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LOCALIZATION AND PARTIAL PURIFICATION OF A NEUTRAL-ACTIVE PHOSPHOLIPASE A_2 FROM BCG-INDUCED RABBIT ALVEOLAR MACROPHAGES

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Summary

The localization and partial purification of a Ca^{2+}-dependent, membrane-associated phospholipase A_2 (phosphatide 2-acylhydrolase, EC 3.1.1.4) from BCG-induced rabbit alveolar macrophages is described. Phospholipase A activity was determined using autoclaved *Escherichia coli*, the phospholipids of which were labelled in the 2-acyl position with [1-^{14}C]oleate. Sonicated macrophages or granule preparations exhibited maximal phospholipase A_2 activity at pH 7.0, with 5 mM Ca^{2+}. Activity was quantitatively recovered in the pellet after centrifugation of homogenates at 100 000 × *g*, indicating that the enzyme is membrane-associated. At least two populations of macrophage granules were separated that contained phospholipase A_2 activity. Plasma membranes enriched 15-fold with respect to alkaline phosphodiesterase I were devoid of phospholipase activity.

The enzyme was purified 1278-fold in a yield of 34%, was active over a broad pH range, and was extremely sensitive to low concentrations of Ca^{2+}. Mg^{2+} and Mn^{2+} would not substitute for Ca^{2+}, 1 mM EDTA completely inhibited enzymatic activity. Absolute specificity for the 2-position was demonstrated using 1-[1-^{14}C]stearyl-2-acyl 3-*sn*-glycerophosphorylethanolamine as substrate. Phospholipase A_2 activity was inhibited by the nonsteroidal anti-inflammatory agent indomethacin; the amount of drug required for 50% inhibition was $5 \cdot 10^{-4}$ M.

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Introduction

Neutral-active, Ca^{2+}-dependent phospholipases A_2 (phospholipase 2-acyl-hydrolase, EC 3.1.1.4) accumulate in inflammatory sites and are thought to be important mediators of inflammatory reactions [1]. These enzymes hydrolyze membrane phospholipids at the 2-acyl position releasing 1-acyl-lysophospholipids and predominantly unsaturated free fatty acids. Lysophospholipids are cytotoxic and produce intense inflammation when injected intraperitoneally [2,3]. Free fatty acids are known to be membrane lytic and some, such as arachidonic acid, serve as precursors of prostaglandins and endoperoxides, compounds with a myriad of effects on membranes during inflammation.

Macrophages, like other phagocytic cells produce prostaglandins during inflammation [4] and the origin and release of arachidonic acid by these cells and its regulation by anti-inflammatory agents is under intense investigation [5]. The Bacillus Calmette Guérin (BCG)-induced alveolar macrophage is a cell from a chronic inflammation of lung the enzymology of which has been studied extensively [6]. This cell contains phospholipases active at acid pH which are Ca^{2+}-independent, and are localized in the soluble fraction of granules [7]. By contrast, a Ca^{2+}-dependent phospholipase A_2, active in the neutral to alkaline pH range, has also been described but not localized or extensively characterized [8].

A similar phospholipase A_2 in rabbit and human polymorphonuclear leukocytes is associated with both azurophilic and specific granules and may contribute to the bacteriocidal activity of neutrophils by hydrolyzing microbial phospholipids [9,10]. Interestingly, the rabbit leukocyte phospholipase A_2 is inhibited by non-steroidal anti-inflammatory agents; such as indomethacin and the sensitivity to inhibition in vitro appears to be a function of the availability of Ca^{2+} [11,12].

In view of its potential role during inflammation, and possible involvement in lung surfactant metabolism, we described the localization and partial purification of a Ca^{2+}-dependent phospholipase A_2 from BCG-induced rabbit alveolar macrophages and its inhibition by indomethacin.

Materials and Methods

Cell preparation. New Zealand white rabbits (2.5—3.5 kg) were twice injected intravenously at 1-week intervals with 0.15 mg BCG in mineral oil. 2 weeks after the second injection they were killed by air embolism; the lung was removed and lavaged three times with 40 ml isotonic saline to harvest the aveolar macrophages. The macrophages were sedimented at $900 \times g$ for 10 min (2—6 ml packed cells/rabbit) washed twice with saline, and resuspended in 0.25 M sucrose 25 mM Tris-HCl pH 7.2 ($5 \cdot 10^6$ cells/ml) for cell fractionation or were frozen in saline for acid extraction.

Cell fractionation. The resuspended cells were homogenized with a motor driven Potter-Elvehjem homogenizer. Cell disruption was monitored by light microscopy and was discontinued when 70—90% of the cells were disrupted. The homogenate was diluted with 2 vol. buffer and centrifuged at $900 \times g$ for 10 min to remove nuclei and intact cells. The supernatant fraction was centrif-

uged at 12 000 × *g* for 20 min to obtain a granule pellet. This pellet was gently resuspended in buffered 0.25 M sucrose by hand homogenization, and an aliquot (4-ml) was layered onto 26 ml of a 30—55% (w/w) linear sucrose gradient and centrifuged at 90 000 × *g* for 6 h in a SW 25.1 rotor. After centrifugation, 15 fractions (2 ml/fraction) were collected by puncturing the bottom of the centrifuge tube and each fraction was assayed for protein and enzymic activities. Plasma membrane from BCG-induced macrophages were prepared by previously published methods [13].

Enzyme assays. Phospholipase A_2 was measured by established methods using [1-^{14}C]-oleate-labelled autoclaved *Escherichia coli* [14] and 1-acyl-2-[1-^{14}C]-linoleoyl 3-*sn*-glycerophosphorylethanolamine as substrates [15]. To determine positional specificity, 1-[1-^{14}C]-stearyl-2-acyl 3-*sn*-glycerophosphorylethanolamine was used as a substrate. With [1-^{14}C]-oleate-labelled *E. coli*, incubation mixtures contained, in a total volume of 0.5 ml, 5 mM $CaCl_2$/100 mM Tris-maleate (pH 7.5)/$2.5 \cdot 10^8$ cells of autoclaved *E. coli* (corresponding to 6000 cpm and 5 nmol phospholipid)/sufficient enzyme to produce 5—30% hydrolysis (0.25—1.25 nmol) of *E. coli* phospholipid. The lysosomal membrane marker β-glucosidase [16] and η-acetylglucosaminidase [17] were assayed using the *p*-nitrophenyl substrates in the presence of 0.1% Triton X-100. NADPH-cytochrome *C* reductase (microsomal) and cytochrome *c* oxidase (motochondrial) were assayed by method of Sottocasa et al. [18] as previously described. Alkaline phosphodiesterase I, a plasma membrane marker was assayed by the method of Wang et al. [13]. Proteins were determined by the method of Lowry et al. [19] for cell homogenates and subfractions or by the method of Bradford [20] modified by incubating at 37°C for 5 min prior to reading absorbance at 595 nm. Bovine serum albumin was used as the protein standard.

Purification of phospholipase A_2. Fresh or frozen macrophages collected as described above were resuspended with 2 vol. ice-cold 0.18 M H_2SO_4 to a final concentration of 0.12 M and were gently homogenized at 4°C. After 20 min of storage on ice the preparation was rehomogenized and centrifuged at 20 000 × *g* for 20 min. The supernatant fraction (SUP I) was removed and dialyzed exhaustively against 25 mM Tris-HCl, pH 7.0/0.15 M NaCl. The dialysate was centrifuged at 20 000 × *g* for 20 min and the resultant pellet, containing most of the phospholipase activity, was resuspended in 25 mM citrate (pH 5.0)/0.15 M NaCl. After 20 min at 4°C, the pellet was resuspended and centrifuged as above. The salt concentration of this supernatant fraction (SUP II) which now contained phospholipase activity was adjusted to 0.1 M NaCl and the fraction was applied to a CM-BioGel (BioRad) column equilibrated with 0.1 M NaCl/25 mM citrate, pH 5.0. The column was washed with 250 ml buffer and the phospholipase was eluted with 120 ml of a linear NaCl gradient (0.1—2.0 M)/25 mM citrate, pH 5.0, and collected in 3 ml fractions. These fractions were then assayed for phospholipase A_2 activity and protein. Sodium concentrations were determined by flame photometry.

Results

The optimal pH for hydrolysis of autoclaved *E. coli* by macrophage sonicates and granule preparations is shown in Fig. 1. In the presence of 5.0 mM Ca^{2+}

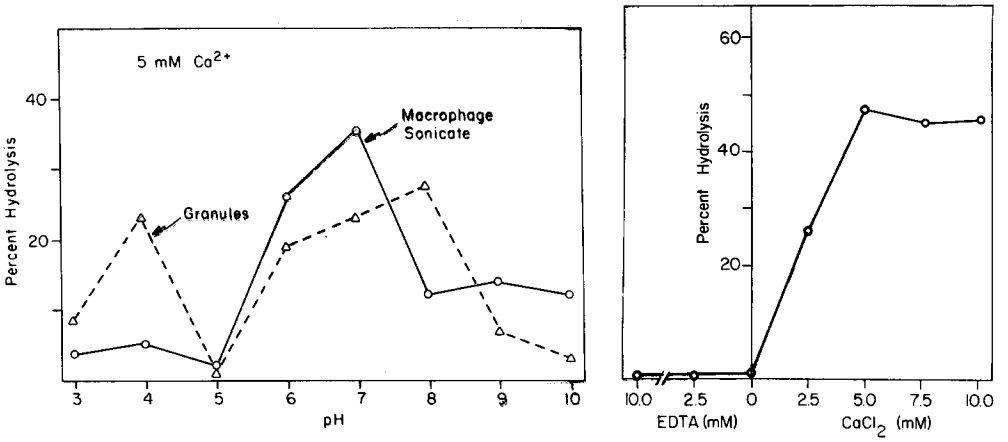

Fig. 1. Optimal pH for macrophage phospholipase A_2 activity. Macrophage sonicates (○———○) and granules (△ — — — △) were prepared as described in Materials and Methods. Standard reaction mixtures in a total volume of 0.5 ml contained $2.5 \cdot 10^8$ cells of autoclaved *E. coli* (6000 cpm)/5 mM $CaCl_2$/100 μg protein/50 μmol of the appropriate buffer (sodium acetate, pH 3.0—5.5; Tris-maleate, pH 5.5—8.5; Tris-HCl, pH 8.5—10.0) and were incubated for 30 min at 37° C. Phospholipase A activity is expressed as percent hydrolysis/30 min. (100% = 5 nmol phospholipid hydrolyzed).

Fig. 2. Ca^{2+} requirement for the membrane-associated phospholipase A_2. Standard reaction mixtures (see legend to Fig. 1) containing the indicated concentrations of $CaCl_2$ or EDTA, were incubated for 30 min at 37° C.

both preparations exhibit a peak of phospholipase A activity between pH values 6.0 and 8.0. The only radioactive product formed is [1-^{14}C]-oleate presumably due to the action of a phospholipase A_2. Differential centrifugation of disrupted macrophages or granules demonstrated that more than 95% of the phospholipase A_2 activity was sedimentable at 100 000 × *g* and, therefore, was probably tightly membrane associated (data not shown). By contrast, phospholipase A active at pH 4.0 was found in homogenates and granule preparations but not in sonicated macrophages. Because the soluble, acid-active enzymes of lysosomes have been studied extensively [21], in this report we describe the characterization and partial purification of the membrane-associated phospholipase A_2 active at physiologic pH.

The phospholipase A_2 associated with the membranes of macrophage granule preparations was most active at pH 7.0 and had an absolute requirement for calcium (Fig. 2). No activity was noted in the presence of EDTA or in the absence of added Ca^{2+}. Maximal activity was found in the presence of 5 mM Ca^{2+}; Mg^{2+} or Mn^{2+} would not substitute for calcium in the reaction; and higher concentrations of calcium (up to 25 mM) were not inhibitory.

To demonstrate the intracellular localization of the membrane-associated enzyme, intact granules were subjected to isopycnic sucrose density gradient centrifugation and the distribution of phospholipase A_2 and organelle marker enzymes was determined. As shown by the distribution of the granule marker enzymes β-glucosidase (Fig. 3a) and η-acetylglucosaminidase (Fig. 3b), at least two granule populations were found (fractions 7 and 12). Phospholipase A_2 exhibited an apparent bimodal distribution, corresponding closely to that of the granule marker enzymes β-glucosidase, and *N*-acetylglucosaminidase. The

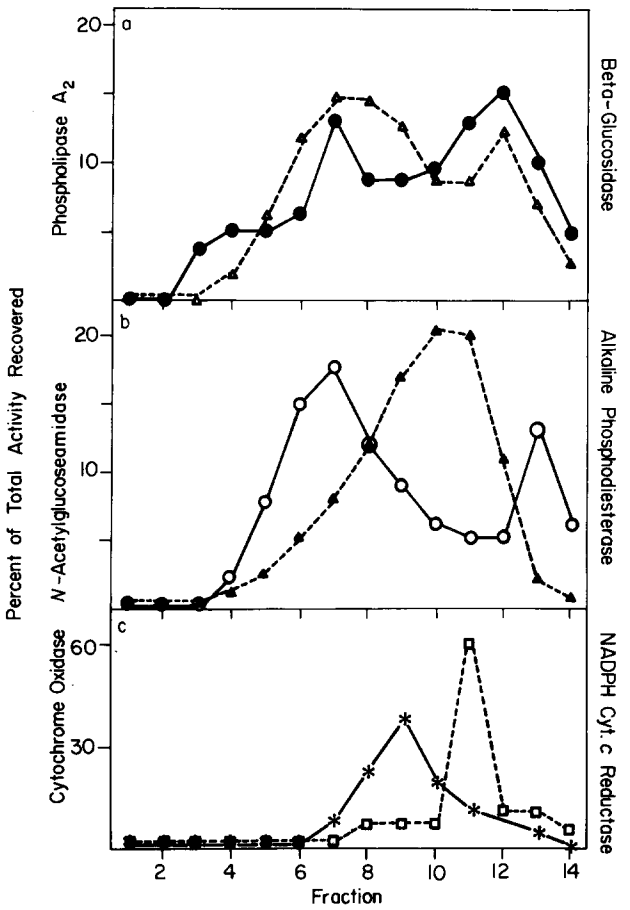

Fig. 3. Distribution of organelle marker enzymes after isopycnic sucrose density gradient centrifugation of macrophage granules. An aliquot (4.0 ml) of resuspended macrophage granules was applied to a linear sucrose gradient and the sample was centrifuged and the fractions collected as described in Materials and Methods. Fraction 1 refers to the bottom of the gradient and fraction 14, the top. The enzymes measured are: ●———●, phospholipase A_2; △ — — — △, B-glucosidase (panel a); ○———○, η-acetylglucosaminidase; ▲ — — — ▲, alkaline phosphodiesterase (panel b); and *———*, cytochrome oxidase; □ - - - - - □, NADPH-cytochrome *c* reductase (panel c). Each point represents the average of duplicate determinations; enzyme recoveries ranged between 80 and 120%.

mitochondrial (cytochrome oxidase) and microsomal (NADPH-cytochrome *C* reductase) markers each distributed as discrete symmetrical peaks of activity in fractions 9 and 11, respectively, and may contain some neutral-active phospholipase A_2 activity. Because the plasma membrane marker, alkaline phosphodiesterase, was broadly distributed in the gradient, plasma membranes were isolated using a recently described procedure [13]. Although enriched up to 15-fold in alkaline phosphodiesterase activity, phospholipase A_2 activity was not detected in this fraction but was quantitatively recovered in other particulate fractions. Thus, we concluded that the neutral-active phospholipase A_2 of macrophages is primarily associated with granule membranes.

To solubilize and purify the granule membrane-associated phospholipase A_2,

TABLE I

RECOVERY OF PROTEIN AND ENZYMATIC ACTIVITY DURING PURIFICATION OF PHOSPHOLIPASE A_2 FROM BCG-INDUCED RABBIT ALVEOLAR MACROPHAGE

Fraction	Total protein (mg)	Yield (%)	Total Phospholipase A_2 activity (nmol/h)	Total activity (%)	Specific activity (nmol/h per mg)	Purification (fold)
Homogenate	735.0	100	26 000	100	35	1
Supernate I (acid extract)	141.0	19	26 508	102	188	5
Supernate II (pellet extract)	0.9	0.12	15 989	61	17 765	504
CM-Biogel	0.2	0.03	8947	34	44 737	1278

Macrophages were obtained as described in Materials and Methods. The data is a single experiment from an extraction of $2.6 \cdot 10^9$ macrophages and is representative of four other experiments. Phospholipase A_2 activity is measured with autoclaved *E. coli* as substrate and activity is expressed as nmol free fatty acid formed/h per mg protein. All values are corrected for non-enzymatic hydrolysis which was less than 3% in all experiments.

intact macrophages were extracted with sulfuric acid. The recovery of phospholipase A_2 activity and protein is summarized in Table I. Selective pH precipitation of the acid extract (SUP I) followed by re-extraction of the resultant pellet yielded a supernate fraction (SUP I) which was enriched 504-fold over the homogenate with a recovery of 61%.

Further enrichment of the phospholipase was achieved by ion-exchange chromatography of SUP II on CM-Biogel (Fig. 4). A single, symmetrical peak of

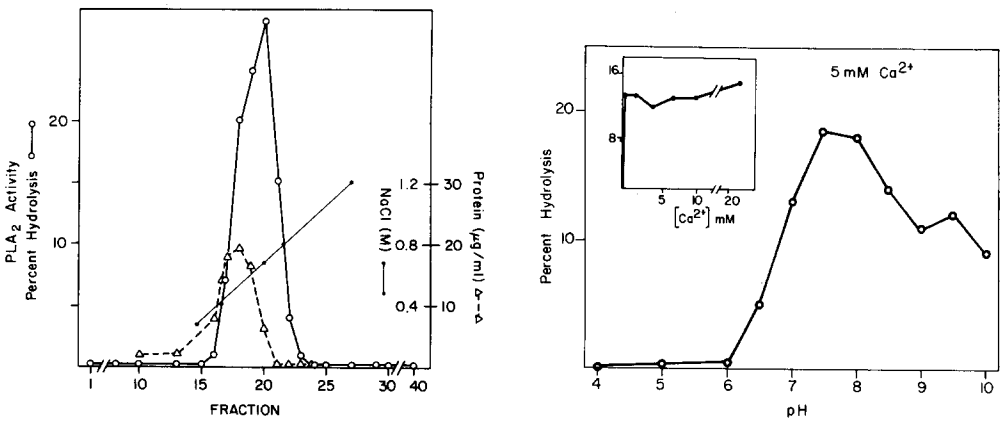

Fig. 4. Ion-exchange chromatography of the acid-solubilized phospholipase A_2. Phospholipase A_2 (PLA_2) activity was eluted from a CM-BioGel column by a linear NaCl gradient (see Materials and Methods) and is expressed as percent hydrolysis/10 μl sample. Each value is the average of determinations done at two levels of protein in the linear range of the assay; the mixtures were incubated at 37° C for 10 min.

Fig. 5. Optimal pH and Ca^{2+} requirements of the partially purified phospholipase A_2. 10 μl of the pooled CM-BioGel fraction were added to standard reaction mixtures containing (5 mM) $CaCl_2$ and at the indicated pH, or at pH 7.5 with the indicated concentration of $CaCl_2$ (insert); the mixtures were incubated at 37° C for 10 min.

enzyme activity eluted between 0.4 and 0.8 M NaCl and contained approx. 0.2 mg protein. The average specific activity of the phospholipase A_2 was 44.7 μmol/h per mg and was enriched 1278-fold with respect to the homogenate in yield of 33%. The specific activity was not constant over the entire elution profile. Thus, the peak fraction (fraction 20) had a specific activity of 108 μmol/h per mg (representing a 3000-fold enrichment over the homogenate), and fractions 21 and 22 contained significant phospholipase activity and no measurable protein suggesting an even further enrichment of the enzyme in this region of the column.

The pH optima and Ca^{2+} requirement of the partially purified enzyme is shown in Fig. 5. The purified phospholipase A_2 is active over a broad pH range. Thus, the solubilized enzyme is similar to the phospholipase A_2 activity of disrupted macrophages and granules (Fig. 1) with respect to the pH required for optimal activity. While, 5 mM Ca^{2+} was required for maximal phospholipase A_2 activity in disrupted granules, less than 1.0 mM Ca^{2+} was required for maximal activity with the solubilized enzyme (insert).

To confirm the positional specificity of the granule-associated phospholipase A_2, the partially purified enzyme was incubated with 1-[1-^{14}C]-stearyl-2-acyl 3-*sn*-glycerophosphorylethanolamine (Fig. 6). The only product formed with increasing concentrations of enzyme was radiolabelled lysophosphatidylethanolamine indicating phospholipase A_2 specificity.

Recent reports have demonstrated that indomethacin inhibits phospholipase A_2 activity of polymorphonuclear leukocytes and human platelets [11,12] but

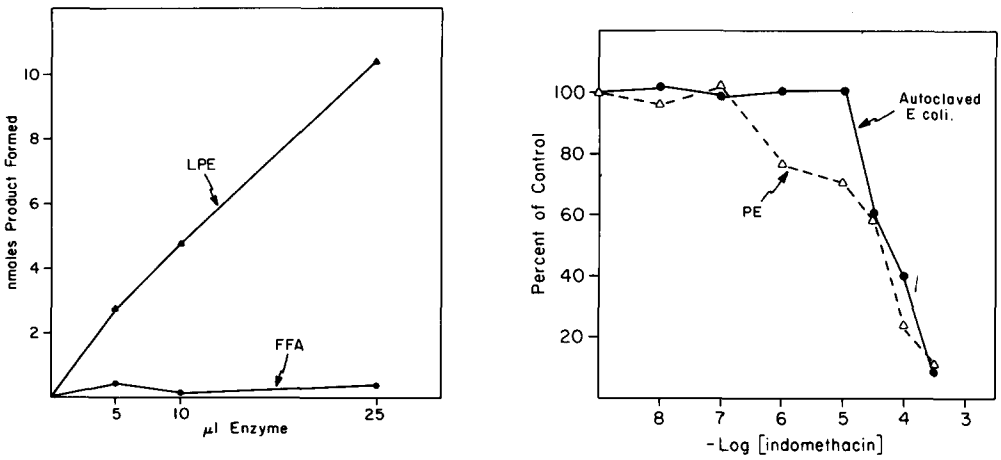

Fig. 6. Positional specificity of the partially purified phospholipase A_2 (PLA_2). Reaction mixtures in a total volume of 1.0 ml contained 2.5 μmol $CaCl_2$/50 nmols 1-[1-^{14}C]-stearyl-2-acyl 3-*sn*-glycerophosphorylethanolamine (9000 cpm)/the indicated amount of enzyme (10 μg protein/ml) and were incubated for 1 h at 37°C. Activity is expressed as nmol product formed: ▲——▲, 1-[1-^{14}C]-stearyl lysophosphatidylethanolamine (LPE); ●——●, 1-[1-^{14}C]-stearate (FFA).

Fig. 7. Inhibition of the partially purified phospholipase A_2 by indomethacin. Phospholipid hydrolysis was measured in reaction mixtures containing 10 μl pooled CM-BioGel fraction (10 μg protein/ml) and either radiolabelled autoclaved *E. coli* (see legend Fig. 1) or 50 nmol 1-acyl-2-[1-^{14}C]-linoleoyl 3-*sn*-glycerophosphorylethanolamine (PE) (see legend Fig. 6) in the presence of varying concentrations of indomethacin. The drug was dissolved in 95% ethanol and appropriate ethanol controls were run.

not enzymes of venom and pancreatic origin. The effect of indomethacin on the purified macrophage phospholipase A_2 using autoclaved *E. coli* or sonicated dispersions of pure phosphatidylethanolamine as substrate is shown in Fig. 7. Activity was inhibited by indomethacin in a dose-dependent fashion and the amount required for 50% inhibition of phospholipase activity was similar regardless of substrate used (approx. $5.0 \cdot 10^{-4}$ M). Inhibition was readily detected at $1.0 \cdot 10^{-6}$ M drug using as substrate pure phosphatidylethanolamine, whereas only concentrations of indomethacin greater than $1.0 \cdot 10^{-5}$ M inhibited activity when *E. coli* was used as substrate.

Discussion

Our results demonstrate that BCG-induced rabbit alveolar macrophages contain a Ca^{2+}-dependent, neutral-active, phospholipase A_2 that is predominantly associated with intracellular granules. Previous studies had suggested that this enzyme was of microsomal origin [18]. Macrophage granules also contain acid-active soluble phospholipases A (pH 3.5—5.5) which are Ca^{2+}-independent and are inactivated by sonication. By comparison, azurophilic and specific granules of human and rabbit polymorphonuclear leukocytes contain a similar Ca^{2+}-dependent, membrane-associated, phospholipase A_2, but lack a soluble, acid-active phospholipase A_2 [10,14].

The bimodal distribution of the Ca^{2+}-dependent phospholipase A_2 after isopycnic density gradient centrifugation of disrupted macrophages resembled that of the lysosomal (granule) marker enzymes β-glucosidase and *N*-acetylglucosaminidase, and probably reflects the presence of different populations of macrophage granules. Lowrie et al. [25] has shown that BCG-induced alveolar macrophages have at least three classes of granules, each with a unique enzyme content. Thus, the appearance of phospholipase activity in fractions 3 and 4 (Fig. 3) could be due to the presence of a dense granule population that contains little β-glucosidase or η-acetylglucosaminidase activity. Similar results were obtained for the intracellular distribution of the phospholipase A_2 in human and rabbit polymorphonuclear leukocytes [10,14], although the leukocytic enzyme unlike the macrophage phospholipase A_2 is probably associated with the plasma membrane as well.

Sulfuric acid extraction, pH precipitation, and ion-exchange chromatography have recently been used to isolate membrane-associated phospholipases A_2 from rabbit polymorphonuclear-leukocytes and human platelets [23,24]. As judged by the almost 10-fold range in the specific activity of the macrophage phospholipase A_2 across the CM-BioGel column (fraction 17: 13 μmol/h per mg vs. fractions 20: 108 μmol/h per mg), the preparation at this stage is substantially contaminated. However, the maximal reliable specific activity in fraction 20 of 108 μmol/h per mg, approaches that of the rabbit leukocyte phospholipase A_2 (300 μmol/h per mg) recently purified more than 8000-fold to near homogeneiety [23].

The partially purified macrophage phospholipase A_2 was extremely sensitive to added Ca^{2+} and low concentrations of detergents. Maximal activity was found with less than 1 mM added Ca^{2+}, whereas the membrane-associated enzyme required at least 5 mM added Ca^{2+} for optimal activity. Concentrations

of Triton X-100, deoxycholate and SDS greater than 0.05% totally inhibited enzymic activity (data not shown). Phospholipases A_2 solubilized from the membranes of rabbit leukocytes and human platelets had similar properties [14,24]. Inhibition of phospholipase activity by detergents in acetone-butanol extracts of macrophages may explain the previously reported lack of a neutral-active phospholipase A_2 in these cells [26].

Earlier work suggested that the neutral-active phospholipase A hydrolyzed the 2-acyl position of phospholipids since incubations containing enzyme and phospholipid labelled with unsaturated fatty acids in the 2-position released only ^{14}C fatty acids as products [8]. However, Sahu and Lynn [26] and Elsbach [27] have described an alkaline-active, lysophospholipase in these cells which in concert with a phospholipase A_1 would yield the same results. Therefore, the enzyme was incubated with 1-[1-^{14}C]-stearyl-2-acyl 3-*sn*-glycerophosphoryl-ethanolamine. The only radioactive product formed was the 1-[1-^{14}C]-stearyl lyso-derivative, confirming the 2-position specificity of this enzyme.

Rabbit alveolar and mouse peritoneal macrophages contain no free arachidonate [28]; they readily incorporate added arachidonate into membrane phospholipids and release arachidonate in response to a variety of stimuli [29,30]. Since arachidonate in phospholipid is almost exclusively esterified at the 2-position, the action of a Ca^{2+}-dependent phospholipase A_2 is thought to mediate the enhanced turnover of unsaturated fatty acids preceding prostaglandin production [31]. Thus, human leukocytes, which contain a Ca^{2+}-dependent, membrane-associated phospholipase A_2 [10], selectively release [^{3}H]-arachidonate, but not palmitate, during phagocytosis of opsonized zymosan particles [32].

Inhibitors of cellular phospholipases could serve to regulate prostaglandin production in both normal and inflammatory processes. In this regard, recent studies by Kaplan et al. [11] have demonstrated that phospholipase A_2 activity of rabbit leukocytes is inhibited by indomethacin while snake venom and pancreatic phospholipases A_2 were unaffected at the concentrations of drugs used. The macrophage phospholipase A_2 was inhibited similarly by indomethacin (Fig. 7) and recently we have demonstrated with the isolated leukocyte phospholipase A_2 that the sensitivity to inhibition by nonsteroidal anti-inflammatory agents is modulated by free Ca^{2+} concentrations [12] and may represent a second site of action for these drugs [5,33].

The role of the neutral-active phospholipase in macrophage functions other than the proposed contribution to membrane phospholipid remodeling and prostaglandin synthesis is not known. In the rabbit leukocyte, the membrane-associated phospholipase A_2 found in azurophilic and specific granules functions synergistically with a bacteriocidal protein also associated with granule membranes, to hydrolyze the phospholipids of intact *E. coli* [23]. The macrophage enzyme with similar physical and chemical properties may have a comparable role. In addition, the Ca^{2+}-dependent phospholipases A_2 may contribute to autolytic and cytolytic events during inflammation [35]. In this regard, a cell-free phospholipase A_2 with similar pH and Ca^{2+} requirements is present in rabbit peritoneal exudates, which appears to be of granulocytic origin [34]. Thus, activation or release of such enzymes could propagate inflammation and injury by generating free fatty acids or lysophospholipids. Clearly, much work is required to elucidate the role of these enzymes in cell function.

Acknowledgements

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