Phospholipid metabolism by phagocytic cells. Phospholipases A_2 associated with rabbit polymorphonuclear leukocyte granules

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Abstract Polymorphonuclear leukocytes obtained from sterile peritoneal exudates in rabbits contain two phospholipid-splitting activities (phosphatidylacylhydrolases EC 3.1.1.4), one most active at pH 5.5 and the other between pH 7.2 and 9.0. Hydrolysis of phospholipid was demonstrated using *Escherichia coli* labeled during growth with [1-^{14}C]oleate and then autoclaved to inactivate *E. coli* phospholipases and to increase the accessibility of the microbial phospholipid substrates.

The acid and alkaline phospholipase activities are both membrane bound, calcium dependent, and heat stable, and they appear to be specific for the 2-acyl position of phospholipids. Evidence was also obtained suggesting that the *E. coli* envelope phospholipids with oleate in position 2 are more readily degraded than those with palmitate. The two activities are associated with azurophilic as well as specific granules (obtained by zonal centrifugation) and with phagosomes (isolated after ingestion of paraffin particles by the granulocytes).

Phospholipase A activities at pH 5.5 and pH 7.5 degrade the two major phospholipids of *E. coli,* phosphatidylethanolamine and phosphatidylglycerol, to the same extent, but the phospholipase activity at acid pH does not hydrolyze micellar dispersions of phosphatidylethanolamine. By contrast, phospholipase A_2 activity at pH 7.5 degrades both types of phosphatidylethanolamine substrates. Heparin and chondroitin sulfate inhibit phospholipase activity at pH 5.5 but have little effect on actvity at pH 7.5.

All detergents tested inhibited phospholipase activity, and both activities are inhibited by reaction products, free fatty acid and lysophosphatidylethanolamine. This product inhibition is only partially prevented by addition of albumin.

Supernatant fractions of granulocyte homogenates contain a heat-labile inhibitor of granule phospholipase activity at pH 7.5. Boiling the fraction not only removes the inhibition but actually results in stimulation of hydrolysis at pH 7.5 as well as pH 5.5.

These granule-associated phospholipase A activities of polymorphonuclear leukocytes differ in several of their properties from granule or lysosomal phospholipases of other phagocytic cells.

Supplementary key words hydrolysis of *Escherichia coli* phospholipids · granules · phagosomes · inhibition by heparin · product inhibition · heat-labile inhibitor · zonal centrifugation

The structure, function, biosynthesis, and turnover of biological membranes have been under intense scrutiny during recent years. The study of phospholipid-splitting enzymes is an integral part of this investigative effort. Of this class of degradative enzymes, those that attack either the 1-acyl or the 2-acyl group of the major glycerophosphatides (the phospholipases A) have received the most attention. Much progress has been made in establishing the characteristics of enzyme–substrate interactions of phospholipases A of different origins (1–3). A number of these phospholipases have been studied in highly purified form (2, 4–7). These studies have provided new insight into the complexities of the interactions between enzymes and nonphysiological water-insoluble substrates (8, 9). Despite these important advances and the reasonable assumption that cellular phospholipases A influence cell function by their effects on cell membranes, in no instance has a specific role been identified for these enzymes in biological events involving cell membranes.

It has been postulated that the extensive membrane alterations accompanying phagocytosis may require the breakdown of phospholipids (10). Indeed, several types of phagocytic cells possess phospholipid-splitting enzymes, including phospholipase A activities (10–15). However, no evidence has yet been obtained showing that the dramatic rearrangements of the phagocyte's membranes during ingestion (16) are associated with increased breakdown of major phospholipid species (14, 15). On the other hand, we have recently demonstrated that the granulocyte's phospholipases do participate in the degradation of phospholipids of ingested microorganisms (17).

As part of a larger investigation of the granulocyte's microbicidal mechanisms and of the role of microbial lipids as determinants in bacterial killing and survival (18, 19), we have undertaken a further examination of the granulocyte's phospholipases.

Abbreviations: PMN, polymorphonuclear leukocytes.

As substrate for the assay of phospholipase activity, we used *E. coli* first grown in the presence of 1-^{14}C-labeled fatty acid and then subjected to autoclaving to inactivate bacterial phospholipases and to render the lipids more accessible to hydrolytic attack (17, 20). The use of autoclaved, radioactive fatty acid-labeled *E. coli* has at least two advantages: (*1*) this substrate is readily broken down under conditions where micellar suspensions of isolated phospholipids are not, and (*2*) the presentation of the lipids as part of a biological structure (although altered by the autoclave procedure) probably provides conditions for the action of phospholipases that are more physiological than those that prevail when the substrate is in micellar form.

In this report we describe some of the properties of two phospholipase A activities, one with an acid and one with an alkaline pH optimum, that are associated with leukocyte granules and phagosomes.

MATERIALS AND METHODS

Labeled substrates

Phospholipids of *Escherichia coli* W were labeled with [1-^{14}C]oleate or [1-^{14}C]palmitate as previously described (20). Generally, 95% of the incorporated label was in phospholipid, and the distribution of the label closely corresponded to the chemical composition of *E. coli* phospholipid: phosphatidylethanolamine, 50–65%; phosphatidylglycerol, 15–20%; and cardiolipin, 10–15%. Radiolabeled *E. coli* were autoclaved for 15 min at 120°C and 2.7 kg/cm^2. As demonstrated by snake venom hydrolysis (*Crotalus adamanteus*), 95% of the [1-^{14}C]oleate was in the 2-acyl position of the phospholipids.

The biosynthetic preparation of 1-acyl-2-[^{14}C]linoleoyl-3-glycerophosphorylethanolamine was carried out as previously described (21).

Cells and cell fractions

Granulocytes were obtained from sterile peritoneal exudates elicited in rabbits by intraperitoneal injection of 0.1% glycogen in isotonic saline. Differential counts showed that more than 95% of the cells were polymorphonuclear leukocytes (PMN), and more than 90% of the cells excluded the vital dye trypan blue. PMN were sedimented by centrifugation at 50 *g* for 10 min, washed once with Hanks' medium, and resuspended in 0.34 M sucrose in a final concentration of 1.0×10^8 cells/ml. The cell suspension was kept at 4°C for 30 min and then homogenized with a Potter-type homogenizer and a motor-driven Teflon pestle until at least 95% of the cells were disrupted. Cell disruption was monitored by phase-contrast microscopy. Granules were isolated by the method of Cohn and Hirsch (22) except that the homogenate was initially centrifuged at 600 *g* (rather than 400 *g*) for 10 min to remove whole cells, nuclei, and debris. The 600 *g* supernatant fluid was centrifuged at 8200 *g* for 20 min to yield the granule pellet, which was resuspended in 0.34 M sucrose (1.0×10^8 cell equivalents/ml). For zonal centrifugation, 8.3×10^9 PMN were homogenized in 16.6 ml of 0.34 M sucrose (5×10^8 cells/ml). The homogenate was centrifuged at 190 *g* for 5 min, and the 190 *g* supernatant fluid containing the granules was applied to the zonal rotor. Fractionation was carried out as described by Baggiolini, Hirsch, and de Duve (23) with the following modifications: (*a*) An AMSE zonal rotor was used, (*b*) the rotor was loaded and emptied at a rate of 25 ml/min while spinning at 1600 rpm in a Mistral 6L MSE centrifuge, and (*c*) the sample was centrifuged at 5000 rpm for 15 min. After centrifugation, the gradient was displaced from the spinning rotor with 2.2 M sucrose, and 30 fractions of 20 ml each were collected. Sucrose concentration was determined with an Abbe refractometer.

Phagosomes were prepared from glycogen-induced rabbit peritoneal PMN by the method of Stossel et al. (24). The phagosome preparation contained 26% of the total granule marker enzyme (β-glucuronidase) activity of the homogenate.

Enzyme assays

Phospholipase A (EC 3.1.1.4) was determined by measuring the release of [^{14}C]oleate in reaction mixtures that contained 2.5×10^8 [^{14}C]oleate-labeled autoclaved *E. coli* cells (containing approximately 5 nmoles of phospholipid [17]), 40 μmoles of the appropriate buffer, 5 μmoles of $CaCl_2$, and 25–50 μg of protein ($1–2 \times 10^6$ cell equivalents) in a total volume of 0.5 ml. Reaction mixtures were incubated for 30 min at 37°C in a shaking water bath. The reaction was stopped by the addition of 3.0 ml of methanol–chloroform 2:1 (v/v). Lipids were extracted overnight at room temperature by the method of Bligh and Dyer (25). ^{14}C-labeled free fatty acid was separated from phospholipid by thin-layer chromatography on commercial silica gel F-254 plates (EM Laboratories, Elmsford, N.Y.) in a solvent system consisting of petroleum ether–ethyl ether–glacial acetic acid 80:20:1 (v/v/v). To separate individual phospholipid species and to detect accumulation of monoacyl derivatives of bacterial and synthetic phosphatidylethanolamine, thin-layer chromatography was carried out in a solvent mixture consisting of chloroform–methanol–0.1 N ammonium hydroxide 80:30:1 (v/v/v). Lipid species were visualized by exposure of the plates to iodine vapors, and the fractions were scraped directly into counting vials for determination of radioactivity by liquid scintillation counting as previously described (12). Phospholipase assays using 1-acyl-2-[^{14}C]linoleoyl-3-glycerophosphorylethanolamine as substrate were carried out also as previously described (21).

The following methods were used to assay for lysosomal marker enzymes. For β-glucuronidase we used the method

TABLE 1. Recovery of protein, β-glucuronidase, and phospholipase A activity at pH 5.5 in granule preparations from rabbit polymorphonuclear leukocytes

Fraction	Protein[a]		β-Glucuronidase[b]		PLA 5.5[c]	
	Total	%	Total	%	Total	%
Homogenate	11.4 ± 0.8	100	486 ± 80	100	167 ± 7.0	100
600 *g* pellet	5.0 ± 0.7	44	150 ± 31	31	42 ± 6.0	25
8200 *g* pellet (granules)	2.6 ± 0.5	23	265 ± 72	54	84 ± 6.4	50
8200 *g* supernatant	2.8 ± 1.5	24	48 ± 2.3	10	21 ± 1.9	13

Results are presented as means ± SEM of four separate experiments. All values have been corrected for nonenzymatic hydrolysis of substrate (less than 2%/hr). Percentage recoveries were 91 for protein, 95 for β-glucuronidase, and 88 for phospholipase A activity at pH 5.5.

[a] Protein is expressed as mg/10^8 cell equivalents.

[b] β-Glucuronidase is expressed as nmoles of substrate hydrolyzed/hr/10^8 cell equivalents.

[c] Phospholipase at pH 5.5 is expressed as nmoles of free fatty acid released/hr/10^8 cell equivalents. Phospholipase activity (1-^{14}C-labeled free fatty acid released from [1-^{14}C]oleate-labeled *E. coli*, calculated as percentage of the total *E. coli* lipid radioactivity) is converted to nanomoles of free fatty acid released by assuming that 2.5 × 10^8 *E. coli* cells contain 5 nmoles of phospholipid (17).

of Talalay, Fishman, and Huggins (26) as modified by Canonico and Bird (27). Peroxidase was measured by the method of Schultz, Shay, and Gruenstein (28), and alkaline phosphatase by the method of Bessey, Lowry, and Brock (29).

Protein was determined by the method of Lowry et al. (30), using bovine serum albumin as standard. The 1-acyl-2-[^{14}C]linoleoyl-3-glycerophosphorylethanolamine and the [^{35}S]heparin were the generous gifts of Dr. B. M. Waite (Bowman Gray School of Medicine), and chondroitin-4- and chondroitin-6-sulfates were given to us by Dr. L. Rosenberg (New York University School of Medicine).

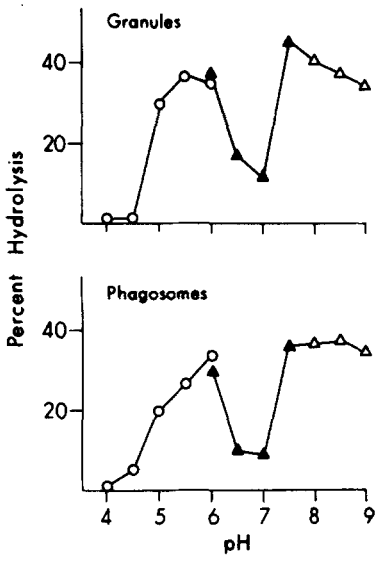

Fig. 1. The pH dependence of phospholipase activities of rabbit polymorphonuclear leukocyte granules and phagosomes. Incubation mixtures in a total volume of 0.5 ml contained 40 μmoles of the appropriate buffer (pH 4.0–6.0, sodium acetate; pH 6.0–7.5, Tris–maleate; pH 8.0–9.0, Tris–HCl), 5 μmoles of $CaCl_2$, 2.5 × 10^8 autoclaved *E. coli* cells labeled with [1-^{14}C]oleate, and 1 × 10^7 cell equivalents of phagosome or 2 × 10^6 cell equivalents of granule preparations. Incubations were carried out at 37°C for 1 hr. Identical results were obtained with granule preparations incubated for 30 min. All values are corrected for nonenzymatic hydrolysis, which did not exceed 2% over the entire pH range.

RESULTS

General properties and intracellular distribution of granulocyte phospholipases

The granule preparations, prepared from rabbit granulocytes, used in these studies contained the same percentages of total protein and β-glucuronidase present in the whole homogenate that have been reported by others (22, 24) (**Table 1**). The phagosome preparations contained 26% of the total β-glucuronidase (granule marker enzyme) activity of the homogenate.

The pH optima for phospholipase activity in these fractions (measured by free fatty acid release from [1-^{14}C]oleate-labeled *E. coli*) are shown in **Fig. 1.** The effects of pH on hydrolysis of the bacterial lipids were almost identical for granules and phagosomes. Two distinct peaks are apparent, one rather discrete between pH 5.0 and 6.0 and the other much broader in the alkaline range. No accumulation of radioactive lyso compounds was detected at any pH.

Figs. 2 and 3 depict optimal conditions for hydrolysis. At both pH 5.5 and pH 7.5 an absolute requirement for Ca^{2+} is evident (**Fig. 2**). No activity was found in the absence of added Ca^{2+} or in the presence of EDTA. Mg^{2+} cannot substitute for Ca^{2+} in this reaction. Phospholipase activity was nearly linear for 30 min if less than 50 μg of granule protein was used per 0.5 ml of reaction mixture at pH 5.5 and less than 25 μg at pH 7.5 (**Fig. 3,** top panel). With increasing numbers of autoclaved *E. coli* at a fixed concentration of granule protein (50 μg at pH 5.5 and 25 μg at pH 7.5), hydrolysis reached a plateau at 2.5 × 10^8 microorganisms. For reasons of economy and

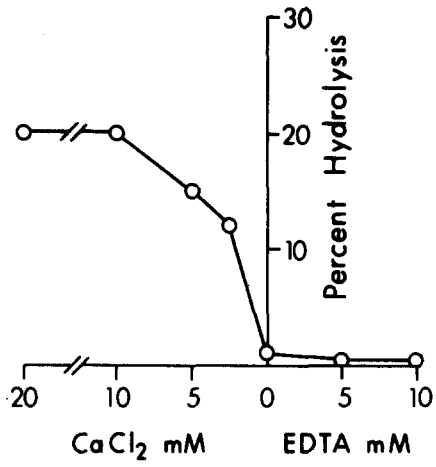

Fig. 2. Calcium requirement for phospholipase activity at pH 7.5. Different concentrations of EDTA and $CaCl_2$ were added to the standard reaction mixture described in the footnote to Table 2. Identical results were obtained for phospholipase activity at pH 5.5.

accuracy of the assay, this number of labeled bacteria was used in the studies reported here.

We have previously reported that during phagocytosis of autoclaved *E. coli* by intact granulocytes, degradation of the two main *E. coli* phospholipids, phosphatidylethanolamine and phosphatidylglycerol, takes place to nearly the same extent (17). This is also the case during breakdown by granule phospholipases at both pH 5.5 and pH 7.5 (not shown). However, whereas micellar dispersions of synthetic phosphatidylethanolamine are broken down at pH 7.5, no degradation was detected at pH 5.5.

The phospholipase activities appeared to be associated predominantly with the granules, as judged by comparing recovery of β-glucuronidase and acid phospholipase activities in the granule fraction relative to the whole homogenate. Table 1 shows that about half of each of these activities is in the granule fraction, with a comparable increase in specific activity. (It should be recalled that PMN contain few mitochondria and other organelles that sediment at 8200 *g*.) Whereas the phospholipase activity at pH 5.5 can be quantitated reasonably accurately, this is not possible at pH 7.5 because the activity in the granule preparations is generally two to three times that in the whole homogenate. This suggests that the whole homogenates contain an inhibitor of the alkaline phospholipase activity that is removed during fractionation. Evidence in support of this suggestion will be presented below.

The subcellular localization of the granule phospholipases was further examined by zonal centrifugation of granule preparations on sucrose gradients. **Fig. 4** depicts the distribution of protein, phospholipase activity at pH 5.5 and 7.5, and marker enzymes for specific (alkaline phosphatase) and azurophilic (peroxidase) granules. As expected, peroxidase activity was largely confined to the denser fractions, marking the azurophilic granules, and

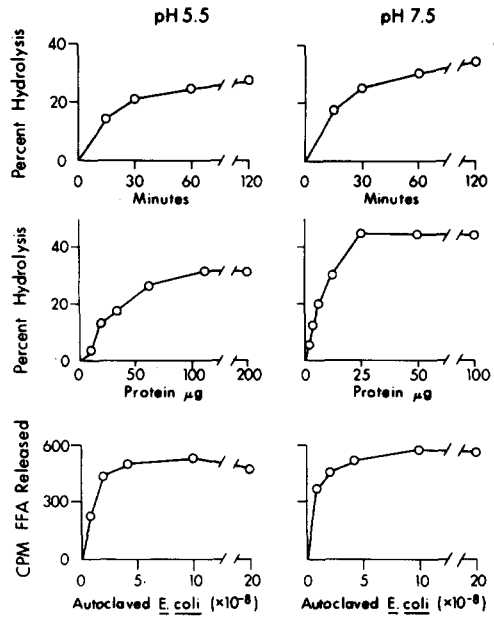

Fig. 3. Effects of time and concentration of protein and substrate on granule phospholipase A activities. Except where indicated otherwise, standard reaction mixtures (Table 2) were incubated for 30 min at 37°C. Phospholipase A activity is expressed as percentage hydrolysis or as cpm of ^{14}C-labeled free fatty acid released.

alkaline phosphatase was concentrated in the lighter fractions. The distributions of the acid and the alkaline phospholipase activities were almost identical and corresponded to the distribution of protein. Both activities were associated with specific as well as azurophilic granules. Considerable phospholipase activity was found in the less

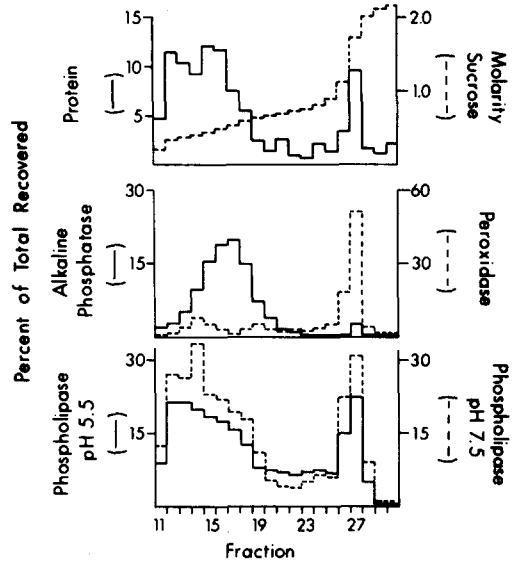

Fig. 4. Zonal centrifugation of granule preparations on a sucrose gradient. Assay procedures are described in Materials and Methods. Protein and enzyme distributions are expressed as the percentage of total activity recovered in each fraction. Percentage recoveries were 89 for protein, 98 for alkaline phosphatase, 88 for peroxidase, and 99 and 105, respectively, for granule phospholipase activity at pH 5.5 and 7.5.

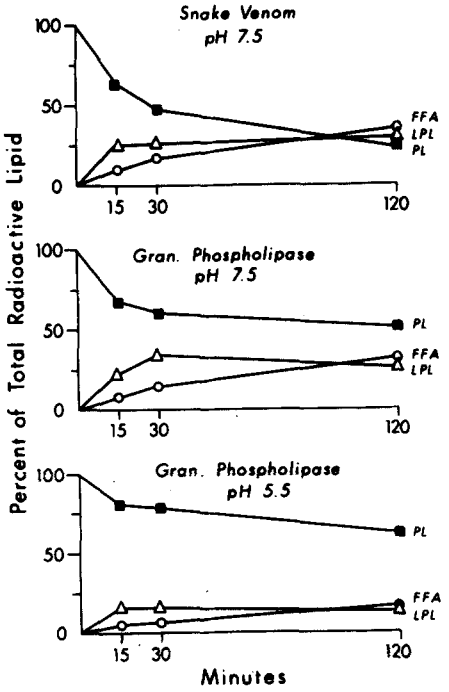

Fig. 5. Comparison of the hydrolysis of [1-^{14}C]palmitate-labeled *E. coli* by snake venom and partially purified granule phospholipases A. The composition of the incubation mixtures was as described in the footnote to Table 2 except that 2.0 × 10^8 cells of [1-^{14}C]palmitate-labeled autoclaved *E. coli* were used as substrate, and a partially purified granulocyte enzyme preparation was used. This preparation was obtained by sulfuric acid extraction of granulocyte homogenates, followed by centrifugation of the extract at 24,000 *g* for 20 min. The supernatant fluid was brought to pH 7.4 by dialysis against 1 mM Tris–HCl, and the dialysate was centrifuged at 24,000 *g* for 20 min to yield a supernatant fluid that contained both phospholipase activities. *Crotalus adamanteus* venom was added in an amount of 200 μg per incubation mixture. PL = phospholipid (PE, phosphatidylethanolamine, and PG, phosphatidylglycerol, representing approx. 85% of the total labeled phospholipid of *E. coli*); LPL, monoacyl derivatives of PE and PG; FFA, free fatty acid.

dense sucrose fractions (fractions 12–15). This does not represent solubilized phospholipase activity because virtually all activity was sedimented by centrifugation of these fractions at 100,000 *g*. It is possible, therefore, that the activity in these fractions was due to the presence of membrane fragments that contain phospholipase activity or to an ill-defined heterogeneous population of granules (23, 31).

Judging by the established positional specificity of snake venom phospholipase A_2 and by its ability to degrade about 85% of the total labeled *E. coli* phospholipids, approximately 95% of the [1-^{14}C]oleate of *E. coli* phosphatidylethanolamine and phosphatidylglycerol occupies position 2. As mentioned earlier, during degradation of *E. coli* lipids by granule preparations, practically no radioactive lyso compounds accumulated either at pH 7.5 or at pH 5.5. However, with this substrate the release of radioactive free fatty acid may result from either phospholipase A_2 activity or the combined activities of a phospholipase A_1 and a lysophospholipase. In an attempt to define more clearly the specificity of the granule phospholipase, *E. coli* labeled with [^{14}C]palmitate was used as substrate because the palmitate occupies both the 1-acyl and the 2-acyl positions of the major *E. coli* phospholipids.

Fig. 5 shows the time course of degradation of [1-^{14}C]palmitate-labeled phosphatidylethanolamine plus phosphatidylglycerol (these two compounds were not chromatographically separated in these experiments because their rate of degradation has always been found to be identical) by snake venom at pH 7.5 and by partially purified granule phospholipases at pH 7.5 and 5.5. It is apparent that the pattern of hydrolysis is very similar for all three activities. In each instance, labeled lyso compounds initially accumulate more rapidly than labeled free fatty acids. At 2 hr, when approximately 80% of the phosphatidylethanolamine plus phosphatidylglycerol has been broken down by snake venom, about half of the radioactivity in the breakdown products is in the lyso derivatives and half in the free fatty acids. (The same distribution was found in experiments in which hydrolysis exceeded 90%.) The different rates of accumulation of labeled 1-monoacyl phosphatide and of the fatty acid stemming from position 2 must reflect the heterogeneity of the substrate. Unless snake venom phospholipase exhibits an acyl-group specificity with respect to these mixed *E. coli* phospholipids, heretofore not detected using micellar substrates with precisely defined fatty acid composition (1), it seems most likely that phosphatidylethanolamine and phosphatidylglycerol subspecies with palmitate in position 2 are initially less accessible to enzymatic attack than other subspecies. Between 30 and 120 min of hydrolysis by granules at pH 7.5, the accumulation of free fatty acid radioactivity exceeded by about 6% the loss of radioactivi-

TABLE 2. Effect of untreated and boiled 8200 *g* supernatant fraction on granule phospholipase A activity

	Phospholipase Activity[a]	
	pH 5.5	pH 7.5
	% hydrolysis	
Granules		
Alone	8.1 ± 0.1	20.3 ± 1.3
+ 8200 *g* supernatant	15.3 ± 3.1	8.2 ± 1.7
+ Boiled 8200 *g* supernatant	13.5 ± 0.5	30.8 ± 3.1
8200 *g* supernatant		
Untreated	7.2 ± 1.3	3.7 ± 0.6
Boiled	0.2 ± 0.2	1.0 ± 0.3

These data are presented as means ± SEM of three experiments. Incubation mixtures in a total volume of 0.5 ml contained 2.5 × 10^8 autoclaved *E. coli* cells labeled with [1-^{14}C]oleate, 40 μmoles of buffer (Tris–maleate at pH 7.5 or sodium acetate at pH 5.5), 5 μmoles of $CaCl_2$, 25–50 μg of granule protein (1–2 × 10^6 cell equivalents), and 2.0 × 10^7 cell equivalents of untreated or boiled 8200 *g* supernatant fraction. Reaction mixtures were incubated at 37° C for 15 min.

[a] Phospholipase A activity (1-^{14}C-labeled free fatty acid released from [1-^{14}C]oleate-labeled *E. coli*) is expressed as percentage of total *E. coli* lipid radioactivity.

ty from phosphatidylethanolamine plus phosphatidylglycerol. Because during this time there was a loss of radioactivity from lyso compounds that corresponded to the excess of radioactive free fatty acid, some lysophospholipase activity must have been present in this purified preparation. This is not surprising because very substantial lysophospholipase activity at alkaline pH has previously been described in granulocyte homogenates (particularly in supernatant fractions [11, 12]).

Notwithstanding the heterogeneity of the substrates used in these experiments, the results strongly suggest that the granules possess mainly phospholipase A_2 activity both at pH 7.5 and pH 5.5.

Modifiers of granule phospholipase A activities

(*a*) *Effects of boiling and an 8200* g *supernatant fraction.* Boiling of whole homogenates of granulocytes caused an increase in phospholipase activity at pH 7.5 but had much less effect on activity at pH 5.5. Boiling of the granule fraction did not affect activity at either pH but, as indicated earlier, activity in the granules at pH 7.5 was two to three times greater than that in the whole homogenate, rendering quantitation of recovery difficult. These findings suggest the presence of a heat-labile inhibitor. Support for this contention is presented in **Table 2.** Granules alone and the 8200 *g* supernatant fraction each exhibited activity at pH 5.5 and 7.5. Whereas at pH 5.5 the two fractions together showed an additive effect, at pH 7.5 the activity of the two fractions together was only half that of the granules alone. Boiling destroyed phospholipase activity in the supernatant fraction. However, addition of boiled supernatant fraction to a granule fraction not only no longer inhibited at pH 7.5 but actually enhanced activity at both pH's.

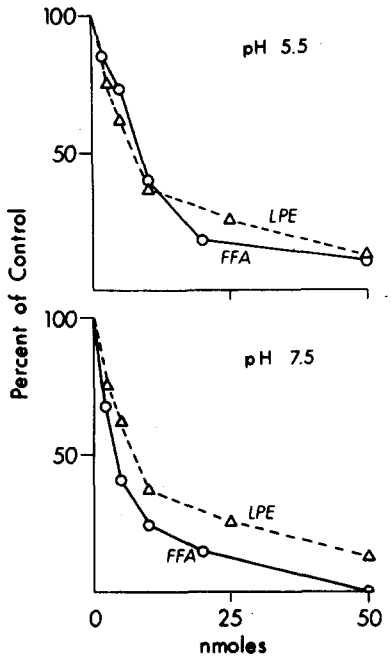

Fig. 6. Product inhibition of granule phospholipases A. Each assay mixture contained 50 μg of granule protein and different concentrations of oleic acid (FFA) and lysophosphatidylethanolamine (LPE) added as aqueous ultrasonic dispersions. Other conditions were as described in the footnote to Table 2.

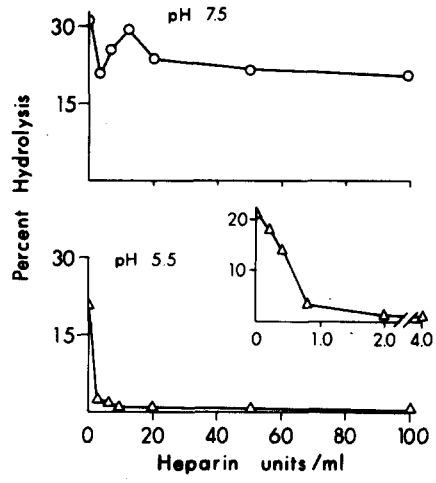

Fig. 7. Inhibition of granule phospholipase A activities by heparin. Standard incubation mixtures contained 40 μg of granule protein and different concentrations of sodium heparin. Incubations were carried out for 1 hr at 37°C.

(*b*) *Product inhibition.* Both products of phospholipase A activity, free fatty acid (oleate) and monoacylphosphatide (lysophosphatidylethanolamine), inhibit degradation of *E. coli* phospholipids by granule preparations (**Fig. 6**). The extent of inhibition at pH 5.5 and 7.5 by increasing concentrations of either oleate or lysophosphatidylethanolamine was approximately the same. Both activities were inhibited at least 50% by 5–10 nmoles of either product. Despite these rather striking inhibitory effects of low concentrations of micellar dispersions of oleate and lysophosphatidylethanolamine, serum albumin devoid of phospholipase activity (32) (at molar concentrations as much as 80-fold higher) failed to markedly stimulate the incomplete degradation of labeled *E. coli* phospholipids. It is possible that the binding affinity of albumin for oleate and lysophosphatidylethanolamine is less than that of the phospholipid-splitting enzymes.

(*c*) *Effects of heparin and detergents.* All detergents tested (the anionic detergent sodium dodecyl sulfate at concentrations of 0.005 g/100 ml and higher and anionic sodium deoxycholate and nonionic Triton X-100 at concentrations of 0.01 g/100 ml or higher) had inhibitory effects on phospholipase activity at both pH's. Heparin at low concentrations profoundly inhibited phospholipase activity at pH 5.5. However, at concentrations as high as 100 U/ml (representing approx. 0.5 mg) it had little effect on the alkaline phospholipase (**Fig. 7**). Inhibition of phospholipase A activity at pH 5.5 is overcome by the addition of enzyme and is readily reversed by protamine sulfate.

Heparin probably exerts its effect on the enzyme rather than on the substrate, inasmuch as it was observed that [^{35}S]heparin did not bind to autoclaved *E. coli* and addition of autoclaved *E. coli* to incubation mixtures that contain heparin did not overcome the inhibitory effect at pH 5.5. Highly purified chondroitin-4-sulfate and chondroitin-6-sulfate also inhibited phospholipase activity at pH 5.5, but at a much higher concentration (25 μg) than heparin.

DISCUSSION

Phospholipase A activity with acid pH optimum has also been found in lysosome-rich fractions of other phagocytic cells, namely Kupffer cells (33) and alveolar macrophages (15). This activity exhibits different properties in each of the three types of phagocytic cells with respect to Ca^{2+} dependence, effect of EDTA, and positional specificity. Neither in liver nor in alveolar macrophages has substantial phospholipase A activity with alkaline pH optimum been found in association with the lysosomes. Thus, distinct differences exist between the phospholipid-splitting enzyme apparatus isolated from different types of phagocytic cells that may reflect differences in cellular function.

Phospholipase A activity at acid and at alkaline pH has previously been demonstrated in rabbit leukocyte preparations using micellar substrates (10, 11, 14). In several respects the activity at acid pH differed from that reported here: the acid phospholipase active towards phosphatidylcholine was unstable and, although associated with the leukocyte granules, was also present in the 8200 *g* supernatant fraction, and, finally, it was not inhibited by deoxycholate. The recently reported alkaline phospholipase activity in granulocyte homogenates (14), which readily hydrolyzes phosphatidylethanolamine (but not phosphatidylcholine), appears rather similar to the activity discussed herein, except that it was less prominently associated with the granule fraction and was less susceptible in inhibition by deoxycholate.

We believe that these differences can be attributed in large measure to the different substrates used. The present characterization of leukocyte phospholipases was carried out with autoclaved *E. coli*. In several studies of phospholipases from various sources, the labeled lipids of these autoclaved organisms have proved to be excellent substrates that are far more easily degraded than substrates in micellar form (15, 20). This is well exemplified in this study by the fact that the phospholipase activity at pH 5.5 could not be demonstrated using purified phosphatidylethanolamine. The harsh autoclave procedure undoubtedly has profound effects on the *E. coli* envelope. However, electron microscopy of autoclaved *E. coli* reveals a remarkably unaltered structure.[1] Moreover, neither thin-layer nor gas–liquid chromatography of, respectively, the main lipid species and the fatty acids of *E. coli* extracted after autoclaving shows recognizable changes. On the other hand, the proteins and other macromolecular constituents of the envelope must have been altered, and we believe it is this alteration in the nonlipid envelope components that renders the *E. coli* lipids accessible to enzymatic attack, because little degradation of unautoclaved *E. coli* takes place (17). Despite the artifactual nature of the autoclaved *E. coli*, the bacterial lipids probably remain arranged within the envelope in a fashion more closely resembling that of natural membranes than is the case for mixed monomeric and micellar lipids.

Although the results show that *E. coli* phosphatidylethanolamine and phosphatidylglycerol as presented in these experiments are readily accepted as substrates by both phospholipase A activities, it is evident that degradation is incomplete, reaching a plateau with time and increasing enzyme concentration. Our evidence suggests that a number of factors contribute to the limited breakdown. These include product inhibition, a heat-labile inhibitory component present in the 8200 *g* supernatant fraction (and also in a 100,000 *g* supernatant fraction),[2] and an apparent selectivity in the breakdown of various subspecies of *E. coli* phosphatidylethanolamine and phosphatidylglycerol by granulocyte phospholipases, which is determined by the fatty acid composition of these two phospholipid species. To what extent each of these factors may operate to regulate phospholipase activity in the intact granulocyte poses a problem for future investigation. In this in vitro system, however, the degradation of the phospholipids of 2.5×10^8 *E. coli* cells (representing approx. 5 nmoles of phospholipid [17]) yields enough free fatty acid and lysophosphatidylethanolamine to reach inhibitory levels (Fig. 6).

Of considerable interest is the inhibitory effect of supernatant fractions of granulocyte homogenates on phospholipase A activity at pH 7.5. Similar observations have been made in brain (34) and in liver (35). In liver, the effect may be nonspecific in that addition of albumin also causes inhibition of lysosomal phospholipase A_1. This is not the case for granulocyte phospholipase A. A regulatory function of constituents of granulocytes on phospholipase activity at pH 7.5 is further suggested by the observation that boiling the supernatant fraction not only removes the inhibitory effect as well as any phospholipase activity but also actually reveals a stimulatory effect.

Although we interpret the marked similarity in degradation of oleate- and palmitate-labeled *E. coli* lipids by snake venom and the two granule phospholipases as indicative of specificity for the 2-acyl position, we recognize that more definitive evidence of positional specificity re-

[1] Zucker-Franklin, D. Unpublished results.
[2] Franson, R., and P. Elsbach. Unpublished results.

quires the use of substrates with precisely defined fatty acid composition. Such further studies are hampered by the fact that micellar substrates are not attacked by the granule phospholipase at pH 5.5 and by the presence of lysophospholipase activity at pH 7.5 (deoxycholate at concentrations that generally inhibit lysophospholipase activity also inactivates phospholipase activity).

The inhibitory effects of heparin at low concentrations and of chondroitin sulfates on acid phospholipase are in line with observations by others on inhibition by heparin of leukocyte granule and liver lysosomal enzymes (35, 36). These effects of heparin in vitro on lipid-splitting enzymes have been attributed to an anionic interaction with the enzyme (36, 37). This appears also to be the case for the acid granulocyte phospholipase activity, because inhibition was overcome by protamine sulfate at concentrations known to neutralize the negative charges of heparin or by addition of enzyme.

Despite the differences in substrate preference and the effect of heparin on the acid and alkaline phospholipases, the two activities have very similar characteristics with regard to stability, requirements for optimal activity, and susceptibility to detergent and product inhibition. Since components present in the 8200 *g* supernatant fraction inhibit phospholipase activity at pH 7.5 but not at pH 5.5, it is conceivable that the bimodal pH curve obtained with homogenate, granule, and phagosome preparations reflects the effects of modifiers of a single enzymic protein rather than two separate enzymes. Further purification of the phospholipases and their inhibitors are needed to resolve this point.

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