Phospholipase A Activity Associated with the Membranes of Human Polymorphonuclear Leucocytes

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Homogenates of human polymorphonuclear leucocytes (granulocytes) contain a Ca^{2+} -dependent phospholipase A with optimal activity at pH 7.0. This enzyme is membrane-bound and is enriched in the crude cytoplasmic-granule fraction. Rate-zonal centrifugation of the cytoplasmic-granule fraction demonstrates that the phospholipase A is associated not only with specific- and azurophilic-granule populations but also with an 'empty' vesicular fraction containing 85% of the total alkaline phosphatase activity of whole homogenate. Thus this phospholipase is associated with granule as well as with other cellular membranes of human granulocytes.

We have described a Ca^{2+} -dependent phospholipase A active in the neutral pH range in rabbit alveolar macrophages and granulocytes (Franson *et al.*, 1973, 1974). The phospholipase A2 of rabbit granulocytes appears to be associated with the membranes of specific and azurophilic granules as well as with an ill-defined heterogeneous population of cytoplasmic granules. A highly purified preparation of this rabbit phospholipase A2 manifests potent bactericidal activity towards *Escherichia coli* and an activity that rapidly increases microbial-envelope permeability (Weiss *et al.*, 1975), suggesting that phospholipid-hydrolysing enzymes of the granulocyte may contribute to membrane alterations during phagocytosis.

As part of a larger effort to study the role of phospholipases in microbe-phagocyte interaction, we describe here the localization and initial characterization of a phospholipase A from human granulocytes.

Materials and Methods

Cells and cell fractions

The blood used in the experiments was obtained from consenting donors 20–40 years old with normal health, haematocrit and total and differential leucocyte counts. Leucocytes were purified by dextran sedimentation (Fallon *et al.*, 1962), and Ficoll/Hypaque-gradient centrifugation (Boyum, 1968) as described by Spitznagel *et al.* (1974).

The purified leucocytes (>95% granulocytes, 1.6×10^9 cells) were resuspended in 5.4 ml of 0.34 M-sucrose and were homogenized with a Teflon pestle in

a glass homogenizer until 60–70% of the cells examined by phase-contrast microscopy appeared to be ruptured. The homogenate was centrifuged at 126g for 15 min. The postnuclear supernatant fraction was pipetted off, diluted to 40 ml with 0.34 M-sucrose and transferred to a Beckman TL-14 zonal rotor ($r_{\text{max.}} = 6.0$ cm, $r_{\text{min.}} = 1.6$ cm) containing 200 ml of 60% (w/w) sucrose as a cushion, 500 ml of a continuous sucrose gradient from 30 to 53% (w/w) sucrose, 40 ml of sample in 0.34 M-sucrose and 40 ml of 0.25 M-sucrose. The rotor was centrifuged until $\int_0^t \omega^2 dt = 3.5 \times 10^{10}$. After centrifugation the rotor was unloaded by sucrose displacement; the first 40 ml (the top 0.25 M-sucrose layer) is fraction 1, the second 40 ml corresponding to the sample applied is fraction 2; thereafter 48 fractions (fractions 3–50) were collected (10 ml/fraction). Sucrose density was determined with an Abbe refractometer. Protein was determined by the method of Lowry *et al.* (1951), with bovine serum albumin as the standard. The marker enzymes for the various granule fractions (peroxidase, lysozyme, lactoferrin, myeloperoxidase, neutral proteinase, alkaline and acid phosphatase and β -glucuronidase) were assayed by standard methods as described by Spitznagel *et al.* (1974). The granule populations obtained by this method are latent and highly enriched with respect to marker enzymes and have been characterized ultrastructurally (Spitznagel *et al.*, 1974).

Phospholipase assay

The phospholipase assays were carried out as previously described with *E. coli* labelled during growth with [$1\text{-}^{14}\text{C}$]oleate and then auto-

claved (Franson *et al.*, 1973, 1974; Laychock *et al.*, 1977). More than 95% of the incorporated label was in phospholipid and, as demonstrated by snake-venom hydrolysis (*Crotalus admanteus*), more than 95% of the [^{14}C]oleate was in the 2-acyl position of the phospholipids (Patriarca *et al.*, 1972). Briefly, phospholipase A activity (EC 3.1.1.4) was determined in reaction mixtures that contained 2.5×10^8 *E. coli* cells (4000 c.p.m.), approx. 5 nmol of *E. coli* phospholipid (Elsbach *et al.*, 1972), 40 μmol of the indicated buffer, 5 μmol of CaCl_2 and 50–200 μg of protein from a given granulocyte fraction (2×10^6 – 8×10^6 cell equivalents) in a total volume of 0.5 ml. Reaction mixtures were incubated for 1–4 h at 37°C in a shaking water bath. The reaction was stopped by the addition of 3.0 vol. of methanol/chloroform (2:1, v/v). Radioactive lipids were extracted (Bligh & Dyer, 1959) and were separated by t.l.c. (Franson *et al.*, 1974), and after detection with iodine vapour were scraped directly into counting vials for determination of radioactivity by liquid-scintillation counting (Franson *et al.*, 1974). Phospholipase activity is calculated as the percentage of non-esterified fatty acid released (c.p.m. of fatty acid divided by the total c.p.m. of radioactive lipid) per unit time. Phospholipase activity was linear with time and protein concentration under the assay conditions used.

Results

The pH for optimal phospholipase activity of human granulocyte homogenates is shown in Fig. 1. Maximal activity was found at pH 7.0 in the presence of 5 mM- Ca^{2+} . Virtually no phospholipase activity was detected below pH 6.0. When the homogenate was centrifuged at 126g for 15 min more than 80% of the recovered phospholipase activity was found in the 126g supernatant fraction, which was also enriched with azurophilic and specific granules (results not shown).

Fig. 2 depicts the optimal conditions for phospholipase activity in the granule-enriched 126g supernatant fraction. Phospholipase A activity was maximal in the presence of 5 mM- Ca^{2+} and was inhibited by EDTA (Fig. 2). Mg^{2+} would not substitute for Ca^{2+} in the reaction (not shown).

The cytoplasmic-granule-enriched preparation was fractionated further by zonal centrifugation into several distinct and well-characterized bands: I, II, III_s and III_r (Spitznagel *et al.*, 1974), with sedimentation increasing in that order. The distribution of phospholipase A activity after zonal centrifugation is shown in Fig. 3. With 85% of the total phospholipase activity recovered, five peaks of enzyme activity were found: in fractions 1–3, 19–20, 22–24, 32–35 and 38–40, corresponding to the well-defined distribution of human cytoplasmic granules as described by Spitznagel *et al.* (1974). Band I (fractions 1 and 2)

Fig. 1. Optimal pH for phospholipase A activity of homogenates of human granulocytes

Standard incubation mixtures as described in the Materials and Methods section contained 40 μmol of buffer (pH 4.0–6.0, sodium acetate; pH 7.0, 7.5, Tris/maleate; pH 8.0, 9.0, Tris/HCl) and 8.0×10^6 cell equivalents of granulocyte homogenate. Reaction mixtures were incubated for 2 h at 37°C. Phospholipase A activity is expressed as percentage hydrolysis/h. All values are corrected for non-enzymic hydrolysis, which was less than 2% in all experiments.

Fig. 2. Ca^{2+} requirement for phospholipase activity at pH 7.0

Different concentrations of EDTA or CaCl_2 were added to the standard reaction mixtures containing 8.0×10^6 cell equivalents of the 126g supernatant fraction (approx. 200 μg of protein).

consists of empty vesicles and contained 85% of the total alkaline phosphatase activity; band II (fractions 22–24), the specific-granule fraction, is peroxidase-negative and contains 50% of the lysozyme and 77% of the lactoferrin; bands III_s and III_r, the azurophilic granules, contain 88% of the myeloperoxidase, 84%

Fig. 3. Distribution of phospholipase activity after zonal centrifugation of granule-enriched preparations from human polymorphonuclear leucocytes

Each gradient fraction was assayed at two different protein concentrations with standard reaction mixtures that were incubated for 4 h at 37°C. Total phospholipase A activity is expressed as units (% hydrolysis/h) per fraction (●); recovery of phospholipase activity was 85%; ■, sucrose concentration (% w/w). The designation of bands I (empty vesicle), II (specific granule) and III (azurophilic granule) refers to the well-defined distribution of subcellular marker enzymes from human granulocytes described by Spitznagel *et al.* (1974).

of the neutral proteinase and 47% of the lysozyme; band III_s (fast) contains more lysozyme. Fractions 19–20 form a heterogeneous membrane fraction enriched in acid phosphatase and β -glucuronidase.

Discussion

The association of phospholipase A activity with specific granules and both slow- and fast-sedimenting azurophilic granules as well as the 'empty' vesicles present in the loading zone of the gradient suggests that the phospholipase of human granulocytes is not restricted to the two granule populations, but is also associated with other subcellular membranes. This is further suggested by the finding that repeated freezing and thawing or osmotic shock had little effect on the solubilization of the enzyme (results not shown).

The phospholipase A activity of human granulocytes resembles phospholipase A activities described in rabbit alveolar macrophages (Franson *et al.*, 1973) and rabbit polymorphonuclear leucocytes (Franson *et al.*, 1974) with respect to pH optima, apparent specificity for the C-2 position of phospholipid, Ca^{2+} requirement and EDTA inhibition. In each case the phospholipases A are membrane-associated, suggesting that migratory phagocytic cells in general may contain similar enzymes. Interestingly, preparations of canine cardiac sarcolemma enriched 8.4-fold

(over the homogenate) for $(\text{Na}^+ + \text{K}^+) - \text{Mg}^{2+}$ adenosine triphosphatase activity also contain a membrane-associated phospholipase A2 that is optimally active at pH 7.0 in the presence of Ca^{2+} (Franson *et al.*, 1976).

We have presented evidence indicating that highly purified preparations of the rabbit granulocyte phospholipase A2, which are also potentially bactericidal towards *E. coli* and cause an almost immediate increase in envelope permeability, attack the *E. coli* phospholipids (Mooney & Elsbach, 1975; Weiss *et al.*, 1975, 1976). The envelope effects can be reversed more than 1 h after practically all microorganisms have irreversibly lost their ability to multiply. The onset of repair of the *E. coli* envelope coincides with the abrupt cessation of net phospholipid degradation and the reincorporation of the labelled products of hydrolysis (lysophosphatides and non-esterified fatty acids) into diacyl phosphatides. Since a phospholipase A similar to the rabbit leucocyte enzyme is associated with human leucocyte membranes, it will be useful to examine the possible relationship between human leucocyte phospholipase A activity and alterations in microbial viability, envelope permeability and phospholipid metabolism.

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