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The Differential Mobilization of Human Neutrophil Granules

Effects of Phorbol Myristate Acetate and Ionophore A23187

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Phorbol myristate acetate (PMA, 2 to 100 ng/ml) and ionophore A23187 (10^{-7} to 10^{-6} M) cause human neutrophils to release up to 50% of the granule-associated enzyme lysozyme extracellularly without release of β-glucuronidase or the cytoplasmic enzyme LDH. When azurophil and specific granules are separated from neutrophil lysates by sucrose density centrifugation, it is found that lysozyme release from neutrophils exposed to PMA or to A23187 reflects a selective disappearance of the small, peroxidase-negative (specific) granules from the cells. These studies demonstrate that neutrophils can mobilize the specific and azurophil granules independently. These studies also demonstrate that under certain conditions the specific granules of human neutrophils behave like the storage granules of secretory cells. Finally, these studies show that techniques of separating neutrophil granules according to their sedimentation characteristics successfully divide these granules into populations that are distinct not only by cytochemical and morphologic criteria but also according to their availability for mobilization and extracellular release. (Am J Pathol 87:273–284, 1977)

CYTOCHEMICAL STUDIES have shown that the cytoplasmic granules of human neutrophils,[1] like those of rabbit neutrophils,[2-5] are of two distinct types: peroxidase-positive azurophil, or primary, granules and peroxidase-negative specific, or secondary, granules. Azurophil granules appear first during neutrophil maturation and are in general larger than the pleomorphic specific granules which are formed later in the development of the cell and at a separate site of the Golgi structure.[2] Peroxidase-positive and peroxidase-negative granules, when recovered from cell ly-

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sates, also differ in their sedimentation characteristics. This difference has been used with rabbit neutrophils [6] and recently with human cells [7,8] to develop methods of separating these granule types, thereby permitting more complete definition of differences in their enzyme content.

Recent reports have indicated that, in addition to differences in their formation, content, and morphology, there are also differences in the way azurophil and specific granules are mobilized by mature neutrophils. Specific granules have been found to fuse with phagocytic vacuoles and discharge their contents more rapidly than azurophil granules.[9] Also it has been found that, while there is degranulation of both granule types during phagocytosis, the contents of specific granules are more accessible for extracellular release.[10] In the following report, we describe studies of two agents, the co-carcinogen phorbol myristate acetate and the ionophore A23187; both agents have been shown to induce neutrophils to degranulate in the absence of phagocytosis.[11-14] Using techniques of separating granules from human neutrophil lysates according to their sedimentation characteristics, we have obtained additional evidence of differences in the mobilization of specific and azurophil granules. Our findings not only support the concept that specific granules are more accessible for mobilization and exocytosis than are azurophil granules, but in addition they indicate that degranulation of these two granule types may occur as independent events. Furthermore, our findings indicate that the granule separation techniques used in these studies provide a means for characterizing granule-associated materials not only according to their granule location but also according to their availability for extracellular release.

Materials and Methods

Preparation and Incubation of Human Neutrophils

Leukocyte suspensions containing 96 to 98% neutrophils were prepared from heparinized blood of healthy adult donors by centrifugation on Hypaque-Ficoll gradients followed by dextran sedimentation and hypotonic lysis of erythrocytes.[15,16] Cells were suspended in modified Hanks' solution (MHS) which contained 1.0 mM $CaCl_2$ and 0.5 mM $MgCl_2$.[17] Neutrophils were then incubated for up to 60 minutes in MHS alone or in the presence of phorbol myristate acetate (Consolidated Midland Co., Brewster, N.Y.) or ionophore A23187 (Eli Lilly Labs., Indianapolis, Ind.). After incubation, cells were centrifuged at 125*g* for 10 minutes, and the incubation media was saved. Cells were then washed in MHS and resuspended either in MHS or in 0.34 M sucrose.

Cell Lysis and Granule Separation

Neutrophil granules were separated by continuous sucrose gradients using methods adapted from previous reports by West and his associates.[7] Neutrophils suspended in 0.34 M sucrose were lysed in the presence of sodium heparin (Upjohn Co., Kalamazoo, Mich.), 1000 units/10^7 neutrophils/ml, by repeatedly aspirating and expressing cell suspensions

through a 20-g, 9-cm, spinal needle. The extent of lysis was followed by phase microscopy and by cell counts until more than 85% of cells were lysed. Lysates were then passed serially through two 13-mm-diameter polycarbonate filters (Nucleopore, Neuroprope, Bethesda, Md.), the first having a pore size of 5 μ and the second a pore size of 2 μ. Aliquots (0.4 ml) of the original cell suspensions and of the granule-rich filtrates were saved for subsequent enzyme determinations to assess the loss of total granule-associated enzyme activity during lysis procedures. For comparison, neutrophils in 0.34 M sucrose (4 × 10^7 cells/ml) were also lysed in the absence of heparin by homogenization in a glass mortar with a tight Teflon pestle.[6] Unbroken cells and nuclear debris were then removed by centrifugation at 150*g* for 10 minutes.

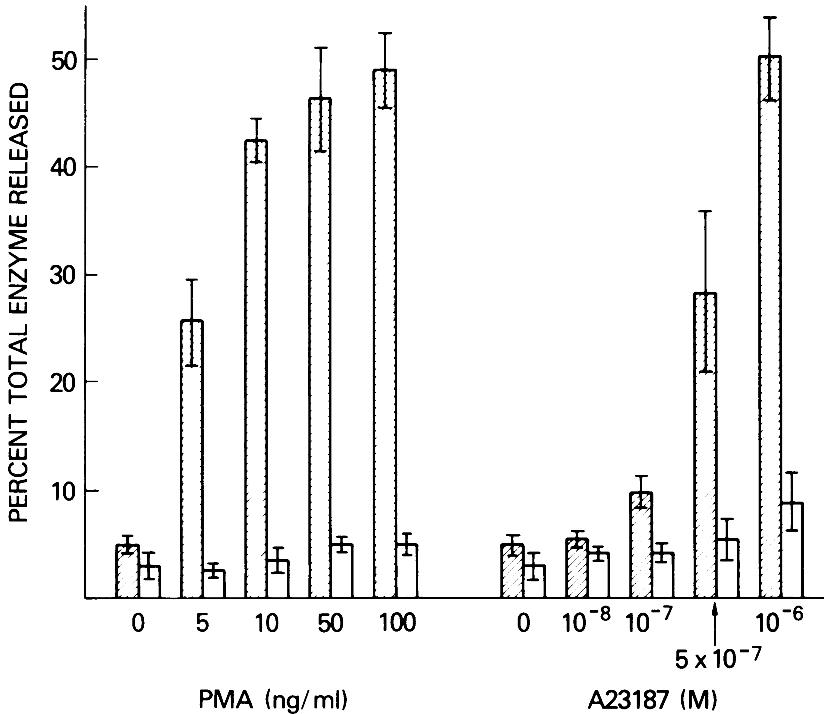
Granule-rich lysates (5 ml) were layered over continuous sucrose gradients in 1 inch × 3½ inches cellulose nitrate tubes (Beckman Instruments, Palo Alto, Calif.) and centrifuged at 95,000*g* for 4 hours at 4 C (SW-27 rotor; Model L2-65B Ultracentrifuge, Beckman Instruments).[7] Sucrose gradients were prepared with a continuous gradient maker (MSE Inc., Westlake, Ohio) using heavy and light sucrose solutions of specific gravities 1.28 and 1.12, respectively. After centrifugation, gradients were photographed and then pumped out from the bottom through a 0.8 mm × 14 cm rigid tube put directly through the gradient and held in a fixed position against the bottom of the centrifuge tubes. Gradients were pumped out at a constant rate (1 ml/min; LKB peristaltic pump-12000, LKB Instruments, Rockville, Md.) and fractionated into 1.2-ml aliquots. Turbidity of the fractions was monitored by measuring optical density at 450 nm.

Enzyme Determinations

Lysozyme activity was determined by measuring the rate of lysis of *Micrococcus lysodeikticus* (Worthington Biochemical Co., Freehold, N.J.) at pH 6.2 according to a turbidometric method.[18,19] Enzyme activity is expressed in terms of micrograms per milliliter egg white lysozyme standard (Worthington). β-Glucuronidase was assayed by measuring the release of phenolphthalein from its β-glucuronate (Sigma Chemical Co., St. Louis, Mo.) after 4 hours' incubation at pH 4.5.[20] Activity is expressed as micrograms phenolphthalein per 10^7 neutrophils per 4 hours. Myeloperoxidase was measured using *O*-tolidine as substrate in the presence of hydrogen peroxide.[6] Activity is expressed in terms of micrograms per milliliter horseradish peroxidase standard (Sigma). Lactic dehydrogenase (LDH) was assayed by measuring the consumption of DPNH during the conversion of pyruvate to lactate. LDH activity is expressed in Wroblewski units.[21] Enzymes were determined after disruption of whole cell or granule suspensions with 0.2% Triton-X-100 (Rohm and Haas Co., Phila., Pa.).

Results

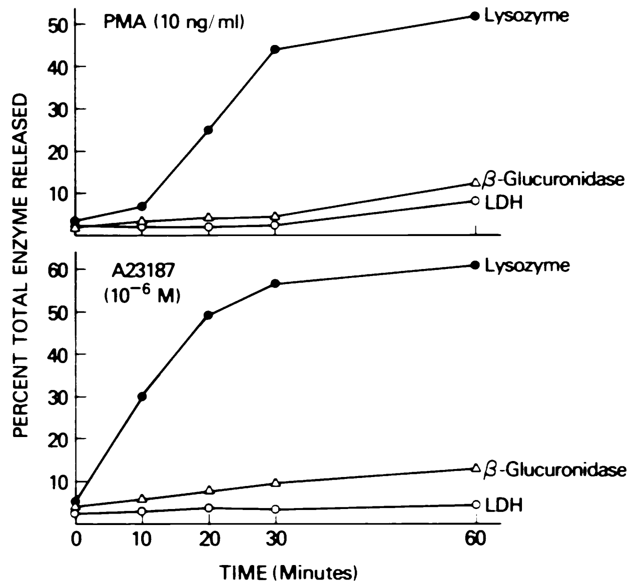
Both phorbol myristate acetate (PMA) and ionophore A23187 induce significant extracellular release of lysozyme from neutrophils with little or no release of β-glucuronidase. This differential release of granule-associated enzymes is observed during 30-minute incubations with dose ranges of PMA and ionophore illustrated in Text-figure 1. These agents at the concentrations shown did not cause release of the cytoplasmic enzyme LDH. The maximum effect of PMA on lysozyme release is achieved at concentrations of 10 to 20 ng/ml. Higher concentrations do not cause greater lysozyme release than that observed with these concentrations (about 50% of total cellular lysozyme). At concentrations of A23187 higher than 10^{-6} M, there was greater than 50% lysozyme release, but



TEXT-FIGURE 1—Extracellular release of lysozyme (*shaded columns*) and β-glucuronidase (*open columns*) from human neutrophils when incubated with phorbol myristate acetate (PMA) or ionophore A23187. Neutrophils (10^7 cells/ml) were incubated with drugs for 30 minutes. Total lysozyme = 21.3 − 36.2 μg egg white lysozyme equivalents/10^7 neutrophils; total β-glucuronidase = 7.8 − 18.8 μg phenolphthalein released/4 hours/10^7 neutrophils. Mean values and standard errors of the means from six replicate studies are shown.

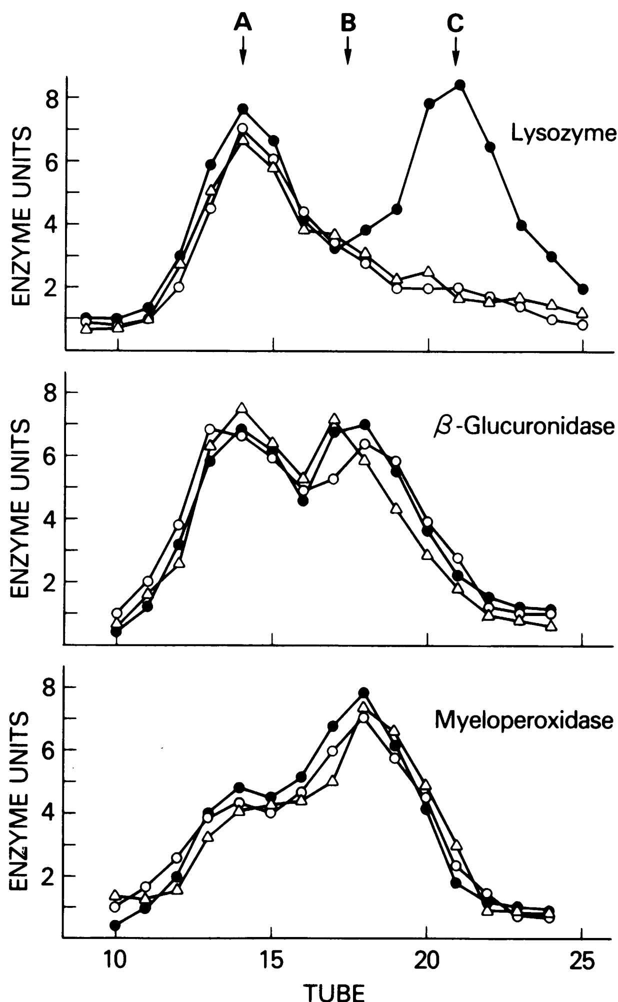
significant release of β-glucuronidase also occurred. At concentrations of 5 × 10^{-6} M or higher, release of LDH was observed, indicating cell damage. The selective release of lysozyme caused by PMA and A23187 (10^{-7} to 10^{-6} M) occurred with similar kinetics (Text-figure 2). With both agents, maximum lysozyme release was observed by 30 minutes of incubation.

β-Glucuronidase has been shown previously to be associated with the peroxidase-positive (azurophil) granules of human neutrophils, while lysozyme is found in about equal amounts in both the peroxidase-positive and the peroxidase-negative (specific) granules.[7,8] The selective secretion of lysozyme, illustrated in Text-figures 1 and 2 and previously shown by others,[12-14] suggests that there is a differential mobilization of the azurophil and specific granules but does not by itself prove that this occurs because of the divided granular distribution of lysozyme. However, by separating granules from neutrophils exposed to these agents, we could demonstrate that a selective mobilization of peroxidase-negative (specific) granules does occur.



TEXT-FIGURE 2 — Extracellular release of lysozyme (*solid circles*), β-glucuronidase (*open triangles*), and lactic dehydrogenase (LDH) (*open circles*) from human neutrophils when incubated with phorbol myristate acetate (PMA) (10 ng/ml) or the ionophore A23187 (10^{-6} M). Neutrophils (10^7 cells/ml) were incubated with drugs for 0 to 60 minutes. Total lysozyme = 28.7 μg egg white lysozyme equivalents/10^7 neutrophils; total β-glucuronidase = 12.3 μg phenophthalein released/4 hours/10^7 neutrophils; total LDH = 931 units/10^7 neutrophils. Results from a representative time study are shown.

Granule separation achieved by sucrose density centrifugation of cell lysates from normal untreated neutrophils is illustrated by a representative gradient shown in Figure 1. The three granule-rich bands are labeled A, B, and C in order of decreasing density, according to the nomenclature adopted by West *et al.*[7] Band C has been shown to contain a purified population of small, pleomorphic, peroxidase-negative granules, while Bands A and B contain larger peroxidase-positive granules.[7] Lysozyme is contained in granules of both Bands A and C, while β-glucuronidase and myeloperoxidase are restricted to granules of Bands A and B. When granules were separated from neutrophils exposed to PMA and A23187 under conditions shown to induce a maximum selective release of lysozyme, and then granule recoveries were compared with those of normal cells, disappearance of the C band granules from the treated cells was observed. Shown in Figure 2 are granule separations from untreated and treated neutrophils in a representative study. The results of enzyme determinations from these gradients once pumped out and fractionated are shown in Text-figure 3. The granule-rich bands were identified by measuring the turbidity of the fractions, and the location of the three peaks of relative optical density are indicated by the labeled arrows. Since the gradients were pumped out from the bottom, the granules of Band A appeared first in the fractions. As shown in the top panel of Text-figure 3, lysozyme associated with the peroxidase-negative granules of Band C was lost from the treated neutrophils. In contrast, the recovery and distribu-



TEXT-FIGURE 3—Fractionation of sucrose gradients depicted in Figure 2. Granule-rich bands (A, B, and C) are located by turbidometric measurements of fractions. Enzyme units: lysozyme = 2.2 μg egg white lysozyme equivalents/ml/unit; β-glucuronidase = 1.7 μg phenolphthalein released/4 hours/ml/unit; myeloperoxidase = 0.09 μg horseradish peroxidase equivalents/ml/unit. Total sedimentable enzyme activities = 81 to 86% enzyme activities recovered from whole cell lysates. [Control, *solid circles*; PMA (20 ng/ml), *open triangles*, A23187 (10^{-6} M), *open circles*]

tion of lysozyme, β-glucuronidase, and myeloperoxidase from the peroxidase-positive granules of Bands A and B were essentially unchanged. Comparable results were obtained when granules were separated from neutrophils lysed by homogenization, after the method of Spitznagel and his associates,[8] rather than by the heparin method used in the experiment shown. However, we found that lysis of cells without concomitant lysis of granules was less complete and more unpredictable with homogenization than that observed with the heparin method.

The selective mobilization of the peroxidase-negative granules of Band C, induced by ionophore A23187, was entirely dependent upon the pres-

ence of extracellular calcium in the incubation media. When neutrophils were incubated with A23187 in modified Hanks' solution with magnesium but without calcium, the recovery of granules and the recovery and distribution of granule enzymes were not different from that of control cells incubated in regular medium but without ionophore. A partial disappearance of C band granules was observed with A23187 (10^{-6} M) in medium containing 0.1 mM calcium, and disappearance of these granules was complete when extracellular calcium concentrations were greater than 0.3 mM. Mobilization of C band granules by PMA, as shown in Figure 2 and Text-figure 3, was reduced by about 40% but not eliminated in the absence of extracellular calcium.

Compared with control neutrophil preparations, neutrophils treated with PMA or with ionophore A23187 showed both morphologic and functional changes. Electron microscopy of the cell preparations showed that these agents induced ruffling of the external membrane, intracellular vacuole formation, and a reduction in the number of cytoplasmic granules.[11] Cells treated with these agents also showed a markedly diminished capacity for directed migration in response to chemotactic stimuli, and this functional change could be related to calcium fluxes into the cells induced by these agents.[22]

Discussion

Although degranulation of both azurophil and specific granules occurs as neutrophils phagocytize opsonized particles, differences in the sequence of degranulation [9] and extent of exocytosis [10] of the two granule types have been observed. Recently, Estensen, White, and Holmes observed that the co-carcinogen phorbol myristate acetate induced a selective release of lysozyme from human leukocytes (85% neutrophils),[19] and this finding, in conjunction with morphologic studies that showed relative depletion of peroxidase-negative granules in neutrophils treated with this agent,[11] led to the conclusion that degranulation of the azurophil and specific granules could occur independently. Our studies, using the approach of granule separation, affirm this conclusion. In addition, we show that ionophore A23187 at certain concentrations, like PMA, can induce a selective calcium-dependent mobilization of peroxidase-negative granules. The ionophore A23187 has been shown by others to induce exocytosis of storage granules in a variety of secretory cells: mast cells,[23] pancreatic cells,[24] and adrenergic neurons,[25] and it is of interest that in neutrophils treated with A23187 or PMA, there is a distinct population of granules that appears to behave like the granules of these secretory cells. Wright and Malawista found that extracellular release of lysozyme from

phagocytizing human neutrophils could be demonstrated readily, in contrast to the release of certain granule-associated acid hydrolases, and they concluded that lysozyme release during phagocytosis might reflect a secretory function of neutrophils important in normal inflammatory responses.[15] The present studies are consistent with the concept that the specific granules of neutrophils may function as secretory granules. Substances localized to the peroxidase-negative specific granules include lysozyme and lactoferrin, which together or individually may have important extracellular bactericidal functions.[8,10,26] Recently we have shown that, under conditions in which lysozyme is released, phagocytizing human neutrophils also release a material that activates the complement system in serum to produce the low-molecular-weight chemotactic factor C5a,[16] and this complement activator—contained in the slow sedimenting, peroxidase-negative granules (Band C)—may function as a secretory product that enables neutrophils to localize and amplify an acute inflammatory response.

Although small peroxidase-negative granules that are isolated from neutrophil lysates by sedimentation techniques (Band C) may be identified as specific granules by morphologic and cytochemical criteria,[7,8] these granules may not represent all "specific" granules as defined in studies of whole cells.[1,9] Nonetheless, our studies show that sucrose density gradient techniques permit the isolation of populations of neutrophil granules that are distinct not only in their content but also in their availability for mobilization and exocytosis. These studies emphasize the importance of localizing neutrophil granule enzymes and mediators to either the specific or the azurophil granules, for this distinction has important implications as to the relative availability of these materials for extracellular release by the cells.

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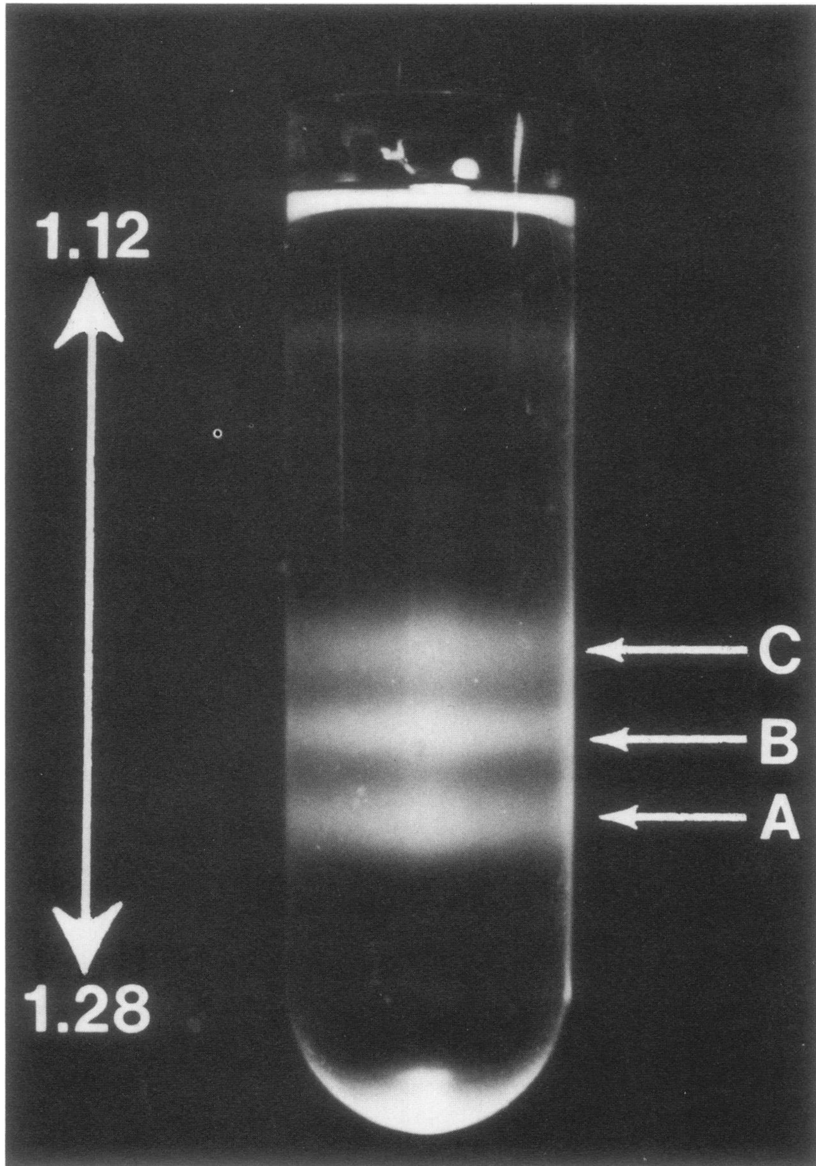

Figure 1—Neutrophil granule separation by sucrose density centrifugation. Continuous sucrose gradient from specific gravity 1.12 to 1.28. Tube size, 1 inch × 3 ½ inches. Granule separation from lysate of 10^8 neutrophils.

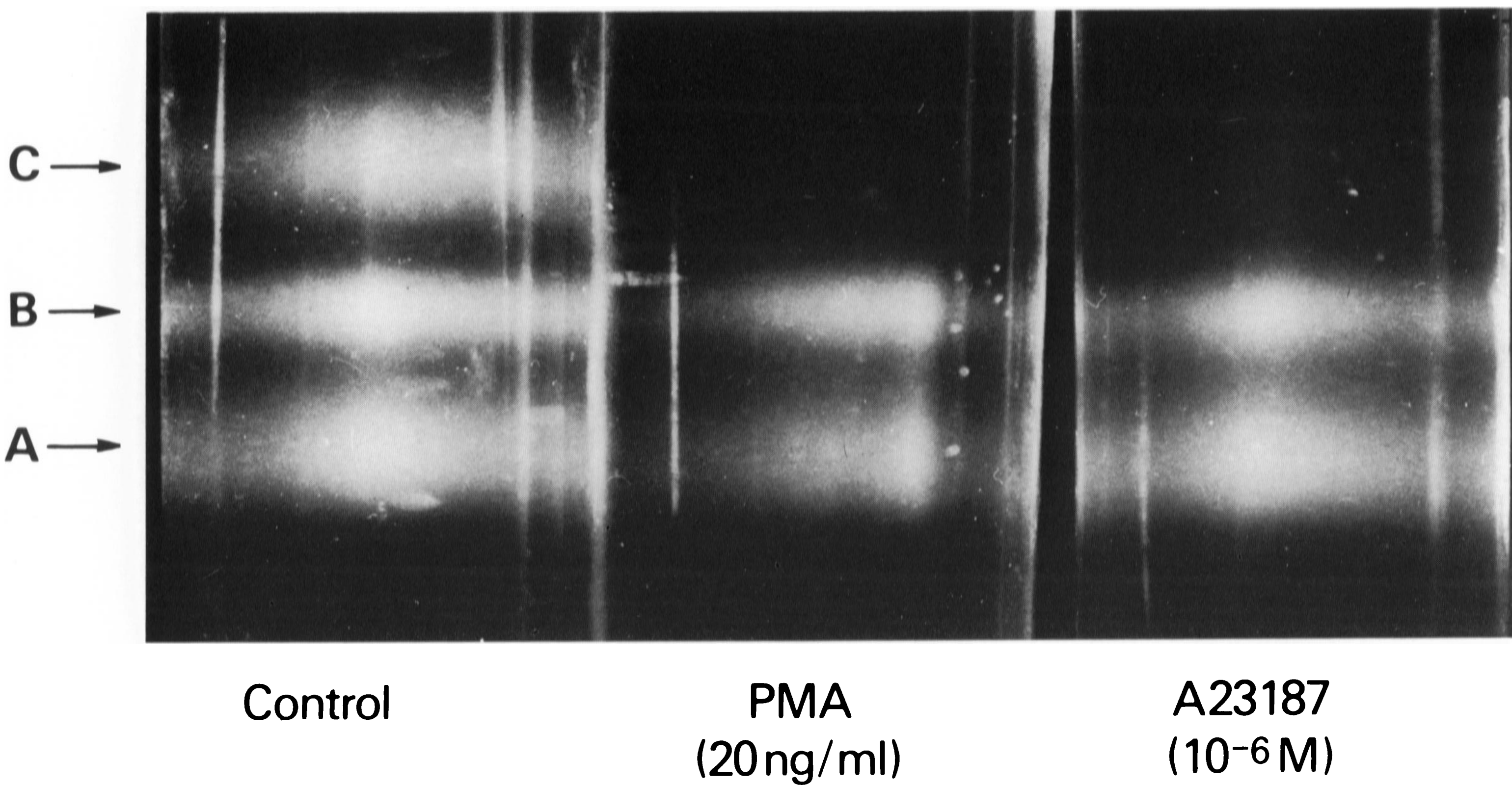

Figure 2—Granule separations from neutrophils incubated 30 minutes with buffer alone, PMA (20 ng/ml), or A23187 (10^{-6} M). Granule separations from lysates of 10^{8} neutrophils in each incubation condition.

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