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An ultracytochemical study on the dynamics of alkaline phosphatase-positive granules in rat neutrophils

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Summary. Alkaline phosphatase (ALPase) activity was examined by cerium-based ultracytochemistry in isolated rat neutrophils following stimulation with phorbol myristate acetate (PMA) or N-formyl-methionyl-leucyl-phenylalanine (fMLP). In control neutrophils, low levels of ALPase activity were detected in small tubular and spherical compartments distributed throughout the cytoplasm. Neutrophils stimulated for 2.5, 5, 15, and 30 min with 50 ng/ml PMA or 10^{-7} M fMLP displayed a time-dependent increase in ALPase activity. At 2.5 min, an increase in activity was first identified in compartments that were aggregated in the central regions of the cell. By 15 min, a dense precipitate was seen in tubular or elongated bead-like structures that extended to and made contact with the plasma membrane. Large enzyme-positive vacuoles were also observed in regions near the plasma membrane. At the longer stimulation times, a fine precipitate was present on the cell surface of the neutrophil in regions where subplasmalemmal ALPase activity was present. The results of this study indicate that an increase in activity and a redistribution of ALPase-positive structures occurs in neutrophils in response to stimulation with PMA and fMLP. It is likely that these compartments are latent pools of ALPase which, upon stimulation, fuse and mobilize the enzyme activity to the cell surface.

Key words: Neutrophil, Alkaline phosphatase, Upregulation, Cytochemistry, Rat

Introduction

Neutrophils play an important role in the first line of defense against invading microbes and are significant mediators of the acute inflammatory response. They can be stimulated by a wide range of agents including chemoattractants and cytokines which produce a variety of responses in neutrophils such as chemotaxis, phagocytosis, degranulation, and the respiratory burst (Estensen et al., 1974; Huang et al., 1984; Irita et al., 1984; Papini et al., 1985; Borregaard et al., 1987; Sha'afi and Molski, 1988; Baggiolini and Wyman, 1990; Gomez-Cambronero and Sha'afi, 1991; Thompson et al., 1994). Recently, it has been shown that the neutrophil is a very sophisticated cell capable of communicating with its environment by mobilizing different types of intracellular granules, some having crucial interactions with the endothelium and migration process, whereas others are for stimulating phagocytosis and defense against microorganisms (Borregaard et al., 1993).

The hallmark of this cell type is the collection of intracellular granules that are present in its cytoplasm (Bainton and Farquhar, 1966; Baggiolini, 1972; Bainton, 1975; Watanabe, 1980). In human neutrophils, the azurophil granules are large spherical compartments that contain a typical array of lysosomal enzymes such as acid phosphatase (Goldstein, 1976; Borregaard et al., 1993) as well as myeloperoxidase, an enzyme that acts in conjunction with hydrogen peroxide for microbicidal activity (Bainton, 1975; Borregaard et al., 1993). Specific granules, on the other hand, are characterized by their oval-shaped appearance and unique contents which include lactoferrin and vitamin B_{12}-binding protein (Baggiolini, 1972; Borregaard et al., 1993).

In addition to these two well-known kinds of cytoplasmic granules, Kobayashi and Robinson (1991) have demonstrated the existence of small slender rod-shaped or rounded compartments in human neutrophils that differ from the azurophil and specific granules. These compartments exhibit ALPase activity that is easily upregulated with both PMA (an activator of protein kinase C) (Borregaard et al., 1983) and fMLP (a chemotactic peptide) (Berger and Medof, 1987; Borregaard et al., 1987; Sengelov et al., 1992). Recently, interest has focused on the kinetics of granule exocytosis and upregulation of ALPase at the plasma membrane (Borregaard et al., 1983, 1987; Berger and Medof, 1987) in order to more clearly elucidate its secretory pathways within the neutrophil. Commonly used as a plasmalemmal marker in cell fractionation studies of human neutrophils (Dewald et al., 1972; Ohno et al., 1985; Parkos et al., 1985; Krause and Lew, 1987), the origin and dynamics of ALPase activity within

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neutrophils are now becoming more fully understood.

The present study was undertaken to confirm the existence of these ALPase-positive granules in rat neutrophils and to examine their dynamics by comparing cells in their resting state and in experimentally-induced activated states. Particular attention was directed to the ultracytochemical differences in localization of ALPase activity in cytoplasmic structures and at the plasma membrane.

Materials and methods

Reagents

Dimethyl sulfoxide (DMSO), propylene oxide, sodium chloride, ethyl ether, potassium chloride, sodium hydroxide, sucrose, disodium hydrogenphosphate 12-water, potassium ferrocyanide, glutaraldehyde (25% aqueous), cacodylic acid sodium salt, and cerium (III) chloride were purchased from Nacalai Tesque Inc. (Kyoto, Japan). N-formyl-methionyl-leucyl-phenyl-alanine (fMLP), phorbol 12-myristate 13-acetate (PMA), p-nitrophenylphosphate (p-NPP), Histopaque-1077, poly-L-lysine, N-tris[hydroxymethyl]-methylglycine (Tricine), Saponin, Triton X-100, and L[-]2,3,5,6-tetrahydro-6-phenylimidazo[2,1-b]thiazole (Levamisole) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Magnesium sulfate, potassium phosphate, monobasic lead (II) acetate trihydrate, uranyl acetate and calcium chloride were obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan), citric acid from Kanto Chemical Co. Inc. (Tokyo, Japan), osmium tetroxide from PGM Chemicals (PTY) Ltd. (New Germany, RSA), and vinyl cyclohexene dioxide (ERL 4206) from TAAB Laboratories Equipment Ltd. (Berkshire, England).

Purification of neutrophils

Neutrophils were obtained from specific pathogen-free adult male Sprague-Dawley rats (250-350g; Sankyo Lab Services, Shizuoka, Japan). All surgical procedures were performed under ether anesthesia.

Neutrophils were purified from either whole blood samples or from peritoneal fluid. Whole blood was obtained by heart puncture and collected in plastic syringes followed by the addition of acid citrate (1mg/10ml blood) to prevent coagulation in blood samples. A Ca^{++}- and Mg^{++}-free phosphate-buffered solution (PBS, pH 7.4) was then added to the sample prior to purification.

For the peritoneal samples, neutrophil accumulation was first induced by intraperitoneal injection of 7.5% sodium caseinate (4ml/100g body weight). After 12 hrs, peritoneal fluid was slowly withdrawn using a syringe followed by addition of a small volume of Ca^{++}- and Mg^{++}-free PBS in the peritoneum and use of a negative pressure bottle to facilitate the removal of remaining neutrophils in the cavity.

Purification of isolated rat neutrophils in both samples was performed with Histopaque 1077 by centrifugation at 110 g for 10 min at 40 °C. Any remaining erythrocytes were removed by hypotonic lysis with a small volume of distilled water for 30 sec. Samples were then rinsed in an isotonic solution and washed in PBS before use. All procedures were performed in plastic tubes.

Cell stimulation

Purified neutrophils were stimulated with either PMA or fMLP. PMA and fMLP were first dissolved in DMSO as stock solutions and stored at -20 °C until use. The final working concentration of 10^{-7} M and 50 ng/ml for fMLP and PMA respectively in all assay cases. Cell stimulation was performed for 2.5, 5.0, 15 and 30 min at 37 °C and then stopped by rinsing samples with Ca^{++}- and Mg^{++}-free PBS at each time interval. For controls, unstimulated cells were maintained at 4 °C until fixed.

Fixation

Both stimulated and unstimulated neutrophils were fixed in suspension with 2% glutaraldehyde in 0.1M sodium cacodylate (pH 7.4) with 5% sucrose for 10-30 min at 4 °C. Following centrifugation at 110 g for 10 min at 4 °C, cells were resuspended in a small volume of cacodylate buffer and then layered for 5 min over round glass cover slips (12 mm in diameter) coated with 0.2% poly-L-lysine. Cover slip preparations were then washed 3 times in 200 mM TAPS buffer (pH 9.4) and processed for detection of ALPase activity.

Cytochemistry

Neutrophils were incubated in the cerium-based reaction medium of Kobayashi and Robinson (Kobayashi et al., 1987; Kobayashi and Robinson, 1991) for detection of ALPase activity. The reaction medium contained 50 mM Tricine, 100 mM TAPS, 2 mM cerium chloride, 2 mM p-NPP, 5 mM magnesium sulfate, 0.006% Triton X-100 and 0.004% Saponin adjusted to pH 9.4. Incubations were performed for 60 min in a shaking water bath at 37 °C. Control cytochemical procedures consisted of 1) omission of substrate (p-NPP) from the reaction medium; 2) omission of Mg^{++} from the reaction medium; and 3) preincubation with 2 mM Levamisole (a potent inhibitor of ALPase) in TAPS buffer for 20 min at room temperature followed by incubation with 2 mM Levamisole in the full reaction medium.

Spectrophotometrical assay of ALPase activity

ALPase activity was determined spectrophotometrically by measuring the absorbance of p-nitrophenol produced by the hydrolysis of p-NPP in the reaction

mediums. Absorbance changes were measured at 410 nm with a Hitachi 220A spectrophotometer (Hitachi Ltd., Tokyo, Japan) equipped with a 37 °C thermostated cuvette holder. A total of $1x10^6$ neutrophils/ml was used in each sample and each experiment was performed 3 times (n=3) and represented as an average. Enzyme activity, expressed as the change in optical density/$1x10^6$ neutrophils/ml, was measured for unstimulated neutrophils inhibited with 2 mM Levamisole, unstimulated neutrophils in the full reaction medium, and both fMLP- and PMA-stimulated neutrophils.

Electron microscopy

After the cytochemical procedures, neutrophils were postfixed with 2% osmium tetroxide in 0.1M cacodylate buffer (pH 7.4) containing 1.5% potassium ferrocyanide (Robinson et al., 1982) for 15 min at room temperature. Samples were then dehydrated in a graded series of ethanols and embedded in Spurr's epoxy resin. Ultrathin sections, 50-75 nm in thickness, and thick sections, 0.50-0.75 μm in thickness were collected on copper grids and observed unstained in a JEM-1200EX electron microscope (JEOL Co., Tokyo, Japan) operated at 80 kV.

Results

Spectrophotometry

The change in optical density of the ALPase reaction media for control and stimulation experiments is seen in the histogram in Figure 1. Unstimulated neutrophils displayed moderate activity when incubated in the full reaction medium and this activity could be inhibited almost entirely with 2 mM Levamisole. Little or no reaction was detected in substrate-free media or Mg^{++}-free media in cytochemical control experiments (data not shown).

The values for 15 min stimulation of neutrophils with either fMLP or PMA are given in Figure 1. At this stimulation time, the change in optical density increased by greater than twofold for both fMLP and PMA. PMA exhibited a slightly higher stimulation when compared with fMLP. By 15 min, the maximum activity of ALPase was attained and did not change significantly with longer neutrophil stimulation times.

Ultracytochemistry

Unstimulated neutrophils

Rat neutrophils isolated from blood and peritoneal fluid were identified according to the description of Watanabe (1980). Under electron microscopy, these cells were rounded in appearance and contained a multilobulated nucleus with a mixture of hetero- and euchromatin. Numerous rounded or ovoid granules were observed in the cytoplasm. Few organelles such as mitochondria, rough-surfaced endoplasmic reticulum, Golgi complex and glycogen granules were present. Morphologically, rat peritoneal neutrophils elicited by intraperitoneal injection of sodium caseinate appeared similar to those of blood neutrophils except for a higher amount of glycogen granules in the cytoplasm (see Fig. 3b). This uptake of glycogen during inflammation has been described in detail by Robinson and others (1982).

Unstimulated blood and peritoneal neutrophils incubated in the ALPase reaction medium displayed electron-dense reaction product localized primarily in slender rod-shaped structures and in small vesicles and vacuoles which were scattered throughout the cytoplasm (Fig. 2). In some cases, ALPase activity was also seen in the Golgi complex of the unstimulated neutrophils (Fig. 2 inset). No reaction product was observed on the plasma membrane surface of unstimulated blood or peritoneal neutrophils (Fig. 2).

Monocytes, lymphocytes, and eosinophils were also found occasionally in the samples processed for detection of ALPase activity. For the most part, these cells were negative for ALPase activity, however, discrete ALPase-positive granules, in the form of the short rod-shaped structures, were seen in the cytoplasm of the eosinophils (data not shown).

Cytochemical controls incubated in a substrate-free or Mg^{++}-free medium were devoid of reaction product, whereas inhibition with 2 mM Levamisole significantly reduced the staining in neutrophils (Fig. 3). Blood (Fig. 3a) and peritoneal (Fig. 3b) neutrophils displayed similar results in all control experiments.

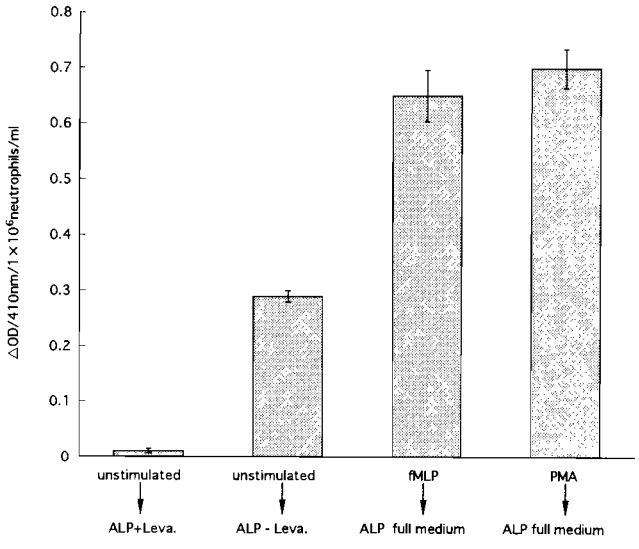

Fig. 1. Histogram showing the differences in ALPase activity in rat blood neutrophils in control and experimental conditions. Assays were performed by spectrophotometric analysis of reaction media at 410 nm and represented as a change in optical density (DOD). All samples contained $1x10^6$ neutrophils/ml. Incubation in 2 mM Levamisole inhibits the enzyme activity whereas stimulation of neutrophils with 0.25% of either fMLP or PMA dramatically increased its activity (n=3, $p<0.01$).

PMA- and fMLP-stimulated neutrophils

At 2.5 min post-stimulation, ALPase-positive structures were seen aggregated at the center of both the PMA-stimulated (Fig. 4a) and of the fMLP-stimulated (Fig. 4b) isolated neutrophils. They appeared as small spherical or slender rod-shaped granules that occupied regions between the lobes of the nucleus. The plasma membrane of these cells displayed a small amount of reaction product appearing as intermittent electron dense regions along its surface.

At 5 min post-stimulation with PMA or fMLP, the positive-staining granules became more prominent and were distributed in clusters throughout the cytoplasm. A denser reaction product within the granules was observed and larger vacuoles became more evident. These larger vacuoles appeared to be formed by fusion of neighboring granules since connections were often observed between the compartments (Fig. 5a,b).

After stimulation for 15 min, ALPase-positive granules and vacuoles appeared to coalesce forming long tubular and bead-like structures or large vacuoles (Fig. 6a,b). Within the large vacuoles, punctuate densities of reaction product were observed on the inner surface. At high magnifications, these enzyme-positive structures were seen in close proximity to the plasma membrane some in contact or continuous with the neutrophil surface (Fig. 7).

The intensity and concentration of ALPase activity became more evident 30 min after stimulation with PMA or fMLP (Fig. 8). Dense reaction product was seen in vacuoles and elongated tubular structures which aggregated in subplasmalemmal regions and made contact with the plasma membrane.

Discussion

ALPase is a highly ubiquitous enzyme found in almost all species from bacteria to man (Bosse et al., 1993; Simopoulos and Jencks, 1994). Since its

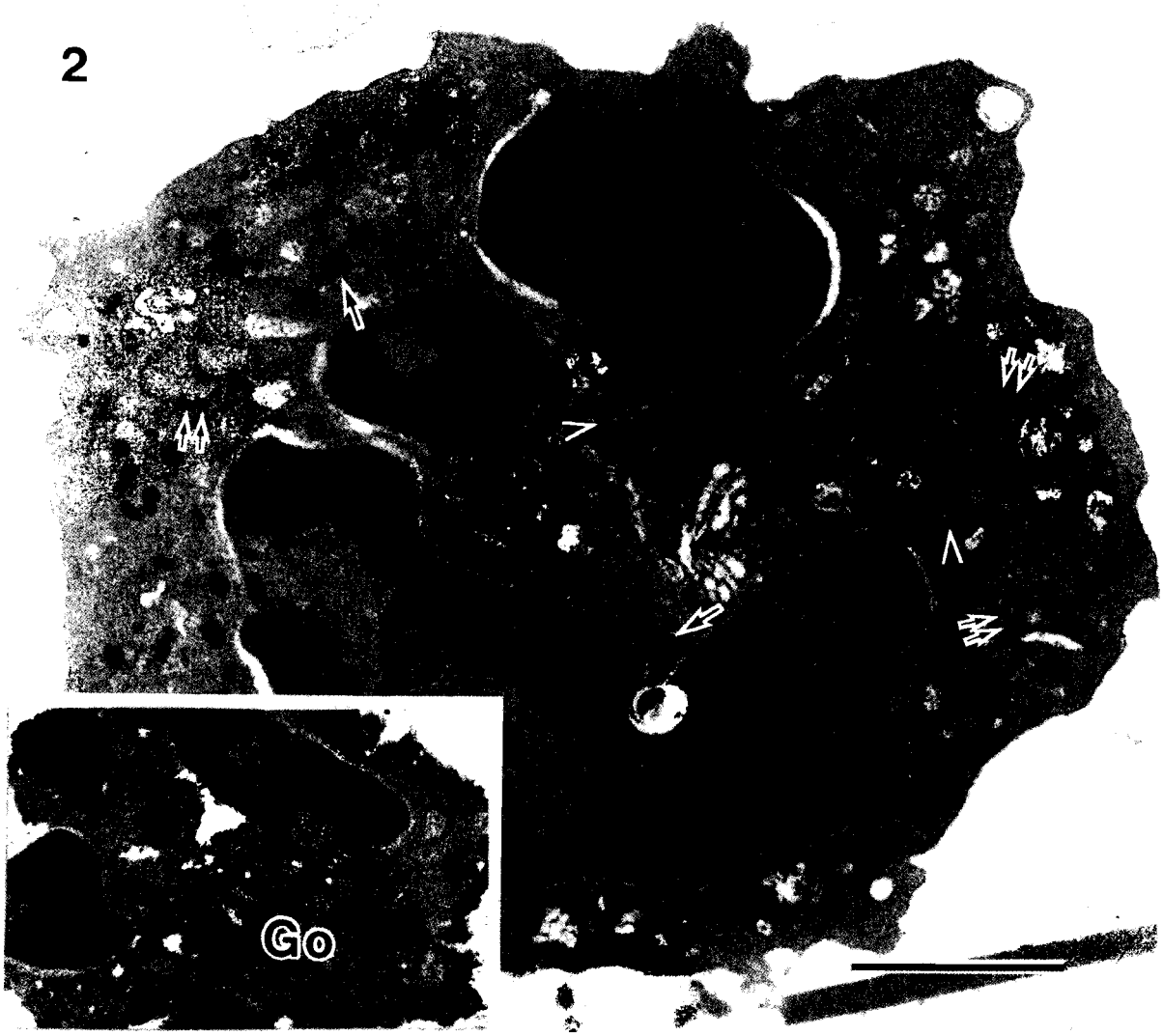

Fig. 2. Electron micrograph of an unstimulated rat blood neutrophil incubated in the cerium-based ALPase reaction medium. Electron dense staining is restricted to slender rod-shaped structures (double arrows), and small vesicles (single arrows). The Golgi complex (Go) also shows some ALPase activity in the unstimulated neutrophils (inset). Bar: 1μm.

distribution in vertebrate tissues is so diverse and varies markedly in disease states, this enzyme has been the choice for study by many biochemists, pathologists and histochemists.

We have utilized ALPase activity as a marker for an intracellular compartment in rat neutrophils. This enzyme activity was visualized by cerium-based electron microscopic cytochemical methods and was present in a small population of spherical or rod-shaped granules in the unstimulated neutrophils. These findings in rat neutrophils coincide with those observed in human blood neutrophils (Kobayashi and Robinson, 1991). In addition, we demonstrated that this ALPase activity can be upregulated upon stimulation with PMA and fMLP.

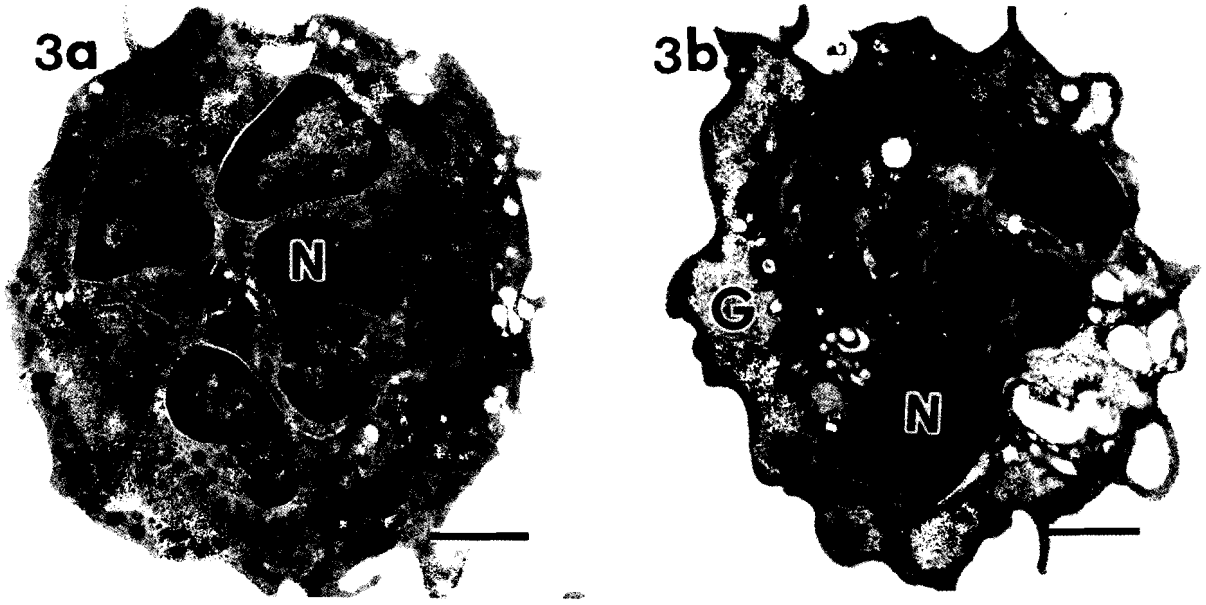

Fig. 3. Cytochemical control experiments for ALPase activity. Negative reactions are seen in a blood neutrophil incubated in a substrate-free medium **(a)**, and in a peritoneal neutrophil incubated in a reaction medium containing 2 mM Levamisole **(b)**. Abundant glycogen granules (G) are present in the cytoplasm of a peritoneal neutrophil. Bar: 1μm.

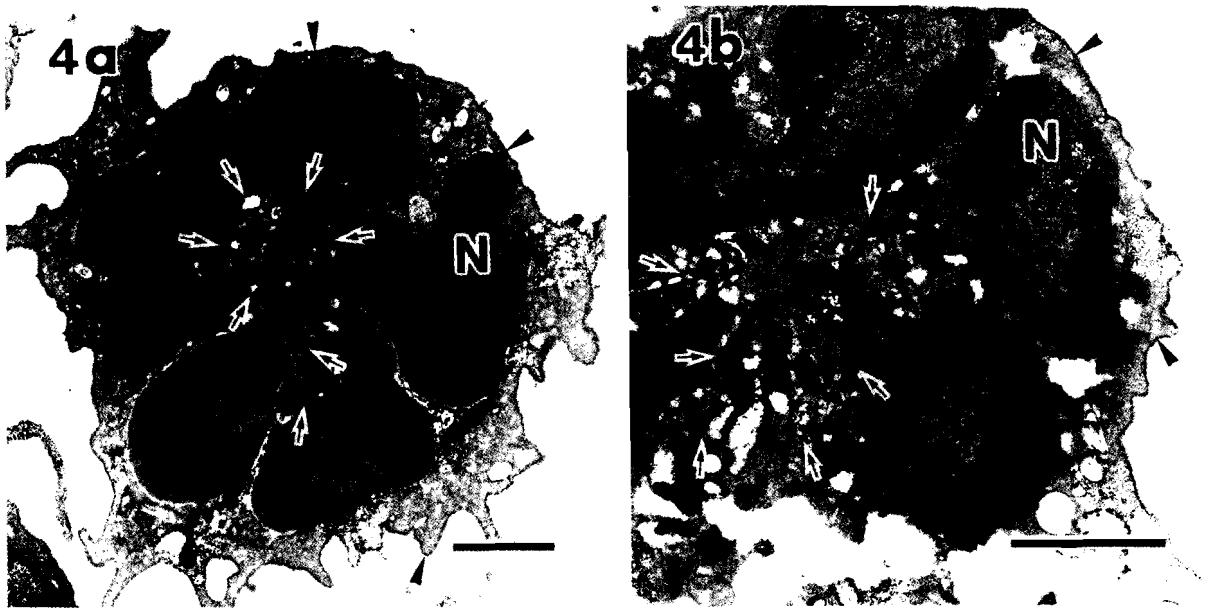

Fig. 4. Rat blood neutrophils incubated in the ALPase reaction medium after stimulation for 2.5 min with 0.25% PMA **(a)** or 0.25% fMLP **(b)**. ALPase-positive granules are accumulated in the center of the the neutrophil (single arrows). A small amount of ALPase activity in both cells is also seen on the surface of the plasma membrane (arrowheads). Bar: 1μm.

When rat neutrophils were stimulated with PMA for 2.5 min, ALPase-positive granules aggregated in the central region of cells. A small amount of reaction product also appeared on the plasma membrane surface of neutrophils at the same time. This phenomenon implies that the upregulation of this enzyme leads to an increase in activity at the cell surface which may be derived from the translocation of ALPase-positive granules from the cytoplasm of neutrophils. After prolonged periods of stimulation (i.e. 15-30 min) with either PMA or fMLP, the ALPase-positive granules fused and became elongated bead-like compartments or large vacuoles which became closely associated with or in contact with the plasma membrane.

PMA-stimulated rat neutrophils differed only slightly from fMLP-treated ones; the former eliciting a faster

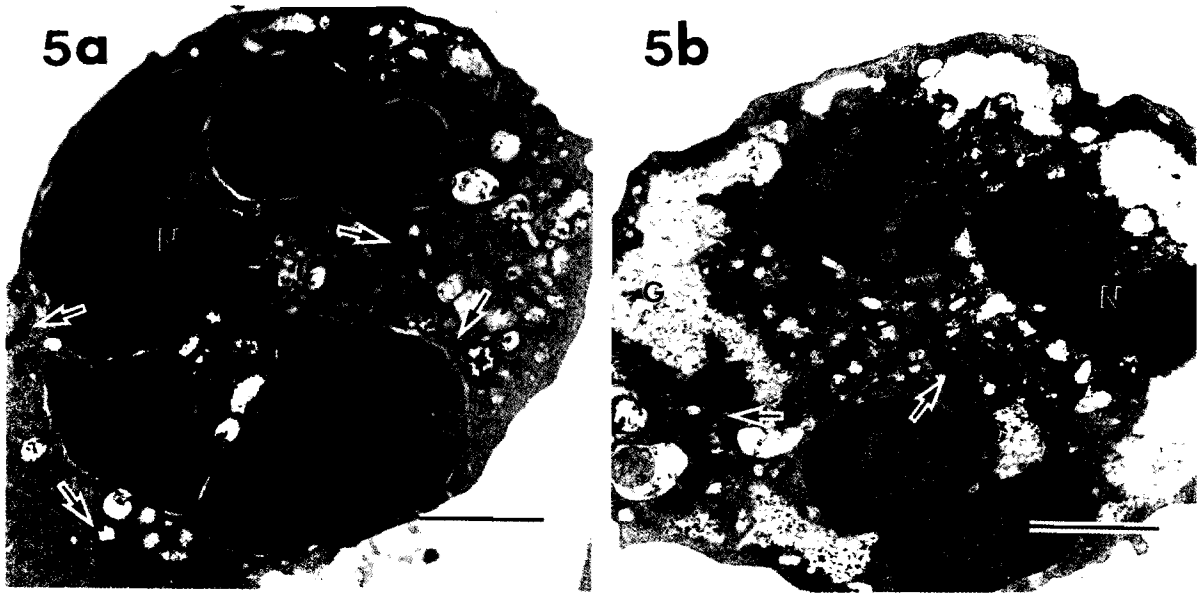

Fig. 5. Blood (**a**) and peritoneal (**b**) neutrophils incubated in the ALPase reaction medium after 5 min stimulation with PMA. The ALPase-positive compartments are fused with each other (arrows in a) around the nucleus (N) or appear as clusters of enzyme-positive structures (arrows in b). Weak ALPase activity is present on the cell surface (arrowheads). G: glycogen. Bar: 1μm.

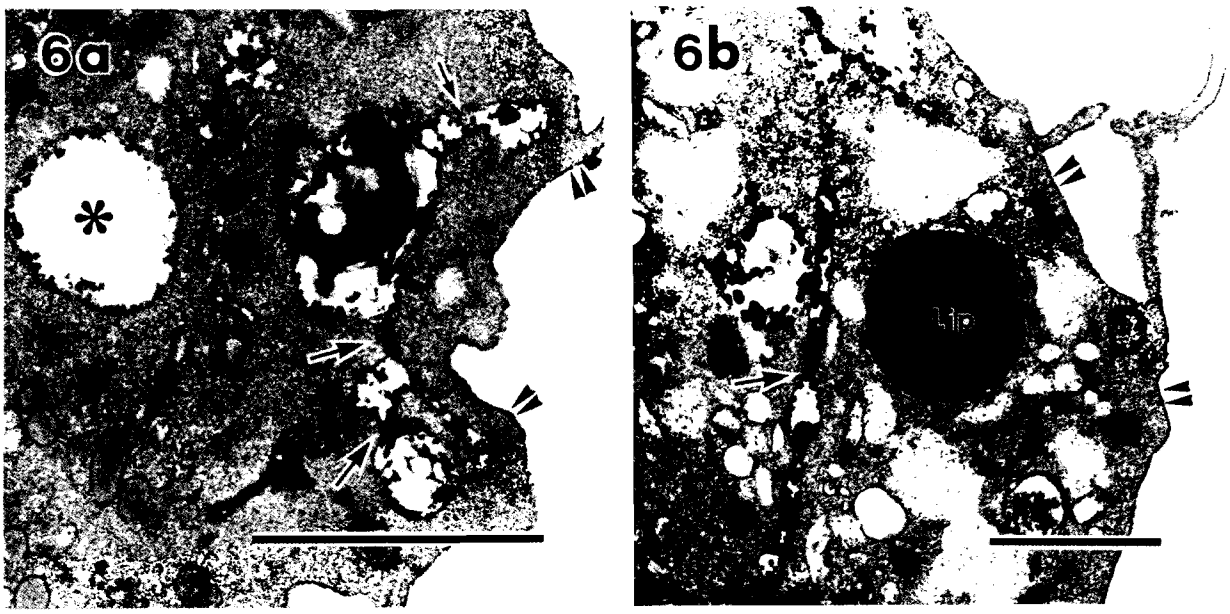

Fig. 6. Blood (**a**) and peritoneal (**b**) neutrophils incubated in the ALPase reaction medium after 15 min stimulation with PMA. Activity is localized within large vacuoles (asterisk in a) which, in some cases, appear to be interconnected (arrow in a). Long tubular structures (arrow in b) also display dense reaction product. A fine precipitate is seen along the surface of the plasma membrane (arrowheads). Lip: lipids. Bar: 1μm.

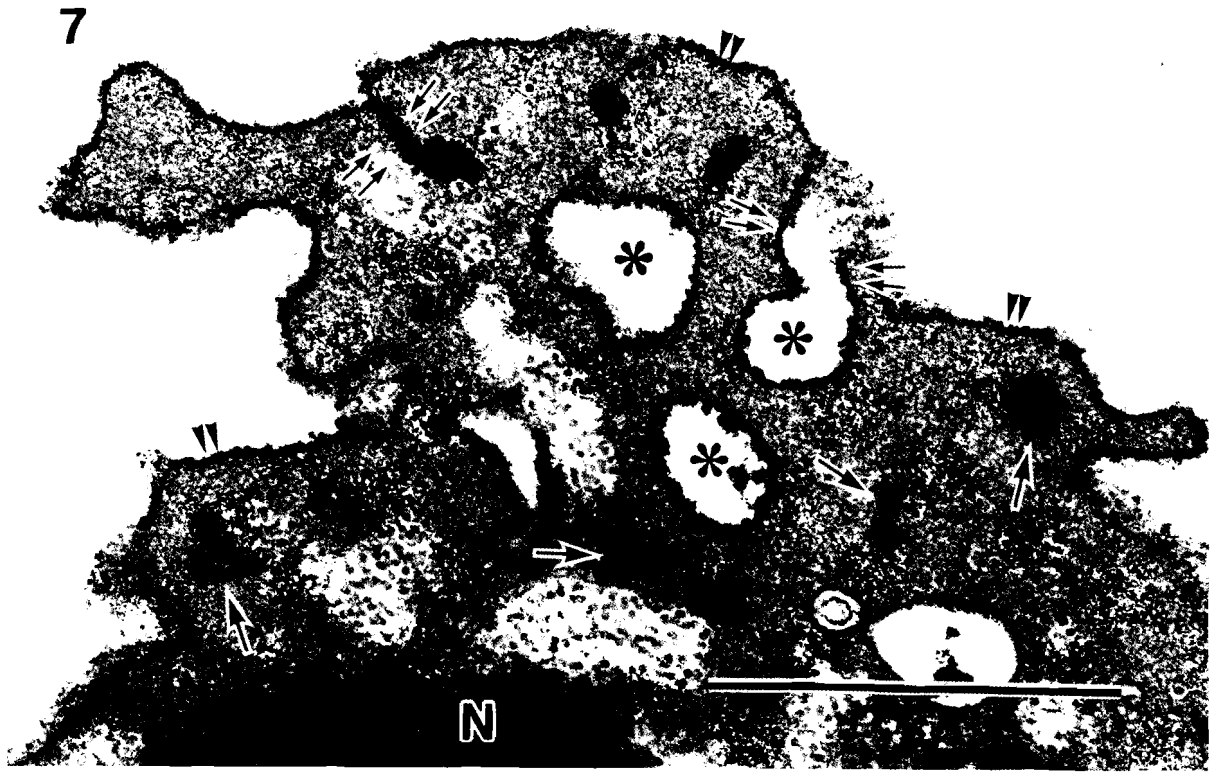

Fig. 7. High magnification electron micrograph of a peritoneal neutrophil incubated in the ALPase reaction medium after stimulation with PMA for 15 min. Reaction product is seen in cytoplasmic granules (single arrows) and in vacuoles (asterisk). Some ALPase-positive structures are connected with the cell surface (double arrows) on which the reaction product is also localized (double arrowheads). N: nucleus. Bar: 1μm.

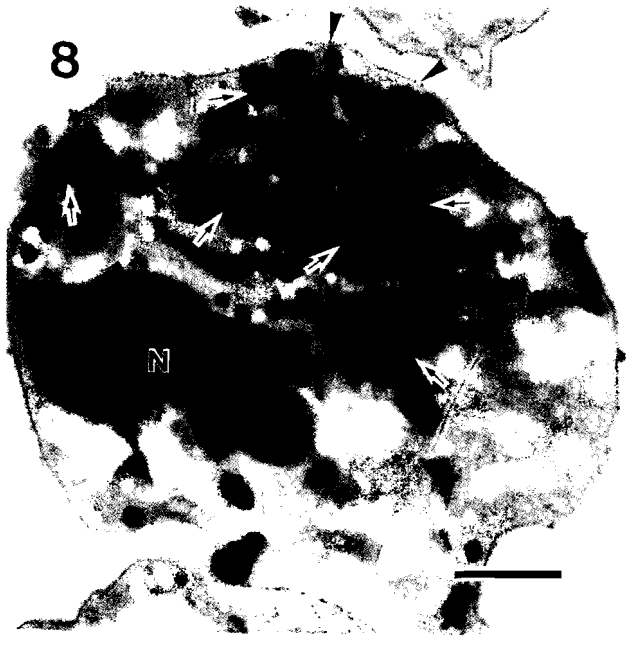

Fig. 8. Rat peritoneal neutrophil incubated in the ALPase reaction medium after stimulation with fMLP for 30 min. Dense accumulations of ALPase-positive granules are present in the cytoplasm and appear to be fused to each other (single arrows). Reaction product is also present on the cell surface (arrowheads). Bar: 1μm.

response and more obvious and numerous ALPase-positive granules than the latter. This may be due to different signal transduction mechanisms within neutrophils that are activated by PMA and fMLP. PMA has been shown to be an activator of protein kinase C, whereas fMLP functions as a chemotactic peptide. Since the dynamics of these granules can be upregulated by both PMA and fMLP, it may be deduced that the increased preponderance of enzyme-positive structures is related to protein kinase C activation (White et al., 1984; Papini et al., 1985; Omann et al., 1987; Baggiolini and Wyman, 1990). Functionally, protein kinase C may modulate cellular responses indirectly by one or more steps in the excitation response mechanism which may include receptor binding, a coupling protein, the activity of the IP3-specific phosphatase (regulator of calcium levels), as well as phosphorylation of some kinds of proteins (Huang et al., 1984; White et al., 1984; Papini et al., 1985; Foy and Simchowitz, 1989). It has been confirmed recently that fMLP at micromolar concentrations can directly activate the NADPH oxidase and cause both calcium releases from stores and influx (Al-Mohanna and Hallett, 1990; Thelen et al., 1993). The calcium ions activate protein kinase C (PKCγ) which is responsible for the phosphorylation of proteins and is important in neutrophil activities, such as NADPH

oxidase components (respiratory burst) and vimentin (cyto-skeleton) (Pettit et al., 1997).

Structurally, ALPase is a dimeric metalloprotein that has two Zn^{++} ions and a Mg^{++} ion in each active site (Simopoulos and Jencks, 1994), and is anchored to the plasma membrane via covalent linkages to glycosyl-phosphatidylinositol (Smith et al., 1985; Low, 1987; Cain et al., 1993). The ALPase found in neutrophils has been reported to belong to the so-called "liver-type" isoenzyme (Borgers et al., 1978). In unstimulated human neutrophils, the majority of ALPase is present as a cytoplasmic pool (80%) and to a lesser extent on the cell surface (20%) (Cain et al., 1993). Although this enzyme may be masked or the activity levels too low to be detected at the plasma membrane of neutrophils in unstimulated conditions, stimulating factors such as those used in this experiment enabled detection of plasma membrane-associated ALPase activity (Thompson et al., 1994). In addition, observations in our study revealed ALPase activity in the Golgi complex of unstimulated cells which may suggest that the mature neutrophils can synthesize this enzyme and store it as a latent ALPase-positive cytoplasmic pool.

While the existence of azurophil and specific granules in the mature neutrophil has been demonstrated by many researchers (Bainton and Farquhar, 1966; Baggiolini, 1972; Bainton, 1975), the secretory nature of granules has only recently been recognized (Borregaard et al., 1983, 1987, 1993; Kobayashi and Robinson, 1991; Cain et al., 1993). In neutrophils, the tracking of an intracellular mobilizable membrane compartment which could account for the upregulation of surface membrane proteins, such as the adhesion protein, Mac-1, CR1, and acceleration factor, in response to stimulation with inflammatory mediators that did not by themselves mediate exocytosis of other well-known granules (Berger and Medof, 1987; Gomez-Cambronero and Sha'afi, 1991), resulted in the discovery of a light and highly mobilizable vesicular compartment identified by ALPase (Borregaard et al., 1987, 1993; Kobayashi and Robinson, 1991; Sengelov et al., 1992; Cain et al., 1993). A novel, mobilizable intracellular compartment in human neutrophils with storage ability (Borregaard et al., 1987) has been shown to contain CD14, a glycosylphosphatidylinositol-anchored protein that binds and initiates cellular responses bacterial LPS. By gradient fractionation and electrophoresis, it was shown that the intracellular CD14 ran precisely with ALPase activity and that these receptors could be deployed rapidly to the cell surface in response to stimulation with a variety of agonists (Detmers et al., 1995). Approximately 70% of the latent ALPase activity was colocalized with these protein receptors within a unique population of compartments which were distinct from primary and secondary granules (Kobayashi and Robinson, 1991). This colocalization of ALPase activity with receptor expression suggests that these two proteins may be functionally associated with each other during neutrophil stimulation.

Clinically, ALPase activity is useful as a diagnostic marker in hematology (Baggiolini, 1972). Reports have shown a marked decrease of ALPase activity in neutrophils of pregnant women with AIDS (Grozdea et al., 1988) and in patients with chronic myeloid leukemia (Bottomley et al., 1969; Rustin and Peters, 1979a; Rustin et al., 1979b; Wilson et al., 1981). In contrast, increased ALPase activity has been reported in many bacterial infections, osteomyelofibrosis, polycythemia vera, some leukocytic disorders, and in Down's syndrome (King et al., 1962; Alter et al., 1963). It has become clear that due to these particular disease conditions, neutrophils of such patients show different activity and serve, therefore, as diagnostic evidence for a variety of infections or disorders.

In summary, this study has demonstrated the existence of ALPase-positive compartments in rat neutrophils which can be upregulated by PMA or fMLP; a similar finding to that observed in human neutrophils. We propose that these ALPase-positive granules in rat neutrophils be considered a unique type of secretory granule since their activity could be upregulated experimentally resulting in migration to areas nearby and in contact with the cell surface. Further elucidation of the mechanisms that contribute to enzyme translocation and regulation in neutrophils will provide for a better understanding of this cell both in health and disease.

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