{14}C]-palmitoyl-*sn*-glycero-3-phosphorylcholine under standard assay conditions utilizing the TLC extraction method. Using a set amount of purified $iPLA_2$, the apparent rate of release of the *sn-2* radiolabeled fatty acid from the single labeled phospholipid was found to be about 540 pmol/min, while the concomitant rate of formation of 2-[1-^{14}C]palmitoyl-lyso-PC, due to PLA_1 activity, was found to be about 87 pmol/min. Thus, it would appear that the PLA_1 activity is about 15% of the PLA_2

TABLE III
Specificity of iPLA$_2$ toward various substrates

Peak I from the hydroxyapatite column was assayed with 100 μM indicated phospholipid (in the presence of 0.8 mM ATP) using standard assay conditions. Activities were normalized to that of dipalmitoyl-PC.

Substrate	Activity
	%
Dipalmitoyl-PC	100
1-Palmitoyl-2-arachidonyl-PC	27
1-Palmitoyl-2-arachidonyl-PE	43
1-*O*-Hexadecyl-2-arachidonyl-PC	6
Palmitoyl-lyso-PC	5

activity. However, the sequential action of a PLA$_1$ followed by a lysophospholipase would have underestimated the PLA$_1$ activity observed in this experiment. Therefore, an identical experiment was carried out utilizing the double-labeled phospholipid. In this case, the combined apparent rate of release of fatty acids from both the *sn-1* and *sn-2* positions was found to be about 630 pmol/min. This rate is the same as the combined rate of the PLA$_2$ and the PLA$_1$ activity observed with the single-labeled phospholipid ruling out a significant contribution from sequential PLA$_1$ and lysophospholipase activity.

Comparison to Myocardial iPLA$_2$ Model—Of particular relevance to the data presented in this paper was the report that the crude myocardial iPLA$_2$ enzyme (M_r = 40,000) is modulated by a high molecular weight, ATP binding, regulatory protein (20) suggested to be phosphofructokinase (21), a tetrameric enzyme composed of M_r = 85,000 subunits. Accordingly, we tried to determine if the M_r = 80,000 P388D$_1$ iPLA$_2$ is actually a phosphofructokinase regulatory subunit that is associated with a M_r = 40,000 iPLA$_2$ catalytic subunit.

In some of the enzyme preparations, SDS-PAGE of peak I samples gave a very lightly stained M_r = 40,000 band. Using the catalytic/regulatory model, this band could represent the iPLA$_2$. It appears that the majority of this band elutes in peak II, which contains only a small fraction of the iPLA$_2$ activity (Figs. 1*D* and 2). However, it is possible that an M_r = 80,000 regulatory protein dissociates from the catalytic iPLA$_2$ on the hydroxyapatite column and elutes in peak I, while the majority of the catalytic iPLA$_2$ elutes in peak II. The low amount of iPLA$_2$ activity observed in peak II would then be due to a limited amount of the regulatory protein. To investigate this possibility, peak II from the hydroxyapatite column was examined in more detail. We found that the magnitude of ATP activation (2–6-fold) and the nucleotide specificities of peak II were identical with peak I. Gel filtration chromatography of peak II resulted in a profile similar to peak I, giving a broad peak centered at M_r = 400,000. When fractions from peak II were added to fractions from peak I, no additional increase in iPLA$_2$ activity was observed. Furthermore, the total recoveries observed for the hydroxyapatite column were typically 76% (n = 13), which is inconsistent with the separation of two proteins that are necessary to support full catalytic activity. Therefore, it is unlikely that a regulatory and catalytic subunit separate on the hydroxyapatite column. However, we cannot rule out the possibility that the iPLA$_2$ elutes as a functional regulatory/catalytic complex in both peaks I and II, and the M_r = 40,000 band observed in peak II is a contaminating protein.

In order to determine if the M_r = 80,000 protein is phosphofructokinase, Western analysis was carried out utilizing rabbit muscle phosphofructokinase antibody kindly provided by Richard Gross, Washington University School of Medicine. Under the conditions employed, no cross-reactivity was observed with ~100 ng of the purified P388D$_1$ iPLA$_2$ even though ~5 ng of rabbit muscle phosphofructokinase did cross-react with the antibody. Of course, this is not definitive because there could be species or isoform differences to prevent cross-reactivity (29–31).

Alternatively, attempts were made to elute the P388D$_1$ iPLA$_2$ from the ATP-agarose column using affinity elution conditions unique to phosphofructokinase isoforms (32) and which were used by Hazen and Gross (21) to demonstrate the association of the catalytic myocardial iPLA$_2$ with the M_r = 85,000 phosphofructokinase. Utilizing conditions shown to bind the phosphofructokinase/myocardial iPLA$_2$ complex, rabbit muscle phosphofructokinase bound to the ATP-agarose column and the majority eluted with a combination of ADP and fructose 6-phosphate as expected. However, under these conditions, the P388D$_1$ iPLA$_2$ activity failed to bind to the column. When 1.0 mM Triton X-100 was added to the buffers, ~21% of the iPLA$_2$ activity did bind to the affinity column. After sequential washes of fructose 6-phosphate, ADP, fructose 6-phosphate + ADP, and ATP, we found that ~9% of the iPLA$_2$ activity eluted with ADP alone, and the remaining 12% eluted with ATP. Finally, the column was run under the standard conditions employed in the purification of the P388D$_1$ iPLA$_2$ utilizing octyl-Sepharose fractions and again the iPLA$_2$ activity did not elute with the combination of ADP and fructose 6-phosphate, but did elute with 1 mM ATP. Thus, the P388D$_1$ iPLA$_2$ does not appear to behave identically with the myocardial phosphofructokinase/iPLA$_2$ complex, even though the existence of a P388D$_1$ phosphofructokinase/iPLA$_2$ complex cannot be excluded.

DISCUSSION

Macrophage iPLA$_2$—The purification of a cytosolic iPLA$_2$ from the macrophage-like cell line P388D$_1$ is reported. We have found that it is activated by nucleoside di- and triphosphates and is stabilized by and shows maximal activity in the presence of Triton X-100. Interestingly, the observed nucleotide activation is sensitive to the concentration of Triton X-100 present in the assay with no activation observed in the complete absence of Triton X-100. Additionally, the P388D$_1$ iPLA$_2$ will not bind to the ATP affinity column in the absence of Triton X-100. One explanation for these results is that Triton X-100 is stabilizing or facilitating a conformational change in the enzyme that is necessary for nucleotide binding. However, independent of nucleotide activation, Triton X-100 alone increased the observed iPLA$_2$ activity in a concentration-dependent manner. The apparent activation by Triton X-100 implies that the P388D$_1$ iPLA$_2$ prefers mixed micelles over vesicles, with the decrease in activity observed at higher concentrations of Triton X-100 consistent with surface dilution kinetics. Thus, the lack of ATP activation observed with vesicles may suggest that the nucleotide activation is sensitive to the physicochemical structure of the substrate. Because we are dealing with an enzyme that acts on a lipid-water interface, it is very difficult to distinguish between enzyme-detergent solubilization and substrate conformational effects. In fact, both of these factors may contribute to the observed phenomenon.

In attempting to determine the molecular mass of the iPLA$_2$, we obtained two different estimates. The purified enzyme preparation yielded a single major band on SDS-PAGE at M_r = 80,000 using standard silver staining techniques. The fact that the intensity of the M_r = 80,000 band correlated with the iPLA$_2$ activity in each of the fractions from the hydroxyapatite column suggests that it is likely to be associated with iPLA$_2$ activity, either as a regulatory protein or as a catalytic protein. However, Superose 6 FPLC gel filtration chromatography and membrane filtration indicated a M_r = 300,000–400,000 for the iPLA$_2$. The radiation inactivation studies also indicated a size of 337,000 for the catalytically active form of the enzyme and not 80,000. Since the irradiated samples were assayed under activating conditions (*i.e.* in the presence of Triton X-100 and

ATP), the single exponential inactivation curve reflects the survival of the highly active form of the enzyme. Thus, the 337-kDa target size must be interpreted as the mass required for the expression of this activated state. The simplest interpretation of the above findings would be that the P388D_1 iPLA_2 functions as a tetramer of homologous M_r = 80,000 subunits. This would also mean that the M_r = 80,000 peptides are responsible for the observed nucleotide activation. However, alternative interpretations include a complex containing four M_r = 80,000 regulatory peptides and one M_r = 40,000 catalytic peptide. In either case, it is possible that the mechanism of nucleotide activation is the functional coupling of these individual monomers into a more efficient oligomeric structure, and that Triton X-100 may play some role in these processes.

Other iPLA_2s—The P388D_1 iPLA_2 appears to be distinct from the other iPLA_2s that have been reported in the literature. The majority of these other enzymes can be easily distinguished from the P388D_1 iPLA_2 by their substrate specificities and enzyme characteristics. For example, Chap and co-workers (17) have reported the purification of an iPLA_2 from guinea pig intestine which has equal amounts of both PLA_2 and lysophospholipase activities, efficiently removing both fatty acid chains thereby converting diacyl-PC to glycerol phosphorylcholine. This enzyme was tightly associated with the membrane and appears to be a M_r = 97,000 protein. Another iPLA_2 has been reported in hamster heart (16). This cytosolic enzyme hydrolyzed both *sn-1* and *sn-2* fatty acids at equal rates, was inhibited by detergents, and migrated on SDS-PAGE as a M_r = 14,000 protein. Horrocks and co-workers (19) have reported two iPLA_2s from bovine brain, which displayed similar sensitivities to Triton X-100 as the P388D_1 enzyme. However, these enzymes were purified only 1,000-fold, with no reported substrate specificity studies or conclusive molecular weights, making it difficult to ascertain their relationship with the enzyme reported in this study.

The best characterized iPLA_2 is the plasmalogen-specific canine myocardial enzyme reported in an intriguing series of papers by Gross and co-workers (18, 20, 21). They have purified an ATP-insensitive, highly active iPLA_2 that is a M_r = 40,000 protein by SDS-PAGE using iodination techniques (18). This enzyme has a substrate preference for plasmalogen > *sn-1* alkyl-ether PC > diacyl PC, and it displayed a fatty acid preference for arachidonyl > palmitoyl. The myocardial iPLA_2 is inhibited by various detergents including Triton X-100, and, therefore, assays using mixed micelles were unsuccessful. In contrast, mixed micelles of DPPC and Triton X-100 seem to be the preferred substrate for the P388D_1 enzyme. Positional specificities of the two enzymes also differ. The P388D_1 iPLA_2 displayed ~5% lysophospholipase activity and ~15% PLA_1 activity, while the myocardial iPLA_2 had less than 1% lysophospholipase and no detectable PLA_1 activity. Finally, the P388D_1 iPLA_2 has a pH optimum of ~7.5, while the myocardial iPLA_2 pH optimum is ~6.5. Thus, it appears that the P388D_1 iPLA_2 and the myocardial iPLA_2 have distinct properties.

In addition, several experiments were carried out in order to ascertain whether the reported phosphofructokinase regulatory/iPLA_2 catalytic model proposed by Hazen and Gross (21) was relevant to the P388D_1 iPLA_2. Attempts to identify a phosphofructokinase protein in our preparations through Western analysis and specific affinity elution were unsuccessful. Although this model cannot be ruled out, we have been unable to obtain any direct evidence for the presence of separate catalytic and regulatory proteins in our system. However, it is intriguing to speculate that the data reported herein represent the purification of an enzyme similar to the purified myocardial iPLA_2, but which exists in a stable, functional complex with phosphofructokinase. If this is the case, our data demonstrate that, in P388D_1 cells, the formation of this complex is necessary for the full expression of the iPLA_2's ATP-activated state and that it has a functional size of about 340 kDa. In addition, our data raises several interesting questions as to the true substrate specificity of this enzyme, the importance of Triton X-100 in the stability and in the observed nucleotide activation, and the exact relationship between it and the myocardial iPLA_2.

Conclusion—Obviously, a better understanding of the mechanisms and components involved in nucleotide activation and oligomerization of the P388D_1 iPLA_2 is required. Such insight may help us better understand the mode of regulation utilized by this enzyme and its importance in modulating the iPLA_2 catalytic activity. In addition, because PLA_2s are thought to be responsible for the release of arachidonic acid and the subsequent formation of many important secondary messengers as well as to play a vital role in lipid metabolism, understanding the control mechanisms that may be relevant to these processes is of great interest. Indeed, the complex regulation observed with the P388D_1 iPLA_2 suggests that it may play a central role in phospholipid metabolism.

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