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Solubilization, Purification, and Characterization of a Membrane-bound Phospholipase A₂ from the P388D₁ Macrophage-like Cell Line*

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The release of free arachidonic acid from membrane phospholipids is believed to be the rate-controlling step in the production of the prostaglandins, leukotrienes, and related metabolites in inflammatory cells such as the macrophage. We have previously identified several different phospholipases in the macrophage-like cell line P388D₁ potentially capable of controlling arachidonic acid release. Among them, a membrane-bound, alkaline pH optimum, Ca²⁺-dependent phospholipase A₂ is of particular interest because of the likelihood that the regulatory enzyme has these properties. This phospholipase A₂ has now been solubilized from the membrane fraction with octyl glucoside and partially purified. The first two steps in this purification are butanol extractions that yield a lyophilized, stable preparation of phospholipase A₂ lacking other phospholipase activities. This phospholipase A₂ shows considerably more activity when assayed in the presence of glycerol, regardless of whether the substrate, dipalmitoylphosphatidylcholine, is in the form of sonicated vesicles or mixed micelles with the nonionic surfactant Triton X-100. Glycerol (70%) increases both the V_{max} and the K_m with both substrate forms, giving a V_{max} of about 15 nmol min⁻¹ mg⁻¹ and an apparent K_m of about 60 μM for vesicles and a V_{max} of about 100 nmol min⁻¹ mg⁻¹ and an apparent K_m of about 1 mM for mixed micelles. V_{max}/K_m is slightly greater for vesicles than for mixed micelles. The lyophilized preparation of the enzyme is routinely purified about 60-fold and is suitable for evaluating phospholipase A₂ inhibitors such as manoalide analogues. Subsequent steps in the purification are acetonitrile extraction followed by high performance liquid chromatography on an Aquapore BU-300 column and a Superose 12 column. This yields a 2500-fold purification of the membrane-bound phospholipase A₂ with a 25% recovery and a specific activity of about 800 nmol min⁻¹ mg⁻¹ toward 100 μM dipalmitoylphosphatidylcholine in mixed micelles. When this material was subjected to analysis on a Superose 12 sizing column, the molecular mass of the active fraction was approximately 18,000 daltons.

It is generally accepted that the biosynthesis of the prostaglandins and leukotrienes is dependent on the availability of free arachidonic acid derived from membrane phospholipids where it is normally found esterified in the sn-2 position (1, 2). Therefore, phospholipase A₂, which catalyzes the hydrolysis of the fatty acid in the sn-2 position of phospholipids, is likely to play a central role in the biosynthesis of the oxygenated products of arachidonic acid (3). Upon exposure to inflammatory stimuli, a variety of these oxygenated products has been shown to be released from macrophages, cells that are of paramount importance in inflammation and immune responses (4-7). Although phospholipase A₂ activities have been demonstrated to be present in various macrophage preparations (8-11), in general there is less information available about the enzymatic mechanism of arachidonate release from macrophages than from platelets (12, 13). To understand completely how arachidonic acid release is regulated, it is important to characterize the biochemical and enzymatic properties of the phospholipases that participate in this process. An ideal source of such enzymes is a macrophage-like cell line, because this source provides sufficient numbers of cells for the isolation of membrane-bound enzymes. In studies reported here, we have used the P388D₁ macrophage-like cell line because it is a homogeneous source of cells that can be grown in large numbers for enzyme preparation, and it can also be grown in monolayers for the study of ligand-induced prostaglandin generation.[1]

Previous work (14) on the phospholipases in the P388D₁ macrophage-like cells revealed that at least four different phospholipase A activities and at least one lysophospholipase activity (11) exist in various subcellular fractions of the cells. Of particular interest is the membrane-bound, Ca²⁺-dependent, alkaline pH optimum phospholipase A₂ because of its possible involvement in the regulation of prostaglandin and leukotriene production. Particular focus on membrane-bound phospholipases is warranted because of the high content of phospholipids containing arachidonic acid in the macrophage's membrane (4). Furthermore, stimulation of macrophages with immune complexes or zymosan, which are thought to bind to specific membrane receptors, shows an increased release of oxygenated arachidonic acid products (5-7). It has also been reported that an Fc receptor found on P388D₁ and murine macrophage cells possesses an intrinsic phospholipase A₂ activity which is activated when bound to aggregated IgG₂b (15, 16).

We have now succeeded in solubilizing this membrane-bound phospholipase with octyl glucoside and have prepared a partially purified, stable lyophilized enzyme preparation.

[1] E. A. Dennis, unpublished observations.

This preparation has no other phospholipase activities; it hydrolyzes only the fatty acid at the *sn*-2 position and has an absolute requirement for Ca^{2+}. As such, it provides a convenient and reliable source of membrane-bound phospholipase A$_2$ from a cell involved in inflammatory responses and prostaglandin production, and it is suitable for inhibitor studies. Kinetic characterization of this enzyme is described herein; an analysis of its activity toward arachidonoyl-containing substrates will be presented elsewhere. A preliminary report of these findings has been presented (17). This enzyme preparation was also a suitable starting point for high performance liquid chromatography (HPLC)[2] purification and size determination. These results are described herein. However, the amount of pure protein that could be reasonably obtained from the cell line source without undo labor made a detailed study of the highly purified enzyme less attractive at this time.

EXPERIMENTAL PROCEDURES[3]

RESULTS

We report here the development of a routine scheme for the solubilization and partial purification of a membrane-bound phospholipase A$_2$ from the macrophage cell line P388D$_1$. The scheme is summarized in Fig. 1 and the details are provided under "Experimental Procedures." Results of a typical purification are summarized in Table I. Novel or unusual aspects of certain steps are described in more detail below.

Solubilization of LP-1 with Octyl Glucoside—To solubilize the membrane proteins, octyl glucoside was employed as a detergent with the membrane-enriched fraction (LP-1) (14). The recovery of proteins in HS-1 was greater as the concentration of octyl glucoside was increased. However, the best yield and highest specific activity of phospholipase A$_2$ were obtained when 10 mM octyl glucoside was used. The enzyme activities of HS-1 and LP-1 were suppressed when the concentration of octyl glucoside in the assay mixture was at or above 10 mM, as shown in Fig. 2. Therefore, octyl glucoside must be dialyzed out of the HS-1 preparation to obtain enzyme activity under standard assay conditions. The enzyme activities of both preparations were found to be stable at −20 °C for at least 6 months. Octyl glucoside up to 100 mM in the assay did not affect fatty acid extraction in the Dole assay.

Extraction of HS-1 with Butyl Alcohol—After dialysis, HS-1 was mixed with 25% butanol and then centrifuged to separate the emulsion into two phases. Although the aqueous phase contained 70–75% of the protein, no phospholipase activity was found. However, when the butanol residue phase was suspended in ice-cold Hepes buffer to dissolve excess butanol, a protein precipitate was observed. This protein was pelleted by centrifugation and resuspended in 6 M urea buffer (Butanol Extract I or BE-I). About 20 to 25% of the protein and 40 to 60% of the enzyme activity originally in HS-1 was recovered in this solubilized fraction. The extraction of the enzyme into BE-I was more efficient when vortexed at room temperature for 30 s than when mixed at 4 °C for 30 min.

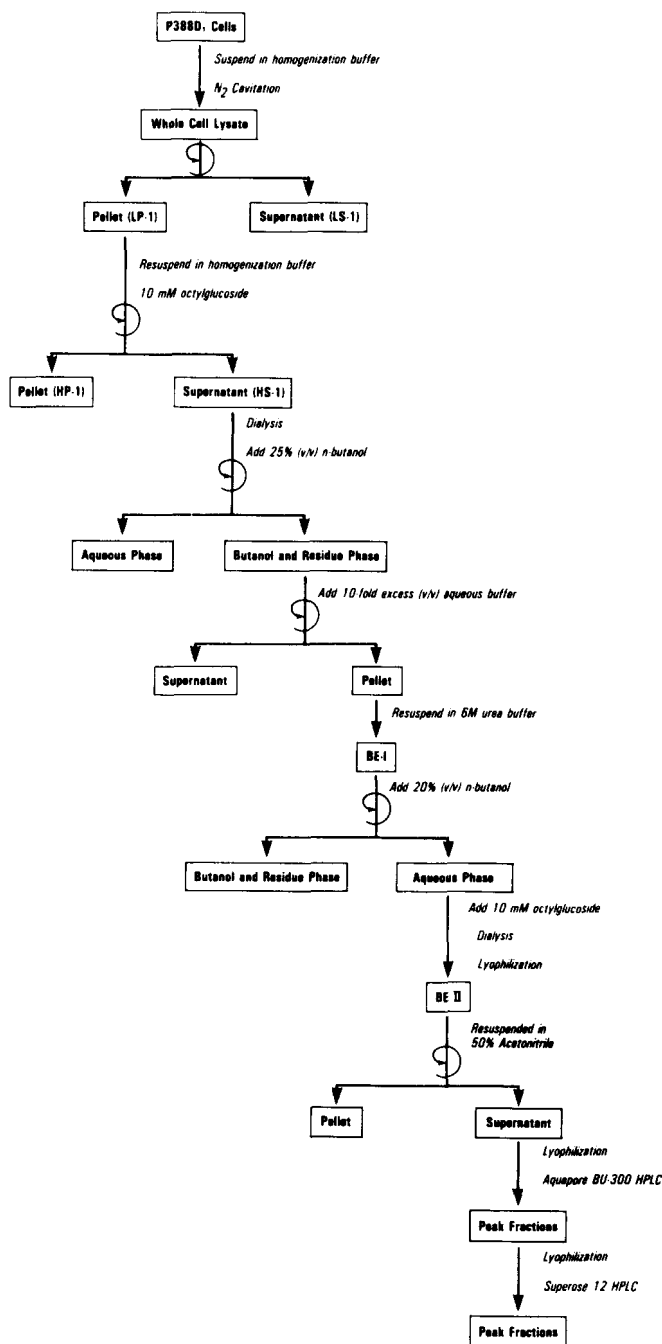When BE-I was mixed with 20% butanol at room temper-

FIG. 1. **Scheme for the octyl glucoside solubilization and butanol extraction of the membrane-bound phospholipase A$_2$ from the P388D$_1$ macrophage-like cell line.**

ature and centrifuged, unlike the previous extraction, over 100% of the enzyme activity was found in the aqueous phase and none in the butanol residue phase. The aqueous phase (containing 10 mM octyl glycoside to help in enzyme solubility) was passed through a membrane filter to remove floating debris, dialyzed against lyophilization buffer and lyophilized (Butanol Extract II or BE-II). The presence of EDTA in the lyophilization buffer was necessary to prevent precipitation in the dialysis bag. Routinely, 3–4% of the protein and 50–60% of the enzyme activity from HS-1 was recovered in BE-II when the activity was assayed with glycerol (see below). When BE-II was assayed without glycerol, the apparent enzyme activity was somewhat lower and varied from 15 to 40% of that in HS-1.

TABLE I

Purification of phospholipase A₂ from P388D₁ macrophage-like cells

Cell fraction[a]	Protein	Total activity[b]	Specific activity	Purifi-cation
	$mg/10^9$ cells	micro-units/ 10^9 cells	micro-units/mg protein	-fold
Whole cell lysate	179	58,260	325	1.0
LP-1	78	78,240	1,000	3.1
HS-1	50	65,180	1,300	4.0
BE-I	12	36,610	3,050	9.4
BE-II	2.0	41,650	20,800	64
Acetonitrile extract	0.23	41,300	180,000	550
Aquapore BU-300 peak	0.030	16,460	549,000	1,690
Superose 12 peak	0.018	14,580	810,000	2,490

[a] Cell fractions are defined in Fig. 1 and under "Experimental Procedures." Protein samples containing octyl glucoside urea, butanol, etc. were dialyzed and/or lyophilized before they were subjected to assay.

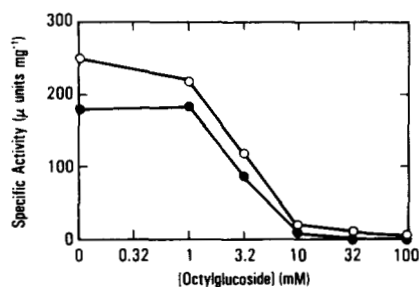[b] The Dole assay described under "Experimental Procedures" was employed.

FIG. 2. **Effect of octyl glucoside on the phospholipase A₂ activity of the low speed pellet LP-1 (●) before solubilization and of the high speed supernatant HS-1 (○) after solubilization with octyl glucoside.** In the case of HS-1, it was dialyzed against buffer lacking octyl glycoside before incubation with the specific concentration of octyl glucoside indicated.

Extraction of the Enzyme with Acetonitrile—When BE-II was simply mixed with 50% acetonitrile in deionized water and centrifuged, the supernatant contained 80 to 100% of the enzyme activity and 10 to 20% of the protein. This supernatant was lyophilized in polypropylene tubes and yielded a final enzyme recovery of over 80% that found in BE-II. In contrast, when the acetonitrile was removed by dialysis, the enzyme recovery was less than 50% and often as low as 20%.

Separation of the Phospholipase A₂ by Reverse Phase HPLC—The lyophilized acetonitrile extract was suspended in HPLC buffer (containing 30% acetonitrile), centrifuged, and filtered. Over 90% of the protein and phospholipase A₂ activity was recovered in the filtrate. The filtrate was applied to a reverse phase Aquapore BU-300 column and eluted with a 30 to 60% acetonitrile gradient. The enzyme activity was eluted at about 40–50% acetonitrile in the gradient as shown in Fig. 3. Enzyme activity could be determine directly from the column fractions, even though the presence of acetonitrile in the assay (about 4.5% after dilution) appeared to suppress the activity of BE-II somewhat.

Estimation of the Molecular Mass of the Phospholipase A₂—Pooled Aquapore BU-300 fractions were lyophilized in octyl glucoside, resuspended in buffer, and applied to a Superose 12 column. The molecular mass of the protein was estimated as about 18,000 by comparison with standard proteins as shown in Fig. 4. An essentially identical molecular mass was obtained when the column was run with a peak fraction from the Aquapore column not containing octyl glucoside or with a 6 M urea, 0.5 M NaCl buffer either with or without 10 mM
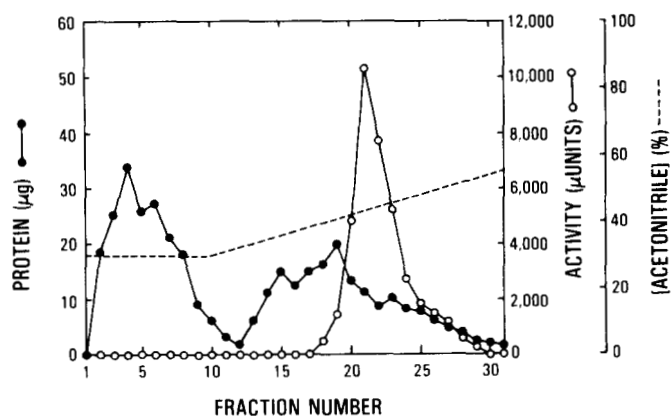

FIG. 3. **Typical purification of phospholipase A₂ by reverse phase HPLC.** The acetonitrile extract (1.9 ml) was applied to an Aquapore BU-300 HPLC column and eluted with a 30 to 60% acetonitrile gradient (- - -) at flow rate of 1 ml/min. Phospholipase A₂ activity (○) of the fractions was measured directly on the fractions using the Dole assay. Protein concentration (●) of the fractions was measured on aliquots which were lyophilized first.
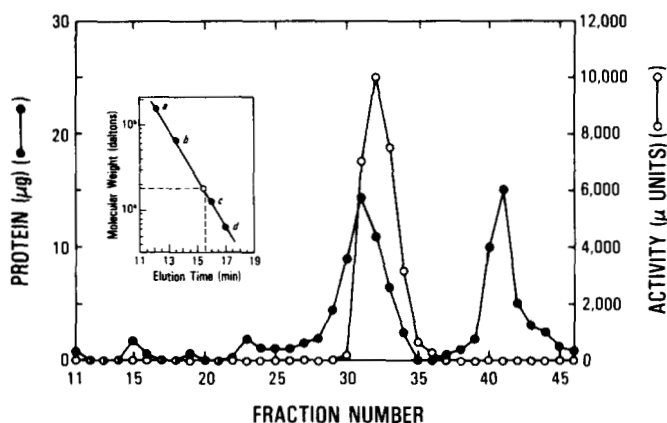

FIG. 4. **Typical purification and molecular mass estimation of phospholipase A₂ on a Superose 12 HPLC column.** Phospholipase A₂ activity (○) and protein (●) were determined as in Fig. 3. In a separate experiment, the molecular mass of the protein was estimated against standard proteins: *a*, IgG; *b*, bovine serum albumin; *c*, cytochrome *c*; and *d*, bovine pancreatic trypsin inhibitor as shown in the *inset*.

octyl glucoside (data not shown). However, when octyl glucoside was omitted from the lyophilization of either the Aquapore or the Superose pooled fractions, much of the activity was lost.

Purification—As shown in Table I, an overall purification of 2,500-fold was obtained with a 25% yield and a specific activity of 810,000 microunits mg⁻¹. After lyophilization in octyl glucoside, the peak fraction (fraction 32) from Superose 12 had a specific activity of 1.7 μmol min⁻¹ mg⁻¹ and was obtained in 17% overall yield, representing a 5,200-fold purification. This peak gave a major band on sodium dodecyl sulfate-polyacrylamide gel electrophoresis with a molecular mass of about 18,000 when compared with standards. Because of the small amounts of protein obtained after the HPLC column and the labor that would have been required to prepare the large amounts of pure protein for the kinetic experiments, all kinetic analyses were done on the intermediate BE-II. Typically, BE-II has a specific activity between 10,000 and 20,000 microunits mg protein⁻¹ and can be easily obtained with a fair yield. This preparation is stable when stored as a lyophilized powder at −20 °C for several months. However, when the lyophilized powder was dissolved, it sometimes lost
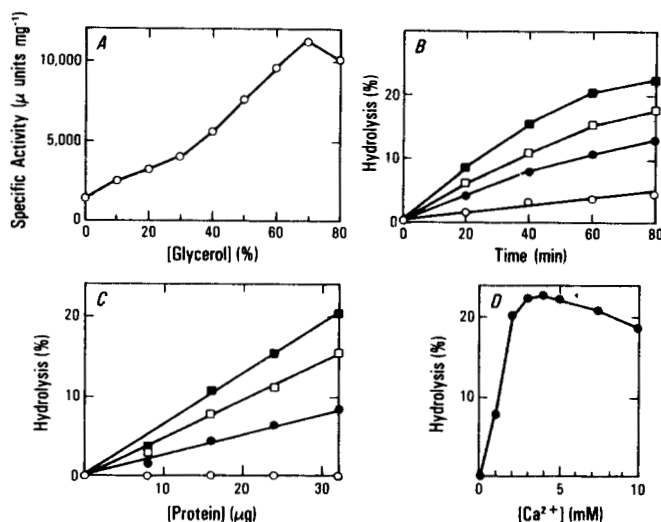
FIG. 5. **Characterization of phospholipase A₂ activity of BE-II.** *Panel A* shows the dependence of the specific activity on glycerol. *Panel B* shows the time course at various protein concentrations: 8 μg (O), 16 μg (●), 24 μg (□), and 32 μg (□). *Panel C* shows the protein dependence at various incubation times: 0 min (O), 20 min (●), 40 min (□), and 60 min (■). *Panel D* shows the Ca^{2+} dependence of the enzyme. Standard assay conditions using the Dole assay were employed.
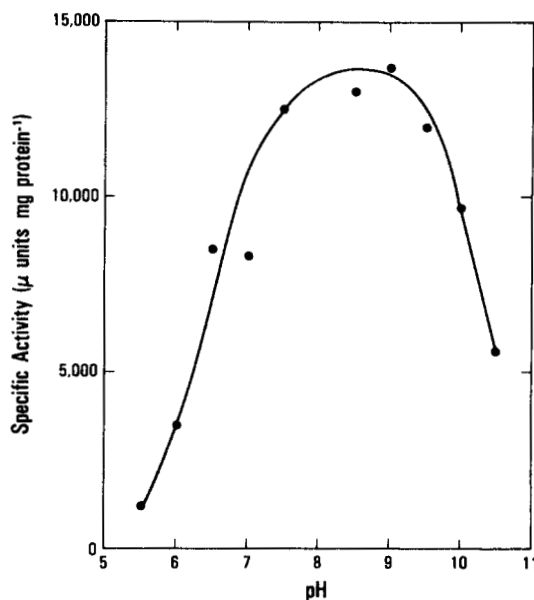

FIG. 6. **pH dependence of phospholipase A₂ activity.** Assays were performed using BE-II and the TLC assay. Standard conditions were used except that imidazole buffer (100 μM) was employed at pH 7.5 and below and glycine buffer (100 μM) at pH 8.0 and above. Ionic strength was maintained at 0.13 M with KCl.

some of its activity when stored at −20 °C for a few days. Therefore, it is important to store BE-II in aliquots such that only the amount of protein needed for the immediate assays is dissolved.

Effect of Glycerol on Phospholipase Activity of P388D₁ Cell Fractions—When glycerol was added to the enzyme assay, the phospholipase activity of BE-II was dramatically increased as the glycerol concentration was raised and reached a 7-fold increase at 70% glycerol in the assay as shown in Fig. 5A. At other stages in the purification procedure, such as the whole cell lysate, LP-1, HS-1, and BE-I, glycerol also increased the enzyme activity, but not always in exactly the same proportion and never as markedly. The time course and protein dependence of the enzyme activity of BE-II are shown in Fig. 5, *B* and *C*, respectively. The time courses were linear to about 8% hydrolysis. The activity was linear with protein above 10 μg/assay; below this it dropped off. The enzyme activity of BE-II shows an absolute dependence on Ca^{2+} as shown in Fig. 5D. Neither the fatty acid extraction assay nor the TLC assay itself was affected by including glycerol in this assay, although 1 to 2 ml of deionized water had to be added into the assay tubes after stopping the reaction to ensure the partitioning of glycerol into the aqueous phase.

pH Dependence of BE-II—The Dole assay was used to follow the enzyme purification, because it is much less laborious than the TLC assay and generally gives comparable results. However, the TLC assay was used for the determination of the kinetic and enzymatic properties, because this assay is much more precise than the extraction assay and this precision is needed for proper substrate dependence experiments. Assays performed by TLC showed that for vesicles and mixed micelles, the percent hydrolysis was linear with time, up to 10–12% for vesicles and 16% for mixed micelles (data not shown).

The pH-rate profile for BE-II is shown in Fig. 6; optimal activity occurs between pH 7.5 and 9.5. For the standard assay, glycine buffer (pH 9.0) was used because it consistently showed the highest activity and minimized the Ca^{2+}-independent phospholipase activity, present in the early steps of

the purification (14), whose pH optimum is 7.5.

Specificity of BE-II—Assays performed on BE-II at both pH 7.0 and 9.0 in the absence of Ca^{2+} and in the presence of 5 mM EDTA showed the absence of any phospholipase activity, demonstrating that there are no Ca^{2+}-independent phospholipases present and that the observed phospholipase A₂ activity has an absolute dependence on Ca^{2+}. Furthermore, no phospholipase A₁ activity was observed throughout the pH range of 3–10.5, as no 2-[1-¹⁴C]palmitoyl-lyso-PC was produced. The incubation of the phospholipase A₂ preparation with 1-[1-¹⁴C]palmitoyl-lyso-PC (125 μM (pH 8.0)) showed the absence of any lysophospholipase activity. The absence of any phospholipase C activity was previously shown in the more crude preparation LS-2 (14). Ionic strength studies at standard assay conditions showed that as the ionic strength increased, the phospholipase A₂ activity decreased. Therefore, to maximize activity, standard assay conditions minimized buffer and CaCl₂ so that the ionic strength was kept constant and as low as feasible (50 mM).

Activity of BE-II toward Substrate—Fig. 7 shows the substrate dependence of BE-II toward vesicles and mixed micelles, each in the presence and absence of glycerol. Clearly, higher enzyme velocities were observed for assays performed in the presence of glycerol (Fig. 7, *B versus A*). Based on the Lineweaver-Burk plots, apparent V_{max} and K_m values as well as specific activities at standard assay conditions were obtained as summarized in Table II. At low substrate concentrations, vesicles had higher velocities than mixed micelles due to their lower apparent K_m. At high substrate concentrations, mixed micelles have higher velocities and therefore a higher V_{max}. However, in the case of mixed micelles in the presence of glycerol (Fig. 7B), the V_{max} was determined by extrapolation of the linear portion since an apparent inhibition was observed at substrate concentrations above 100 μM. At a substrate concentration of 100 μM, the activity of BE-II toward vesicles and micelles was similar. Since the standard assay mixture contains 100 μM phospholipid, the activities determined toward either micelles or vesicles are directly comparable, at least to a first approximation.
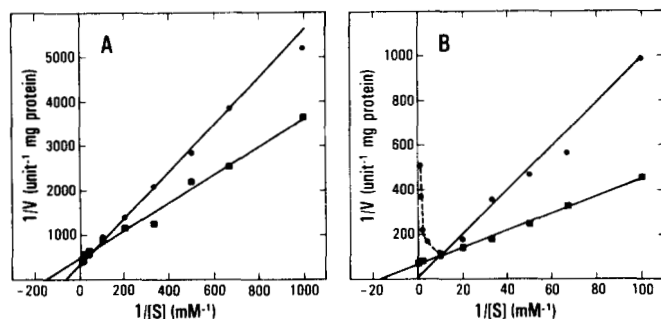
FIG. 7. **Lineweaver-Burk plot of phospholipase A_2 activity toward dipalmitoyl-PC in mixed micelles (●) and vesicles (■).** The TLC assay was employed with BE-II. *Panel A* shows activity in the absence of glycerol and *panel B* shows activity in the presence of 70% glycerol. With mixed micelles, the Triton X-100 concentration was maintained at a molar ratio of 2:1 Triton/phospholipid. Incubation times were varied between 10 and 90 min so as to keep the reaction between 5 and 10% hydrolysis (linear range) at all substrate concentrations.

TABLE II

Kinetic parameters of phospholipase A_2

Apparent K_m and V_{max} values were calculated from data in Fig. 7.

Substrate	Glycerol	Activity[a]	V_{max}	K_m	V_{max}/K_m
		microunits mg protein^{-1}		*μM*	*micro-units mg^{-1} μM^{-1}*
Vesicles	−	2,000	2,100	7	300
Vesicles	+	8,800	15,000	60	250
Vesicles[b]	+	1,600,000	3,600,000	80	45,000
Mixed micelles	−	2,300	2,900	20	145
Mixed micelles	+	8,100	100,000	1,000	100

[a] Activity at 100 μM substrate which corresponds to standard assay conditions as described under "Experimental Procedures" as determined using the TLC assay.

[b] Enzyme purified through Superose 12 procedure as in Table I. Data analyzed by procedure employed in Fig. 7

Activity of Purified Enzyme—The activity of another preparation of enzyme carried through the Superose 12 step (Fig. 1 and Table I) was subjected to kinetic analysis toward vesicles in the presence of glycerol analogous to the experiment on BE-II shown in Fig. 7B. The plot was linear and gave a V_{max} of 3.6 μmol min^{-1} mg protein^{-1} and an apparent K_m of 80 μM as shown in Table II.

Inhibition by Manoalide Analogue—An analogue of manoalide, HDHB, has been studied as an inhibitor of the phospholipase A_2 from cobra venom (18); inhibition of BE-II was also observed with this compound as shown in Fig. 8. Half-inhibition (IC$_{50}$) of about 40 μM was found.

DISCUSSION

The studies described herein provide the means to obtain a membrane-bound phospholipase A_2 using the murine macrophage-like cell line P388D$_1$ as the enzyme source. This enzyme has been partially purified with a minimum number of steps based on simple extraction procedures yielding a stable, soluble, lyophilized preparation referred to as BE-II. Importantly, the enzyme is readily obtained free of other phospholipase activities including phospholipase A_1, phospholipase C, and lysophospholipase with an approximately 60-fold purification. This phospholipase A_2 is Ca^{2+}-dependent and optimally active at alkaline pH which is consistent with other membrane-associated enzymes (reviewed in Ref. 22). At this stage, the enzyme is obtained in relatively good yield and is suitable for unambiguous studies of the kinetic and enzymatic
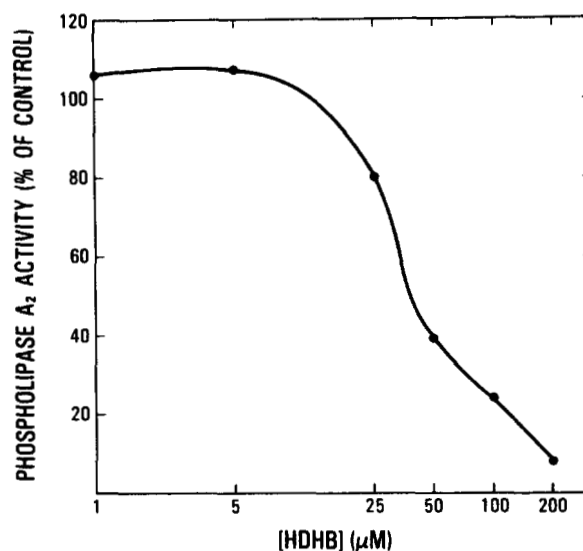

FIG. 8. **Inhibition of phospholipase A_2 (BE-II) by HDHB.** Activity as a function of HDHB is shown on a semi-log scale. The TLC assay was employed with BE-II.

properties of this potentially important protein. Indeed, we have found that HDHB, an analogue of manoalide (23), inhibits BE-II with a similar dose response to the enzyme from cobra venom (18).

Further purification of BE-II could be achieved using HPLC, yielding a 2500-fold purification with a 25% recovery of activity. Throughout the purification steps either detergent, 6 M urea, or organic solvent were required to keep the enzyme solubilized and prevent its aggregation. These requirements are suggestive of an intrinsic membrane enzyme. Furthermore, the finding that the presence of glycerol in the assay medium leads to enhanced activity is also consistent with the membrane-bound nature of the enzyme. Interestingly, as the purity of the fractions were increased, the glycerol effect became somewhat more pronounced. Other similar chemicals such as ethylene glycerol or propylene glycol were also found to activate the enzyme, but not to the same degree as glycerol.

Assays performed at standard condition using dipalmitoyl-PC revealed higher activities when assays were performed in glycerol. Glycerol has previously been used as a stabilizing environment during the purification of membrane-associated proteins. What effect glycerol has on the conformation of the enzyme and the substrate in these assays is presently unclear. The kinetic experiments show that glycerol greatly increases the apparent V_{max}, but at the expense of a higher apparent K_m. Therefore, the overall catalytic efficiencies (V_{max}/K_m) for substrate (whether mixed micelles or vesicles) both in the presence and absence of glycerol are comparable.

An analysis of the substrate forms (mixed micelles *versus* vesicles) shows that vesicles appear to be better substrates when comparing their V_{max}/K_m value, although the apparent V_{max} for micelles (without glycerol) is higher. In the case of mixed micelles in the presence of glycerol, inhibition is observed above 100 μM substrate. One possible explanation might be inhibition of the enzyme by Triton X-100, occurring at high detergent concentrations. However, this apparent inhibition is likely due to an interaction between the glycerol and the detergent which results in a phase change, possibly affecting the solubility of the phospholipids. This is supported by the distinct turbidity observed above substrate concentrations of 100 μM or above Triton X-100 concentrations of 200 μM. Previous reports (8, 10) on phospholipases A_2 from mac-

rophages have suggested Triton X-100 to be inhibitory. However, these determinations were performed at one substrate concentration and may be due to other causes. The substrate dependence experiments do reveal that detergent alters the kinetic parameters, but the extent to which this involves true enzyme inhibition must be determined.

We (24–26) have developed a detailed kinetic analysis to evaluate phospholipases acting on lipid/water interfaces. However, the data presented herein were obtained under limited experimental conditions in order to obtain apparent kinetic parameters which are valid only under the specific experimental conditions employed, but are still useful in comparing the various substrate forms. These parameters (apparent K_m and V_{max}) are the basis for developing a more complete kinetic analysis of the action of this enzyme which will be reported elsewhere.[4]

The specific activity of the most highly purified fraction of the macrophage phospholipase A₂ after HPLC purification was 1.7 μmol min^{-1} mg^{-1} under standard assay conditions, which is within the range of other intracellular phospholipases, generally between 0.2 and 8 μmol min^{-1} mg^{-1}. In contrast, pure extracellular phospholipase A₂ from mammalian pancreas and various snake venoms has typically been in the 1000 μmol min^{-1} mg^{-1} range. Interestingly, kinetic analysis of the purified macrophage enzyme gave a V_{max} of 3.6 μmol min^{-1} mg^{-1} with an apparent K_m (80 μM) similar to that obtained with BE-II (60 μM) under the same experimental conditions, making BE-II a suitable preparation for the enzymatic studies described herein.

The molecular mass estimation by HPLC showed that enzymatic activity coincided with a protein of 18,000 daltons. This enzyme has a molecular mass which is close to that determined for a membrane-bound phospholipase A₂ isolated from sheep red blood cells (27). It is only slightly larger than phospholipase A₂ determined for other intracellular and extracellular enzymes (12,000–15,000 daltons) (reviewed in Ref. 28). The largest membrane-bound phospholipase A₂, isolated from a macrophage (42,000 daltons), has been reported by Nitta *et al.* (16) who contend that this enzyme is an integral part of the Fc receptor, but this has not been substantiated.

In the macrophage, the identity of the enzymes involved in arachidonic acid release for eicosanoid production is not presently known. *A priori*, it is not clear whether the responsible enzyme will show specificity for arachidonic acid in the *sn*-2 position or for a particular head group on the phospholipid. Studies thus far on the BE-II preparation have shown arachidonoyl-PC to function as a substrate at least comparable with dipalmitoyl-PC under conditions different from those found optimal for the saturated lecithin, however. We have found that the kinetics of the macrophage phospholipase A₂ acting on arachidonoyl-containing phospholipid is quite complex and that the dipalmitoyl-PC is a more optimal substrate for the initial kinetic characterization of the enzyme. Indeed an effect of free arachidonic acid on the enzyme has also been found.[4] Since release of free arachidonic acid is presumably the result of ligand-receptor binding, the enzymes responsible must be highly regulated. This effect of fatty acid on the enzyme could be a possible mechanism for such regulation.

To address issues of the identity and regulation of the phospholipases, it is essential to purify and characterize the

various phospholipases of the macrophage, especially those that are associated with the mitochondrial or ribosomal membranes or are plasma membrane-bound. In previous reports, we described the complexities and potential pitfalls of such efforts (14). The present report extends our initial studies and provides a means by which the purification of a phospholipase A₂ can be accomplished. Enzyme recovered from the Aquapore BU-300 and/or Superose 12 columns is sufficiently pure (2500–5000-fold purification) to begin preparation of monoclonal antibodies to this enzyme. This should allow detailed studies of the intracellular localization and function of the protein as well as the development of strategies for obtaining larger quantities of the pure protein.

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Supplementary Material To

SOLUBILIZATION, PURIFICATION, AND CHARACTERIZATION OF A MEMBRANE-BOUND PHOSPHOLIPASE A$_2$ FROM THE P388D$_1$ MACROPHAGE-LIKE CELL LINE

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EXPERIMENTAL PROCEDURE

Materials: Adenosine-5'-triphosphate (ATP), 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (Hepes), immunoglubulin G, cytochrome c, bovine pancreatic trypsin inhibitor and bovine serum albumin were purchased from Sigma Chemical Company (St. Louis, MO.). Ethylenediaminetetraacetic acid, tetrasodium salt, dihydrate (EDTA) was obtained from Mallinckrodt, Inc. (Paris, KY). 1,2-dipalmitoyl-*sn*-glycerol-3-phosphorylcholine (dipalmitoyl PC), Triton X-100, and n-octyl-β-D-glucopyranoside (octylglucoside) were purchased from Calbiochem-Behring (La Jolla, CA). Ultrapure sucrose and ultrapure urea were obtained from Schwarz/Mann (Cambridge, MA). n-Butanol was purchased from MCB Manufacturing Chemists, Inc. (Cincinnati, OH). Acetonitrile, ammonium bicarbonate, calcium chloride, chloroform, diethylether, glycine, n-heptane, sodium chloride, toluene and 2-propanol were obtained from Fisher Scientific (Fair Lawn, NJ). Glycerol (anhydrous) was obtained from J.T. Baker Chemical Co. (Phillipsburg, NJ). Silicic acid (Biosil A, 100-200 mesh) was obtained from Bio-Rad Labs. (Richmond, CA). Liquifluor and 1-palmitoyl,2-[1-^{14}C]-palmitoyl-*sn*-glycerol-3-phosphorylcholine (53 to 55 mCi/mmol) were purchased from New England Nuclear (Boston, MA). HDHB was provided by Dr. Edward Mihelich (18).

Cell Line: The P388D$_1$ cells (19) were provided by Dr. H. Koren (Duke University). These cells were maintained in culture at 37°C and 5% CO$_2$ in RMPI 1640 medium (Scripps Clinic and Research Foundation, La Jolla, CA) supplemented with 10% fetal calf serum (Hyclone Laboratories, Logan, UT), 2 mM L-glutamine (200 mM solution, Whittaker M. A. Bioproduct, Inc., Walkersville, MD), 50 units ml^{-1} of penicillin and 50 μg ml^{-1} of streptomycin (Penicillin-Streptomycin Mixture, Whittaker M. A. Bioproduct, Inc.). The cell culture was started with 1 x 10^5 cells ml^{-1} in 60 ml of culture medium in 150 cm^2 culture flasks (Corning Glass Works, Corning, NY.). The cells generally were confluent in 2 days upon which the culture (usually 0.5 to 1 x 10^6 cells per flask) was innoculated into a 850 cm^3 roller bottle (Corning Glass Works, Corning, NY) containing 450 ml of culture medium and incubated at 0.3 rpm on a bottle roller in a warm room without CO$_2$. After 3 days incubation, any adherent cells were suspended into the *medium by agitation and all cells were harvested by low speed centrifugation* (600 x g, 15 min, 4°C). Routinely, 3.5 to 4.5 x 10^9 cells were harvested from 8 roller bottles with over 90% cell viability.

Preparation of Cell Homogenates: The cells harvested from roller bottles were suspended in homogenization buffer (0.34 M sucrose, 10 mM Hepes, 1 mM EDTA, pH 7.5) containing 1 mM ATP. The suspended cells (1 x 10^9 cells per 30 ml) were continously mixed by a magnetic stirring bar in a plastic cylindrical chamber in a precooled Parr cell disruption bomb (Parr Instrument, Moline, IL) at 4°C for 15 min under 600 psi N$_2$ and lysed by releasing the pressure. The cell lysate was centrifuged (1000 x g, 15 min, 4°C) and the resultant supernatant (LS-1) was removed from the floating foam and pellet (LP-1). LP-1 was enriched in the Ca^{2+}-dependent, alkaline pH-optimum phospholipase A$_2$ associated with plasma membrane, mitochondria or endoplasmic reticulum (14).

Solubilization of the Membrane Fraction with Octylglucoside: The detergent, octylglucoside, was used in order to solubilize the membrane-associated proteins in LP-1. The preparation was resuspended in homogenization buffer (protein from 1 x 10^9 cells brought to 30 ml) to which an aliquot of 1 M octylglucoside was added to bring the final concentration to 10 mM. This suspension was gently mixed in a 250 ml centrifuge tube on a mixer (Super Mixer, Lab-Line Instrument, Inc., Melrose Park, IL) for 1 h at room temperature. It was then centrifuged (134,000 x g, 60 min, 4°C), and the supernatant (HS-1) was removed from pelleted insoluble materials (HP-1). Usually, LP-1 contained 80 to 90% of the Ca^{2+}-dependent phospholipase A$_2$ activity of the whole cell lysate when assayed in the absence of glycerol, but as much as 150% of the activity when glycerol was included in the assay.

Phospholipase A$_2$ Extraction with n-Butanol: Routinely, HS-1 from 8 roller bottles was dialyzed against 4 l Hepes buffer (10 mM, 1 mM EDTA, pH 7.5) overnight at 4°C. The dialysed HS-1 was aliquoted equally into 50 ml centrifuge tubes to which n-butanol (23-25% final concentration, v/v) was added and then vortexed (30 sec). The emulsions were immediately centrifuged (1,700 x g, 20 min, 22°C) to separate the butanol and residue phase from the aqueous phase. After careful removal of the lower aqueous phase by vacuum evacuation through a disposable pipette, the butanol and residue phase in each tube were taken up in ice cold Hepes buffer (10 x volume of butanol-residue phase) and vortexed (20-30 sec). Samples were then kept on ice (1 to 2 hr) to allow for precipitation. The precipitate was centrifuged (2,000 x g, 30 min, 4° C), and the pellet of each tube resuspended in freshly made 6 M urea buffer (20 mM Hepes, 1 mM EDTA, pH 7.5) using 1 ml for protein from 3 x 10^7 cells. This material is referred to as butanol extract I (BE-I).

BE-I was gently stirred with a second addition of n-butanol (20% final concentration, v/v) in 50 ml centrifuge tubes on a mixer (30 min, room temperature). The emulsions were centrifuged (2,000 x g, 30 min, 22°C) and this time, the aqueous phase was collected by vacuum evacuation into a 250 ml centrifuge tube to which octylglucoside (10 mM final concentration) was added. This aqueous phase was then passed through a membrane filter (0.45 μm) to remove debris and dialyzed against 4 l of lyophilization buffer (10 mM ammonium bicarbonate, 0.25 mM EDTA, pH 7.5) overnight at 4°C. The dialysate was aliquoted (each representing protein from about 3 x 10^8 cells), lyophilized, and stored at -20°C. This preparation is referred to as butanol extract II (BE-II).

Acetonitrile Extraction: BE-II was suspended into freshly made 50% acetonitrile in deionized water (1 ml for protein from 3 x 10^8 cells) and centrifuged (8,000 x g, 30 min, 4°C). The supernatant was collected and lyophilized overnight.

Reverse Phase HPLC Chromatography: Chromatography, utilizing an Aquapore BU-300 column (10 cm x 4.6 mm ID, Pierce Chemical Co., Rockford, IL), was carried out on a high performance liquid chromatography (HPLC) system (Perkin-Elmer Corporation, Flanders, NJ). HPLC buffer (50 mM ammonium bicarbonate, 0.25 mM EDTA, pH 7.5) contained 30% acetonitrile (v/v). The acetonitrile extract was suspended in HPLC buffer (protein from 1 x 10^9 cells per ml), centrifuged (32,000 x g, 30 min, 4°C), and the supernatant was filtered through a nylone membrane (0.2 μm, Vangard International, Inc., Neptune, NJ). The filtrate was applied to the column which had been pre-equilibrated with HPLC buffer. Phospholipase A$_2$ was eluted with a 30 to 60% acetonitrile gradient (1.2 % per min) in the same buffer at a flow rate of 1.0 ml per min. Fractions (1.0 ml each) were collected and those containing phospholipase A$_2$ activity were pooled (about 5 ml), octylglucoside added (final concentration 10 mM), and immediately lyophilized.

Gel Filtration HPLC Chromatography: The above pooled and lyophilized Aquapore BU-300 column fractions were resuspended (final concentration, 60μg/ml protein) in column buffer (30% acetonitrile, 0.2 M ammonium bicarbonate, 0.25 mM EDTA, pH 7.5) and 0.5 ml of the sample was applied to a gel filtration HPLC column (Superose 12 HR 10/30, 30 x 300 mm, Pharmacia Fine Chemicals, Upsala, Sweden) at a flow rate of 1.0 ml per min. Eluant was collected in fractions of 0.5 ml. Those containing phospholipase A$_2$ activity were pooled (about 2 ml), octylglucoside added (final concentration 10 mM), and the sample was immediately lyophilized. In order to estimate the molecular weight of the protein, 0.5 ml of each standard protein (concentration, 100 μg/ml), immunoglobin G (IgG), bovine serum albumin (BSA), cytochrome c, and bovine pancreatic trypsin inhibitor, was separately run on the HPLC after suspension in the same buffer.

Phospholipase A$_2$ Dole Assay: This assay is based on a modification (20) of the classic Dole extraction system (21). The following assay was generally utilized during enzyme purification. The assay mixture contained 50 to 400 μl of an enzyme sample from which octylglucoside, urea, and sodium chloride were dialyzed out overnight at 4°C against Hepes buffer (10 mM, 0.25 mM EDTA, pH 7.5) or alternatively lyophilized enzyme samples, that had been dialyzed against ammonium bicarbonate, were resuspended in Hepes buffer. Substrate consisted of 100 μM dipalmitoyl PC containing about 100,000 cpm of 1-palmitoyl-2-[1-^{14}C] palmitoyl PC. Assays also contained 200 μM Triton X-100, 80 mM glycine (pH 9.0), 5 mM CaCl$_2$, and 70% glycerol in a total volume of 0.5 ml. Assay tubes were incubated for 30 min at 40°C in a shaking water bath. The reactions were stopped by adding 2.5 ml of Dole reagent (21) (2-propanol : heptane : 0.5M H$_2$SO$_4$, 400 : 100 : 20, v/v/v) and vortexing.

Into each of the assay tubes containing the Dole reagent, silicic acid (100-200 mg), heptane (1.5 ml) and deionized water (1.5-3.0 ml) were added and then mixed by vortexing (10-15 sec). The tubes were then left to set until the heptane layer completely separated from the aqueous phase (2-5 min). Upon separation, 1.0 ml of the heptane phase was removed from each tube and forced through a Pasteur pipet containing silicic acid (2.0 to 2.5 cm height) with compressed air. The silicic acid was then washed with 1.0 ml of diethylether also under compressed air. The silicic acid removes all phospholipids and lysophospholipids from the heptane, leaving only fatty acid. Samples were collected in glass scintilation vials to which 5.0 ml of scintillation fluid (toluene : Triton X-100 : Liquifluor, 2 : 1 : 0.15, v/v/v) was added and then counted (Delta 300, Searle Analytic, Inc., Des Plaines, IL). Control samples were prepared and treated identically to the assay samples with the exception that the enzyme was omitted. This control measures both non-specific hydrolysis and the amount of radiolabeled fatty acid originally in the substrate. The fatty acid levels in these controls were usually less than 0.4% of the total radioactivity. All assay data is reported as the average of duplicate determinations after substracting the controls. The enzyme activity is expressed in μunits, where 1 μunit is defined as the amount of enzyme required to hydrolyze 1 pmol of phospholipid per min.

Phospholipase A$_2$ TLC Assay: This assay was utilized for kinetic studies on substrate dependence. The standard assay mixture (0.5 ml, final volume) contained 70% glycerol, 5 mM CaCl$_2$, 20 mM glycine buffer (pH 9.0), and 100 μM dipalmitoyl PC containing about 100,000 cpm of 1-palmitoyl,2-[1-^{14}C] palmitoyl PC. Dipalmitoyl PC was prepared as either mixed micelles with 200 μM Triton X-100 or as vesicles. Vesicles were formed by sonicating the phospholipid in buffer containing the CaCl$_2$ in an MSE Model 100-watt sonicator at a temperature above its phase transition until the solution clarified (about 5 min.). The reaction was started by the addition of 50 μl of enzyme solution and was incubated at 40°C for 30 min.

Reactions were quenched by the addition of 0.5 ml of chloroform: methanol : acetic acid (2 : 4 : 1, v/v/v), followed by the addition of 0.25 ml chloroform and 1.0 ml of water to facilitate the partitioning of the glycerol into the aqueous phase. The organic layer, containing the reactants and the products, was dried by vacuum evaporation in an oven (40°C) and the residue was dissolved in 25 μl of chloroform : methanol (2 : 1, v/v). The entire sample was spotted onto one lane (2 cm width) of a 10 cm x 20 cm silica gel G plate (Analtech) and the lipid components were separated over the 10 cm length by elution with 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 and dipalmitoyl PC were scraped directly into scintilation vials to which 6 ml of scintilation fluid was added (Safety-Solve, Research Products International) and counted. Background levels of [^{14}C]-dipalmitoyl PC hydrolysis was measured by substituting enzyme buffer for the freshly prepared enzyme solution in the standard assay and was routinely found to be less than 7 μunits. Averages of duplicate assays are reported.

Protein Assay: Protein concentrations were assayed by a micromethod using BCA Protein Assay Reagents (Pierce Chemical Co., Rockford, IL). Bovine serum albumin was employed as the standard, and the absorbance of the samples was read at 550 nm by an Automated Microplate Reader (El-310, Bio-Tek Instruments, Inc., Burlington, VT).