Solubilization, Purification, and Characterization of a Membrane-bound Phospholipase A$_2$ from the P388D$_1$ 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 P388D$_1$, potentially capable of controlling arachidonic acid release. Among them, a membrane-bound, alkaline pH optimum, Ca$^{2+}$-dependent phospholipase A$_2$ is of particular interest because of the likelihood that the regulatory enzyme has these properties. This phospholipase A$_2$ 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. The first two steps in this purification are butanol extractions that yield a lyophilized, stable preparation of phospholipase A$_2$ lacking other phospholipase activities. This phospholipase A$_2$ 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 $\mu$M 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 A$_2$ inhibitors such as manoolide 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 A$_2$ with a 25% recovery and a specific activity of about 800 nmol min$^{-1}$ mg$^{-1}$ toward 100 $\mu$M 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 A$_{sn}$ 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 A$_2$ 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 P388D$_1$ 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.

Previous work (14) on the phospholipases in the P388D$_1$ 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, Ca$^{2+}$-dependent, alkaline pH optimum phospholipase A$_2$ 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 macrophages’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 P388D$_1$ and murine macrophage cells possesses an intrinsic phospholipase A$_2$ activity which is activated when bound to aggregated IgG$_{\alpha}$ (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.

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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 Ca²⁺. As such, it provides a convenient and reliable source of membrane-bound phospholipase A₂ 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) 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 undue labor made a detailed study of the highly purified enzyme less attractive at this time.

**EXPERIMENTAL PROCEDURES**

**RESULTS**

We report here the development of a routine scheme for the solubilization and partial purification of a membrane-bound phospholipase A₂ from the macrophage cell line P388D₁. 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 A₂ 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-

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2 The abbreviations used are: HPLC, high performance liquid chromatography; dipalmitoyl-PC, 1,2-dipalmitoyl-sn-glycerol-3-phosphorylcholine; HDHB, 3(cis,cis-7,10)-hexadecadienyl-4-hydroxy-2-butenolide; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

3 The "Experimental Procedures" are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are included in the microfilm edition of the Journal that is available from Waverly Press.
TABLE I

<table>
<thead>
<tr>
<th>Cell fraction*</th>
<th>Protein</th>
<th>Total activity</th>
<th>Specific activity</th>
<th>Purification</th>
</tr>
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<tr>
<td></td>
<td>mg/10⁶</td>
<td>micro-</td>
<td>micro-</td>
<td>fold</td>
</tr>
<tr>
<td>cells</td>
<td>cells</td>
<td>units/10⁶ cells</td>
<td>units/mg protein</td>
<td></td>
</tr>
<tr>
<td>Whole cell lysate</td>
<td>179</td>
<td>58,260</td>
<td>325</td>
<td>1.0</td>
</tr>
<tr>
<td>LP-1</td>
<td>78</td>
<td>78,240</td>
<td>1,000</td>
<td>3.1</td>
</tr>
<tr>
<td>HS-1</td>
<td>50</td>
<td>55,180</td>
<td>1,300</td>
<td>4.0</td>
</tr>
<tr>
<td>BE-I</td>
<td>1.2</td>
<td>36,610</td>
<td>3,050</td>
<td>9.4</td>
</tr>
<tr>
<td>BE-II</td>
<td>2.0</td>
<td>41,650</td>
<td>20,800</td>
<td>64</td>
</tr>
<tr>
<td>Acetonitrile extract</td>
<td>0.23</td>
<td>41,300</td>
<td>180,000</td>
<td>550</td>
</tr>
<tr>
<td>Aquapore BU-300 peak</td>
<td>0.030</td>
<td>16,460</td>
<td>549,000</td>
<td>1,690</td>
</tr>
<tr>
<td>Aquapore 12 peak</td>
<td>0.018</td>
<td>14,580</td>
<td>810,000</td>
<td>2,490</td>
</tr>
</tbody>
</table>

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

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 (O) before solubilization and of the high speed supernatant HS-1 (C) after solubilization with octyl glucoside. In the case of HS-1, it was dialyzed against buffer lacking octyl glucoside before incubation with the specific concentration of octyl glucoside indicated.

**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 (O) of the fractions was measured directly on the fractions using the Dole assay. Protein concentration (O) 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 (O) and protein (O) 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.

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 determined 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 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, 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
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 P88D, 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. 5A. At high substrate concentrations, vesicles had higher velocities than mixed micelles, each in the presence and absence of glycerol. Clearly, 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 \( K_a \). 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 \( \mu M \). At a substrate concentration of 100 \( \mu M \), the activity of BE-II toward vesicles and micelles was similar. Since the standard assay mixture contains 100 \( \mu M \) phospholipid, the activities determined toward either micelles or vesicles are directly comparable, at least to a first approximation.

The \( \text{pH} \)-rate profile for BE-II is shown in Fig. 6; optimal activity occurs between \( \text{pH} \) 7.5 and 9.5. For the standard assay, glycine buffer (\( \text{pH} \) 9.0) was used because it consistently showed the highest activity and minimized the \( \text{Ca}^{2+} \)-independent phospholipase activity, present in the early steps of the purification (14), whose \( \text{pH} \) optimum is 7.5.

Specificity of BE-II—Assays performed on BE-II at both \( \text{pH} \) 7.0 and 9.0 in the absence of \( \text{Ca}^{2+} \) and in the presence of 5 mM EDTA showed the absence of any phospholipase activity, demonstrating that there are no \( \text{Ca}^{2+} \)-independent phospholipases present and that the observed phospholipase \( \text{A}_2 \) activity has an absolute dependence on \( \text{Ca}^{2+} \). Furthermore, no phospholipase \( \text{A}_1 \) activity was observed throughout the \( \text{pH} \) range of 3-10.5, as no \( 2\)-[1-\( \text{C} \)]_palmitoyl-lyso-PC was produced. The incubation of the phospholipase \( \text{A}_2 \) preparation with 1-[1-\( \text{C} \)]_palmitoyl-lyso-PC (125 \( \mu M \) (\( \text{pH} \) 8.0)) showed the absence of any lysophospholipase activity. The absence of any phospholipase \( \text{C} \) 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 \( \text{A}_2 \) activity decreased. Therefore, to maximize activity, standard assay conditions minimized buffer and \( \text{CaCl}_2 \) so that the ionic strength was kept constant and as low as feasible (50 mM).
was maintained at a molar ratio. Times were varied between.

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 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 those assays is presently unclear. The kinetic experiments show that glycerol greatly increases the apparent Vmax, but at the expense of a higher apparent Km. Therefore, the overall catalytic efficiencies (Vmax/Km) 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 Vmax/Km value, although the apparent Vmax 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 A2 from mac-

### DISCUSSION

The studies described herein provide the means to obtain a membrane-bound phospholipase A2, using the murine macrophage-like cell line P388D, 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 A2, phospholipase C, and lysophospholipase with an approximately 60-fold purification. This phospholipase A2 is Ca++-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.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Glycerol</th>
<th>Activity* Vmax Kmax Vmax/Km</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vesicles</td>
<td>-</td>
<td>2,000 2,100 7 300</td>
</tr>
<tr>
<td>Vesicles</td>
<td>+</td>
<td>8,800 15,000 66 250</td>
</tr>
<tr>
<td>Vesicles</td>
<td>+</td>
<td>1,600 3,000 80 45,000</td>
</tr>
<tr>
<td>Mixed micelles</td>
<td>+</td>
<td>2,300 2,900 20 145</td>
</tr>
<tr>
<td>Mixed micelles</td>
<td>+</td>
<td>8,100 100,000 1,000 100</td>
</tr>
</tbody>
</table>

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

* 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 Vmax of 3.6 μmol min−1 mg protein−1 and an apparent Km 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 A2 from cobra venom (18); inhibition of BE-II was also observed with this compound as shown in Fig. 8. Half-inhibition (IC50) of about 40 μM was found.

**TABLE II**

Kinetic parameters of phospholipase A2

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Glycerol</th>
<th>Activity* Vmax Kmax Vmax/Km</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vesicles</td>
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<td>8,100 100,000 1,000 100</td>
</tr>
</tbody>
</table>

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

* Enzyme purified through Superose 12 procedure as in Table I. Data analyzed by procedure employed in Fig. 7.
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 \( K_a \) and \( V_{max} \)) are the basis for developing a more complete kinetic analysis of the action of this enzyme which will be reported elsewhere.\(^*\)

The specific activity of the most highly purified fraction of the macrophage phospholipase \( A_2 \) after HPLC purification was 1.7 \( \mu \)mol min\(^{-1}\) mg\(^{-1}\) under standard assay conditions, which is within the range of other intracellular phospholipases, generally between 0.2 and 8 \( \mu \)mol min\(^{-1}\) mg\(^{-1}\). In contrast, pure extracellular phospholipase \( A_2 \) from mammalian pancreas and various snake venoms has typically been in the 1000 \( \mu \)mol min\(^{-1}\) mg\(^{-1}\) range. Interestingly, kinetic analysis of the purified macrophage enzyme gave a \( V_{max} \) of 3.6 \( \mu \)mol min\(^{-1}\) mg\(^{-1}\) with an apparent \( K_a \) (80 \( \mu \)M) similar to that obtained with BE-11 (60 \( \mu \)M) under the same experimental conditions, making BE-11 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 \( A_2 \) isolated from sheep red blood cells (27). It is only slightly larger than phospholipase \( A_2 \) determined for other intracellular and extracellular enzymes (12,000–15,000 daltons) (reviewed in Ref. 28). The largest membrane-bound phospholipase \( A_2 \), 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. \textit{A priori}, it is not clear whether the responsible enzyme will show specificity for arachidonic acid in the \( n-2 \) 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 \( A_2 \) 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 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 \( A_2 \) 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.

**REFERENCES**


\(^*\) M. D. Lister, R. A. Deems, Y. Watanabe, R. J. Ulevitch, and E. A. Dennis, manuscript in preparation.
Macrophage Phospholipase A₂

Supplementary Material

SQUALIZATION, PURIFICATION, AND CHARACTERIZATION OF A MEMBRANE-BOUND PHOSPHOLIPASE A₂ FROM THE T. BACHII, MACROPHAGE (CIL LINE)

Richard J. Ehrlich, Yudachi Yoneda, Masahiko Sato
Mark D. London, Pietro G. Boretti, and Edward A. Hinds

EXPERIMENTAL PROCEDURE

Macrophage Adenylate Cylase (122) and Adenylylcyclase-Cyclonucleotide Phosphodiesterase (222) were purified from bovine lung by the method of Brugge et al. (222). Phospholipase A₂ was purified from bovine lung by the method of Griendling et al. (222). Phospholipase A₂ was purified from bovine lung by the method of Griendling et al. (222).

Cell Culture: The T. baccari cells were provided by the University of Rochester. These cells were maintained in culture in RPMI 1640 medium (Gibco BRL) supplemented with 10% fetal bovine serum (Hyclone Laboratories, Logan, UT), 2 mM L-glutamine, and antibiotics. The cells were grown in 25 cm² flasks in a humidified incubator at 37°C with 5% CO₂.

Preparation of Macrophage Culture: The cells harvested from culture vials were suspended in homogenization buffer (20 mM sodium acetate, 10 mM Hepes, 1 mM EDTA, pH 7.4) and the suspension was homogenized with a Teflon homogenizer at 4°C for 10 min. The homogenate was centrifuged at 30,000 g for 1 h and the supernatant was used as the homogenate.

Determination of Phospholipase A₂ Activity: The assay was performed as described previously (222). The reaction was stopped by the addition of 100 mM EDTA to the assay mixture. The reaction was measured at 400 nm using a photometer.

Phospholipase A₂ Assay: The enzyme was purified from bovine lung and assayed using 4-nitrophenylphosphatidylethanolamine (4-NPP-PE) as substrate. The reaction was performed at 37°C in a medium containing 50 mM Tris-HCl (pH 7.5), 1 mM CaCl₂, and 10 mM MgCl₂. The reaction was terminated by the addition of 100 mM EDTA and the absorbance at 400 nm was measured.

Antiviral Activity: Phospholipase A₂ was assayed for its ability to inhibit the growth of a murine myeloblastoid leukemia cell line (B16). The cells were grown in RPMI 1640 medium supplemented with 10% fetal bovine serum. The cells were injected into the mice and the survival time was determined.

Acute Phase Reactivity: Phospholipase A₂ was assayed for its ability to inhibit the growth of a murine myeloblastoid leukemia cell line (B16). The cells were grown in RPMI 1640 medium supplemented with 10% fetal bovine serum. The cells were injected into the mice and the survival time was determined.

Inhibition of Macrophage Function: Phospholipase A₂ was assayed for its ability to inhibit the growth of a murine myeloblastoid leukemia cell line (B16). The cells were grown in RPMI 1640 medium supplemented with 10% fetal bovine serum. The cells were injected into the mice and the survival time was determined.

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