Tannic Acid-Glutaraldehyde Fixation Reveals Calcium Ionophore-Induced Changes in Rabbit Polymorphonuclear Leukocyte Membranes

W. Allen Shannon, Jr. and Daniel M. Zellmer

Electron Microscopy Unit, Veterans Administration Medical Center (W.A.S., D.M.Z.), and Department of Cell Biology, University of Texas Health Science Center at Dallas (W.A.S.), Dallas, Texas 75235

TAG fixation of normal and Ca^{2+} ionophore-treated rabbit polymorphonuclear leukocytes (PMN) has revealed membrane components not apparent with conventional glutaraldehyde fixation. These included a 30 Å external electron-dense coating on untreated cells. A somewhat thicker coat (40 Å) was observed in ionophore-treated, nondegranulating PMN. In ionophore-treated, degranulating PMN, a 65 Å cell membrane coat was observed. A similar coat was observed on the inner side of the membrane of some azurophil-type granules, but the electron density and thickness were not so pronounced. Cytoplasmic granules were often closely apposed and often protruded outward at the plasma membrane. Extracellular lamellae, sometimes stacked apposed to the plasma membrane, possibly represent remnants of intense granule extrusion. Sequential degranulation of the respective granules was not apparent.

Tannic acid has been previously used with glutaraldehyde followed by osmium tetroxide to enhance the electron density of membranes [1] and membrane surface structure [2–4]. Tannic acid under these conditions has been postulated to react with proteinaceous materials, eg, complexing with basic proteins, glycoproteins, and polypeptides [5–8]. This complex is thought to chelate heavy metal ions, such as lead, osmium, and uranyl ions. The resultant product is highly stable and electron-dense. Saito et al [2] have specifically demonstrated that TAG-visualized membrane asymmetry in sarcoplasmic reticulum is due to Ca^{2+}-binding protein and Ca^{2+} pump protein. Because of the advantages this technique offers for visualizing membrane surface structure, TAG fixation should be especially useful for detecting fine structural cell surface changes in stimulated cells that may accompany established biochemical changes.

The lipophilic, divalent calcium ionophore, A23187 [9], has been used to alter cell membrane permeability to calcium resulting in the increase of intracellular levels of calcium, affecting salivary gland secretion [10], lymphocyte agglutination, and capping [11] under such conditions. A number of investigations have indicated that calcium plays an important role in phagocytosis [12, 13], and the stimulation of human PMN by chemotactic agents has been shown to lead to localized regions of Ca^{2+} flux [14], a phenomenon predicted by Woodin [15]. Biochemical evidence has indicated that Ca^{2+} is required for the enhanced secretion of the specific granule enzyme, lysozyme, in human neutrophils [16, 17] or overall release of cytoplasmic granules in rabbit PMN [18]. The morphological responses to A23187 have been described for microtubules [19], degranulation [12, 18, 20], and membrane changes [12] in the human PMN. It has been demonstrated that A23187 disrupts membrane structure by modifying protein-lipid interactions [21, 22].

In this report, transmission electron microscopy was used to assess the morphological response in rabbit PMN exposed to Ca^{2+} and the ionophore, A23187. Tannic acid in conjunction with glutaraldehyde [2] has been used to reveal PMN membrane structure not apparent in conventional glutaraldehyde and osmium-fixed materials.

METHODS AND MATERIALS

PMN leukocytes were obtained from rabbit peritoneal exudate elicited by intraperitoneal injection of shellfish glycogen and stored overnight at 4°C [23]. The exudate was centrifuged 20 min at 500g, and the pelleted PMN were washed twice with 340 mM sucrose in Hank's balanced salt solution (HBSS) and subsequently centrifuged 5 min at 500g. The PMN were resuspended in HBSS and counted in a Levy chamber.

The incubation mixture contained 1) HBSS, 2) HBSS + 0.1% dimethylsulfoxide (DMSO), or 3) HBSS + 10^{-6} M A23187 + 0.1% DMSO. Incubation was performed at 37°C. All materials were warmed to 37°C, and 10^7 cells/ml were added at time 0. Aliquots of 0.1 ml were transferred immediately (approximately 5 sec to fixative) and 1 and 30 min to glutaraldehyde in 0.1 M sodium cacodylate (pH 7.3) for 2 hours at 25°C or to 2.5% glutaraldehyde, 1% tannic acid (Mallinckrodt analytical reagent, Mallinckrodt, Inc., St. Louis), 0.1 M sodium cacodylate buffer (pH 7.3) following the method of Saito et al [2]. The fixed PMN were washed with 0.1 M sodium cacodylate buffer (pH 7.3) and postfixed with 1% osmium tetroxide, 2.4 mM $CaCl_2$, 0.06 M NaCl in 0.1 M veronal acetate buffer (pH 7.2) for 2 hr. The cells were washed with cacodylate buffer and counterstained with 0.5% aqueous uranyl acetate for 2 hr. After two subsequent washes in the cacodylate buffer, the cells were dehydrated in an ethanol series with a final dehydration in propylene oxide to 50% propylene oxide:50% epon for 30 min. The cells were left in epon overnight in order to permit maximal penetration, and were embedded in fresh epon. Thin sections of approximately 70 nm were made with a diamond knife on a LKB Ultrotome III. Sections were stained with 2% uranyl acetate in 50% ethanol for 10 min followed by 2 min in lead citrate. Observations were made with a Philips 301 electron microscope. Electron micrographs were taken on Kodak 4489 EM film using a 20-μm objective aperture at 60 kV.

The thickness of electron-dense membrane coating was measured on a Summagraphics Bit-Pad 1 digitizer board interfaced with a TRS-80 microcomputer from electron micrographs enlarged to $132,840\times$. An average of the measurements from ten cells (ten measurements per cell) from each procedure examined was calculated. In addition, PMN showing no apparent signs of having degranulated, and PMN apparently having previously degranulated most or all of their granules, were quantified.

RESULTS

TAG-Treated Cells

TAG-fixed cells exhibited many differences from glutaraldehyde-fixed cells, as has been shown for other cells. Membrane structure, especially that of the plasma membrane, appeared more delineated (Fig. 1a,b). The most obvious difference was the enhancement of an external coating of approximately 30 Å, demonstrating an asymmetry of the delineating membrane.

A23187-Treated Cells

An immediate response was observed in A23187-treated cells after addition of the ionophore (Fig. 2). In preparations fixed 5 sec after addition of ionophore, most cells had already begun to lose the small lamellapodia characteristic of untreated or nondegranulating cells (Fig. 3), and had become more spherical. In many cells, the granules had already become peripherally located, and some protruded against the plasma membrane (Figs. 2, 4, 5). Some of the usually more centrally located azurophil granules were observed to cause the plasma membrane to bulge out. Some cells had not begun to degranulate even after 30 min in the incubation medium.

It did not appear that one class of granules approached the cell periphery first. Azurophil granules were as frequently observed at the cell periphery as were the smaller granules (Figs. 2, 4, 5). The medium-sized, heterogeneous population of granules usually designated specific or secondary often remained more centrally located than did the larger azurophil granules. All of the smallest ($\sim$ 100–200 nm diameter) granules appeared to be peripherally located. In extreme instances, the degranulation appeared to be polarized with all granules being confined to one end of the cell (Figs. 2, 6a).

In one study which was quantitated, after 5 sec of ionophore treatment, approximately 59% of the PMN appeared to be nondegranulating, 25% degranulating, and 16% having more or less completed degranulation. These numbers were 20%, 40%, and 40%, respectively, at 1 min, and 5%, 4%, and 91% at 30 min.

Cells fixed with TAG in the process of degranulation demonstrated some unique features. Unlike cells treated with DMSO alone, the plasma membrane of A23187-treated cells was asymmetric with a 35-Å coating on the surface in addition to the 30Å coating observed in untreated cells (Fig. 1d). This was not observed in glutaraldehyde-fixed cells (not shown). This asymmetry was evident over the entire surface of the plasma membrane (Figs. 1d, 4). Ionophore-treated nondegranulating cells (Fig. 3) exhibited a coat averaging 40 Å. The range of this thickness varied greatly, from 24 to 58 Å (Fig. 1c). The membrane of some azurophil-type granules, especially those adjacent to the plasma membrane, was

also asymmetric, however, with the inner aspect appearing to be coated (Fig. 4). Although most of the specific-type granules appeared to have symmetric membranes, some had a slightly thickened outer coating (Fig. 4b).

As granules came into contact with the plasma membrane, several structural phenomena suggested degranulation. The larger azurophil-type granules were in contact with the plasma membrane, where a heptalaminar structure was sometimes observed with the outermost layer being quite dense (Fig. 4). Often the heptalaminar membrane appeared to be bridged. Pentalaminar or trilaminar membrane structures were not apparent. The heptalaminar structures associated with the larger azurophil-type granules were not seen with the smaller specific-type granules; nor were the smaller granules observed to be protruding from the plasma membrane.

In most preparations observed after a 1-minute exposure to the ionophore, the cells were more spherical and had degranulated further. In the experiments reported here, both specific and azurophilic granules were observed in close apposition to the plasma membrane, indicating that there was no apparent sequence of degranulation. Both granules appeared unusually pliable, as they tended to deform to the shapes of surrounding granules or the plasma membrane (Fig. 5).

One unexplained result in these experiments was that numerous laminated membrane lamellae were observed adjacent and external to the plasma membrane (Figs. 2, 4b, 5, 6). These lamellae were stacks of heptalaminar membranes with an outer electron-dense coating. They were flattened in the center, and bulbous on the ends (Figs. 2, 4b, 5, 6). Some of the wider bulbous ends revealed what appeared to be cytoplasmic matrix material (Fig. 6b). Stacks of three or four such lamellae were common. They possibly represent the evaginated plasma membrane having folded back upon itself after degranulation.

Some variations, especially in degranulation phenomena, were seen over four trials.

Controls

Cells incubated in HBSS buffer without additives demonstrated no tendency to degranulate, and were relatively unchanged after 30 min in buffer. Glutaraldehyde-fixed cells appeared as previously described for normal rabbit PMN [23, 24]. DMSO (0.1%) caused no structural changes in the PMN. At higher concentrations (1%), considerable blebbing was induced. Degranulation in response to DMSO was not observed.

Fig. 1.   High magnification of PMN cell membrane. a) Membrane of glutaraldehyde-fixed, untreated PMN exhibiting no apparent coat. b) Membrane of TAG-fixed, untreated PMN exhibiting 30-Å coat. c) Membrane of TAG-fixed, A23187-treated, nondegranulating PMN exhibiting 40-Å coat. d) Membrane of TAG-fixed, A23187-treated, degranulating PMN exhibiting 65-Å coat. × 132,800.

Fig. 2.   TAG-fixed, A23187-treated PMN exhibiting polarity of degranulation. Both azurophil (A) and specific (S) granules are located centrally and peripherally. Some granules protrude from the plasma membrane. Lamellae (arrowheads) adhere to the plasma membrane. Nu = nucleus; M = mitochondrion. × 13,500.

Fig. 3.   TAG-fixed, A23187-treated, nondegranulating PMN. Blebs and lamellapodia are distributed uniformly around the plasma membrane (compare with Fig. 2). Granules appear randomly distributed throughout the cytoplasm. A = azurophil granule; S = specific granule; Nu = nucleus. × 13,500.
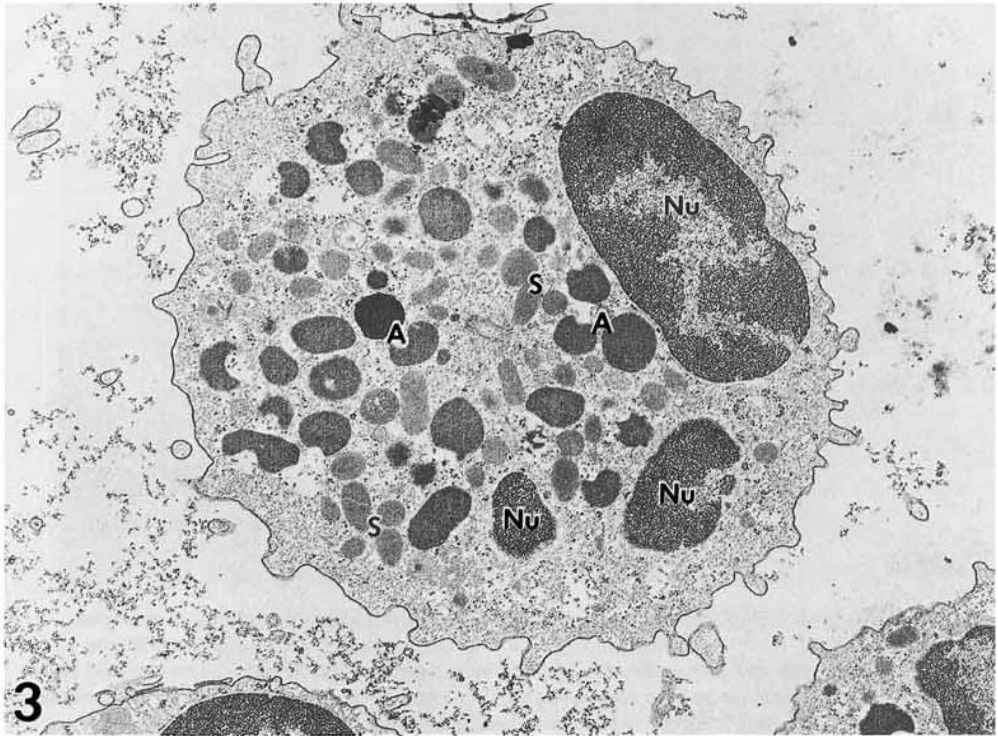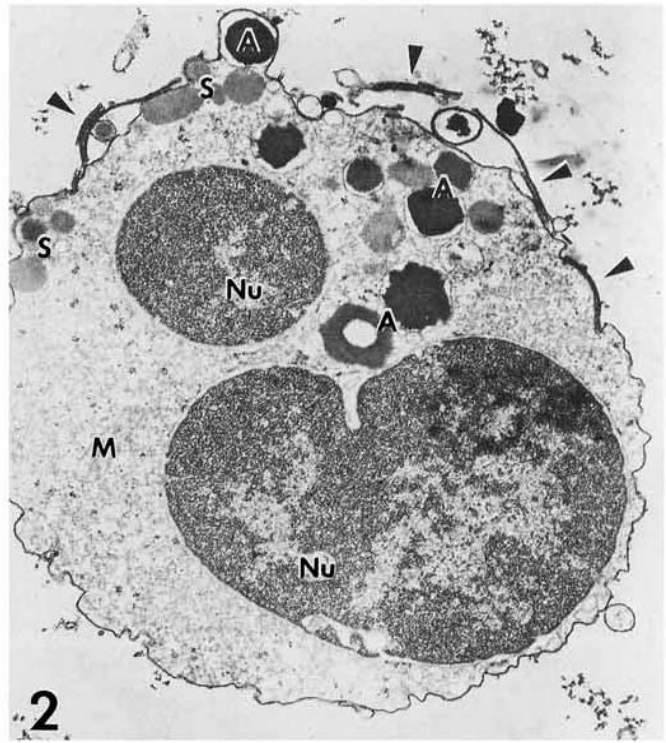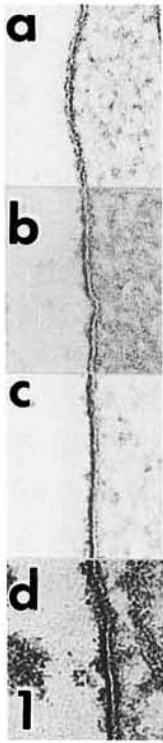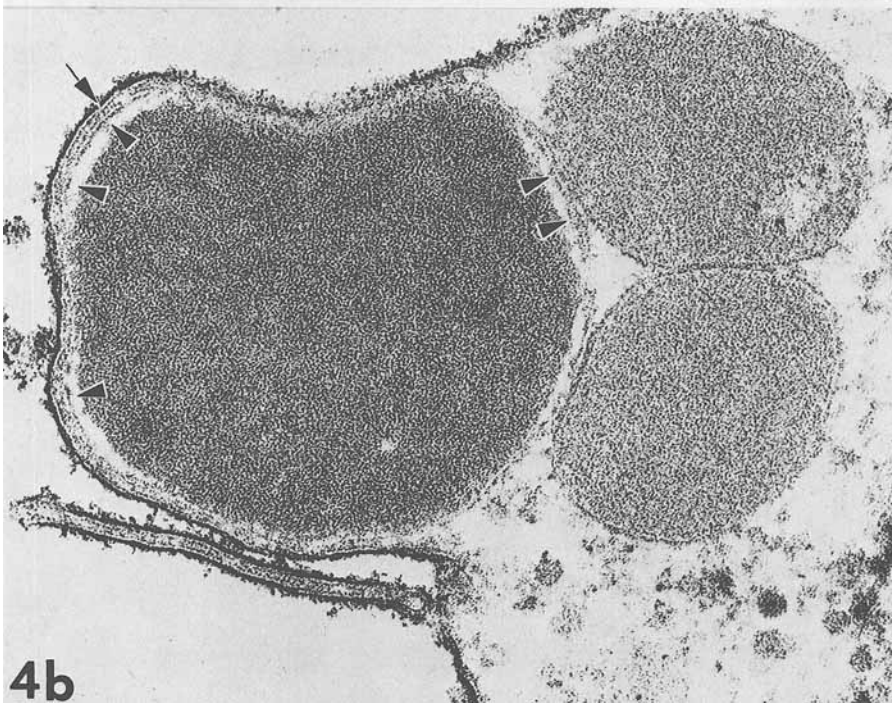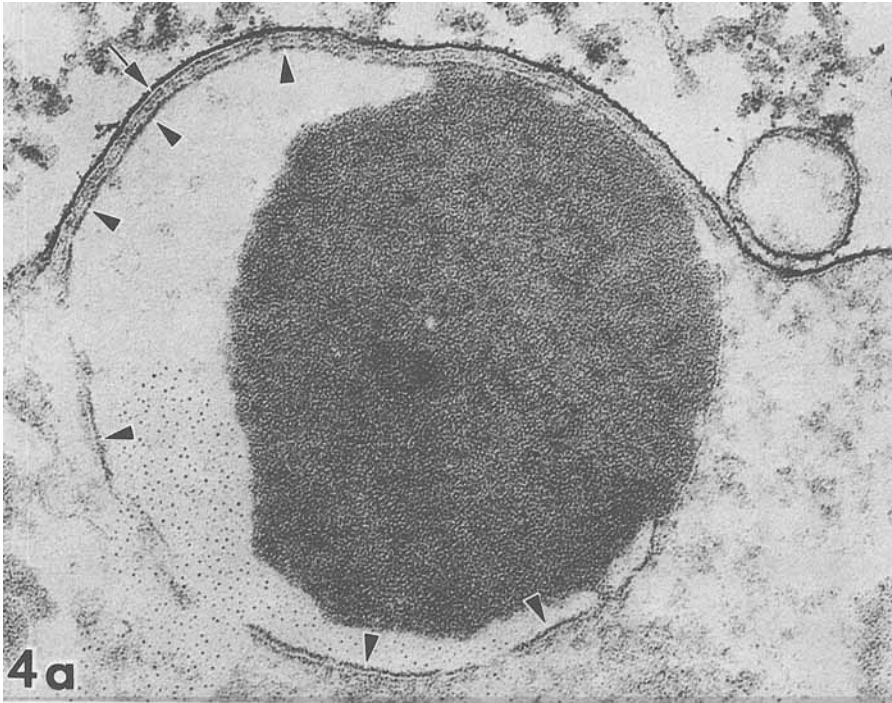
Fig 4   TAG-fixed, A23187-treated, degranulating PMN PMN membrane coating and azurophil
granules bulging out from the plasma membrane a) The inner aspect of the azurophil membrane
(arrowheads) is denser and thicker than the outer aspect Intermembrane bridges can be observed be-
tween the plasma membrane and the azurophil granule membrane (arrow) × 112,800 b) The inner as-
pect of the azurophil granule membrane is denser and thicker than the outer aspect (arrowheads)
Bridging as described in Figure 4a can be observed (arrow) Two specific granules lie adjacent to the
azurophil granule The outer aspect of their membranes is slightly denser and thicker than the inner as-
pect Note the membrane lamella just outside the cell × 112,800

DISCUSSION

The normally symmetric trilaminar plasma membrane of the PMN is seen to be highly asymmetric with TAG fixation. The fixation procedure apparently enhances an outer membrane coating presumed to be of a protein nature [2, 5–8], which cannot be seen in material fixed in conventional glutaraldehyde fixative. TAG fixation has thus provided a morphological correlate to the well-established biochemical data on membrane protein asymmetry [25]. The asymmetry is probably not due to lack of penetration of tannic acid, since the fixation time is considerable. Many granule membranes in the center of most cells, especially nondegranulating ones, are preserved, and TAG-fixed PMN not treated with the ionophore exhibit excellent retention of granule, organelle, and cytoplasmic membrane substructure.

TAG-fixed, ionophore-treated PMN exhibit many differences from glutaraldehyde-fixed cells. The most obvious difference is the asymmetry of the plasma membrane in degranulating cells. The outer perimeter of the membrane is both thicker and more electron-dense after exposure to the ionophore. Since TAG fixation enhances the visualization of proteins, it is probable that one or more proteins became associated with the outer membrane in response to the ionophore. The asymmetry occurs with dense material present on the surface of the plasma membrane and the inner aspect of some azurophil-type granules. The heterogeneity observed within azurophilic-type granules and, indeed, other intracellular components, may reflect the slow penetrating characteristics of the TAG fixative. Those structures closer to the cell exterior would likely be better preserved. Ionophore-treated cells did not appear in general to be as well preserved. Our observations indicate the presence of a thick protein coat on the outermost aspect of the heptalaminar membrane, which occurs at the site of fusion.

The molecular basis or physiological significance of the increased thickness (65 Å coat) of the plasma membrane of the ionophore-treated degranulating PMN is as yet unclear. There are several possible explanations that cannot be distinguished without further experimentation: 1) The changes in coat thickness may be due to direct effects of changes in Ca^{2+} concentration at the cell surface. Saito et al [2] have shown that Ca^{2+}-binding proteins and Ca^{2+} pump proteins can be visualized at membrane surfaces using TAG fixation. Perhaps of more relevance, Hoffstein [14] has shown that there is a dramatic loss of cations (presumably Ca^{2+}) from the surface of ionophore-treated human PMN. Thus, the ionophore-induced changes in plasma membrane morphology reported here may be due to changes in quantity or distribution of cell surface calcium-binding proteins. 2) Since there was probably some cell lysis that occured during the ionophore treatment, and since we have no data that bear on this point, the coat could be due to nonspecific adhesion of cytoplasmic constituents from lysed cells. However, because only the degranulating cells showed the coat, whereas most nondegranulating cells in the same EM section did not to the same degree, we feel this possibility is unlikely. 3) The coat could represent locally released granule contents that selectively adhere to the surface of the cells after degranulation. Lysozyme, which is secreted in response to chemotactic stimuli [26], has been demonstrated to inhibit both chemotaxis and superoxide generation [27], implying

a negative feedback control. In addition, another specific granule component, a protein of 30,000 MW, has also been demonstrated to inhibit chemotaxis and increase surface adherence [28]. This indicates a physiological effect of released granule components upon the cell itself. 4) The appearance of the coat could be due to molecules originally present in the inner surface of the granule membrane which became part of the plasma membrane after degranulation. There is some evidence that azurophil and specific granule membrane proteins are represented in the phagosome membrane [29]. 5) Increased thickness and density of the outer layer of membrane could be due to the effect of the interaction between protein and lipid reported by Klausner et al [21]. Possibly the reported disordering of the lipid structure could cause the morphological changes observed here. A change in the lipid structure might facilitate the fusion of granules observed by Korchak et al [30] in response to A23187 incubation in the presence of anion channel blockers, and might also be responsible for the pliability and adhesion of granules observed in this study.

The increase in coat thickness from 30 to 40 Å in response to A23187 treatment may be due to a direct effect of the ionophore upon the membrane or membrane-binding, since it occurs in nondegranulating cells. It may involve any of the procedures described above except for insertion of endogenous granule membrane proteins, since degranulation has not occurred.

Degranulation of specific and azurophil granules has been thought to be sequential because of the cytochemical evidence of the appearance of marker enzymes in PMN phagosomes containing bacteria [31]. Recently, a kinetic study reported sequential enzyme release in response to A23187 and other stimuli with lysozyme and myeloperoxidase as the specific and azurophil granule markers, respectively [32]. However, early morphological studies [33], as well as recent kinetic studies [34, 35], have challenged the concept of sequential degranulation. Using lactoferrin and β-glucuronidase as specific and azurophil granule markers, nonsequential release was demonstrated in response to A23187 [34], and it was suggested that centrifugal procedures require handling time which misses rapid responses.

Our results indicate that in response to A23187, the fusion and extrusion of azurophil granules and specific granules are apparently quite random. Both granules were observed apparently in the process of extrusion simultaneously even at 5 sec, with both azurophil and specific granules still centrally located. It appeared that some azurophil granules were peripherally located earlier than some of the specific granules, but some specific granules were released, whereas some azurophil granules had yet to approach the plasma membrane. The different

Fig. 5. TAG-fixed, A23187-treated, degranulating PMN. Several protruding granules observed subjacent to the plasma membrane. Specific granules (S) appear in intimate contact and are somewhat deformed, indicating pliability. Note membrane lamellae external to cell. Inset: Both azurophil granules (A) and specific granules (S) are somewhat deformed in conforming to surrounding granules. × 37,400.

Fig. 6. TAG-fixed, A23187-treated, degranulated PMN exhibiting numerous lamellae still attached to the plasma membrane. The granules and lamellae are in the same area of the cell. Inset: three lamellae adjacent to the plasma membrane, the outermost exhibiting an unusually large bulbous end containing cytoplasmic-like material (*). × 135,000.
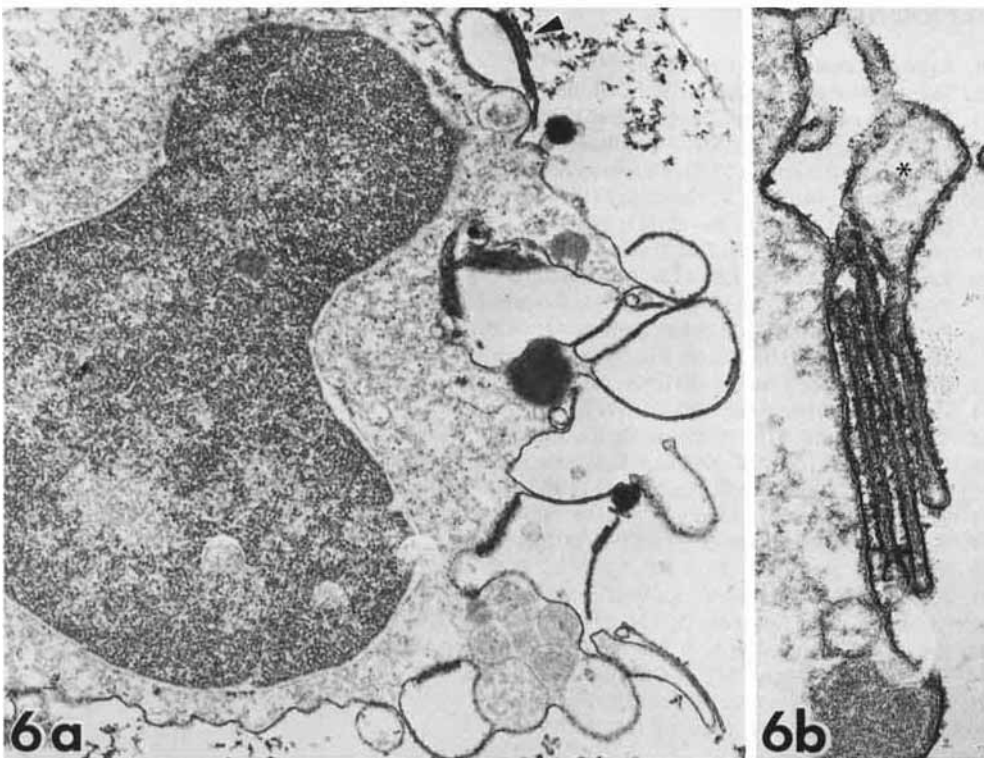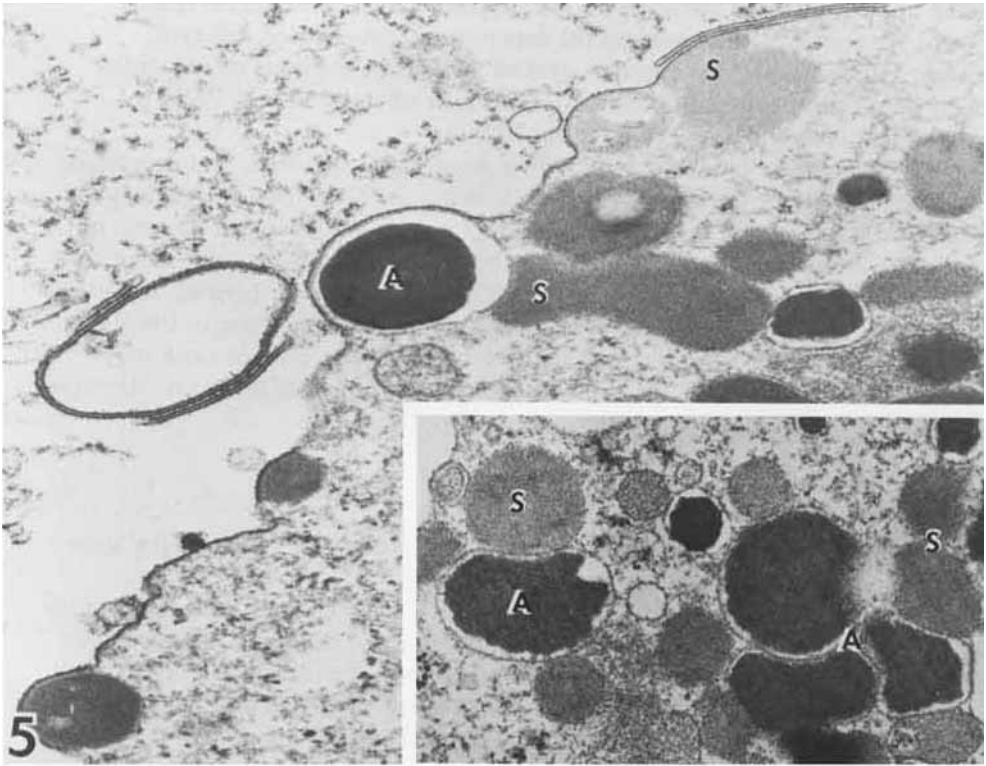
results appearing in the literature on the sequence of granule enzyme release [31–35] are certainly influenced by the experimental procedures, cell type, secretory stimulus, and the enzymes studied. Probably, the issue of sequential versus nonsequential degranulation arises because of the ability of PMN to respond in a variety of ways to different stimuli.

Enzyme release induced by A23187 is an artificial system in that degranulation is induced in the absence of phagocytic stimulus. Therefore, the cellular response to the ionophore may not parallel the response to opsonized bacteria or particulate matter, and experiments using the ionophore must be interpreted with caution. However, the experiments reported here indicate that there are dramatic cell surface changes associated with ionophore-induced granulation in PMN, and TAG fixation has permitted these changes to be visualized. Future work may reveal the biochemical nature and physiological importance of these fine structure changes.

ACKNOWLEDGMENTS

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