

MICROFILAMENTS AND MICROTUBULES IN CALCIUM IONOPHORE-INDUCED SECRETION OF LYSOSOMAL ENZYMES FROM HUMAN POLYMORPHONUCLEAR LEUKOCYTES

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ABSTRACT

Human peripheral blood leukocytes (PMN) are induced to release lysosomal enzymes by the calcium ionophore A23187 in the presence but not the absence of extracellular Ca^{++} . Whereas secretion induced by particulate or immune stimuli is accompanied by an increase in visible microtubules and is inhibitable by colchicine, secretion induced by A23187 and Ca^{++} was not accompanied by an increase in microtubule numbers and was not inhibited by colchicine. Ca^{++} did not appear to regulate microtubule assembly in these cells since resting PMN had a mean of 22.3 ± 2.0 microtubules in the centriolar region as compared to 22.3 ± 1.1 in ionophore-treated cells and 24.9 ± 1.5 in cells exposed to ionophore and 1 mM Ca^{++} . Bipolar filaments, 10 nm thick and 300–400 nm long, were numerous in the pericortical cytoplasm of cells exposed to both reagents. Microtubules in these cells were decorated with an electron-opaque fibrillar material. PMN exposed to A23187 and Ca^{++} were contracted in two directions at right angles to each other: (a) Contractions parallel to the plasma membrane resulted in extensive plication of the cell membrane. The cytoplasm subjacent to the plicae contained dense filamentous webs. Plication was prevented by cytochalasin B or reversed by subsequent exposure to an endocytic stimulus such as zymosan. (b) Contractions perpendicular to the plasma membrane, toward the cytocenter, resulted in the formation of vacuoles in normal PMN and of membrane invaginations in cytochalasin B-treated PMN. Whereas contractions parallel to the plasma membrane could occur in the absence of enzyme release (ionophore alone) and enzyme release could occur in the absence of such contractions (ionophore plus calcium plus cytochalasin B), contraction toward the cytocenter occurred in all experimental conditions in which significant enzyme release was obtained. Thus, lysosomal enzyme secretion in PMN involves contractile movements of the plasma membrane toward the lysosomes rather than the reverse. These calcium-mediated contractile events are mediated by cytochalasin B-insensitive microfilaments but not by microtubule assembly.

KEY WORDS microfilaments · microtubules · The motility of nonmuscle cells, including poly-
calcium ionophore · secretion · lysosomal morphonuclear leukocytes (PMN), has been re-
enzymes · human neutrophils · cytochalasin B lated to several morphologically and/or biochemi-

cally defined entities such as microtubules (20, 22), actin (23), myosin (29-32), and actin-binding protein (4, 14). Indeed, the major biological functions of PMN are related to motility of the cell itself (chemotaxis), of its plasma membrane (phagocytosis), and of its granules and vacuoles (degranulation). Distinct morphological changes occur in the cytoplasm of PMN exposed to chemotactic factors (8, 9), or stimuli for degranulation (10, 11, 17). Cells exposed to such stimuli contain more visible microtubules in their pericentriolar cytoplasm than resting cells. In addition, exposure of PMN to phagocytosable (18) or soluble stimuli (10, 13, 16) for degranulation results in the appearance of microfilaments adjacent to sites such as the phagocytic vacuole into which granules discharge.

Since fluxes of calcium accompany directed mobility and phagocytosis in PMN, it has been suggested that such responses are regulated by local changes in intracellular calcium concentration (1, 3, 8, 19). Furthermore, calcium ions induce enzyme release from specific granules and modulate azurophile granule discharge from PMN prepared in calcium-free media (10, 12), suggesting that calcium fluxes likewise play a role in degranulation. It is possible to manipulate the intracellular concentration of calcium experimentally by means of the calcium ionophore A23187, which is capable of dislodging membrane-bound calcium and of rendering the cell selectively permeable to calcium (25). In the presence of ionophore, the intracellular concentration of calcium can be changed by varying its extracellular concentration (or the concentration of A23187) although intracellular concentrations of ionic calcium will not equal the extracellular concentration. A23187 plus calcium at low concentrations induces discharge of specific granules alone (12) and both specific and azurophile granules at higher concentrations (7, 26, 27).

We have previously shown that microtubule assembly can be partially dissociated from lysosomal enzyme secretion by appropriate pharmacological manipulation and suggested a role for other cytoskeletal elements (11, 36). Because calcium increases leukocyte myosin ATPase activity (29, 30) and has been shown to be necessary for microfilament-related contractions (reviewed in references 15 and 23), we have examined the ultrastructure of PMN, which were induced to secrete enzymes by calcium and ionophore, in order both to determine whether microtubule

assembly could be further dissociated from secretion, and whether microfilaments were more directly involved. In fact, it seemed likely that calcium and ionophore-induced enzyme release from PMN would prove to be independent of microtubules, since microtubules are prevented from assembling *in vitro* by calcium (22) and have been shown to be disassembled *in vivo* by A23187 and calcium in a heliozan (28).

MATERIALS AND METHODS

Preparation of PMN

Venous blood (32 ml) was drawn from healthy adult volunteers and mixed with 8 ml of ACD anticoagulant (NIH formula A) plus 20 ml of 6% dextran in normal saline. The erythrocytes were allowed to sediment for 30-45 min. Portions (15 ml) of the leukocyte-rich supernates were then mixed with 35 ml of 0.87% ammonium chloride and centrifuged for 10 min at 160 g. The cell pellets were resuspended in normal saline, combined in a vol of 50 ml saline, and spun at 160 g for 10 min. The resulting pellet was washed two times with saline and suspended in buffer containing 5 mM *N*-2-hydroxyethyl-piperazine-*N'*-2-ethane sulfonic acid (HEPES) (Grand Island Biological Co., Grand Island, N.Y.) and 150 mM NaCl adjusted to pH 7.4. Aliquots containing 2×10^6 leukocytes were incubated with appropriate compounds as described below and then either fixed for electron microscopy or centrifuged (755 g for 10 min), and the supernate removed for enzyme assays. Cells were incubated at 37°C with or without 10^{-5} M A23187 (kindly supplied by Dr. R. L. Hamill, Eli Lilly & Co., Indianapolis, Ind.) which was prepared as a stock solution in DMSO and diluted in HEPES saline before use. Preincubation with 10^{-5} M colchicine (Sigma Chemical Co., St. Louis, Mo.) was at 37°C for 30 min. Cytochalasin B (5 μ g/ml) (ICI Research Laboratories, Alderley Park, Cheshire, England) in 0.1% dimethyl sulfoxide (DMSO) (Matheson Gas Products, East Rutherford, N.J.) was added for the last 10 min before addition at A23187 and/or calcium. This concentration of DMSO did not influence cell morphology, enzyme release, or enzyme assays.

In some experiments, washed, opsonized zymosan (ICN Nutritional Biochemicals Div., International Chemical & Nuclear Corp., Cleveland, Ohio) was added after 5 min of pretreatment with A23187 and calcium and allowed to incubate for 5-30 min before fixation for electron microscopy.

Enzyme Assays

β -glucuronidase was determined after 18 h of incubation with phenolphthalein glucuronidate as substrate (5). The cytoplasmic enzyme lactate dehydrogenase (LDH) was measured by the method of Wacker et al. (34) and

used as an indicator of cell viability. Total enzyme activities were determined in simultaneously run, duplicate reaction mixtures to which had been added the detergent Triton X-100 (0.2%) (Rohm and Haas Co., Philadelphia, Pa.).

Electron Microscopy

Cell suspensions were fixed for 5 min at room temperature with a combined aldehyde-osmium tetroxide fixative as previously described (38) and embedded in Spurr's low viscosity epoxy. Silver sections were lead stained and viewed in a Zeiss EM 9S. All centrioles visible on five sections cut from each of three experiments were photographed at $\times 16,000$ and printed at $\times 48,000$ on high-contrast paper. Pericentriolar microtubules were counted as previously described (9). A square grid, calibrated so that it outlined an area $2 \mu\text{m}$ by $2 \mu\text{m}$, was superimposed on each micrograph so that a centriole was in its center. Longitudinal profiles of microtubules ($24\text{--}25 \text{ nm}$ wide and at least 50 nm long) were counted within that square. The number of visible microtubules varies to some extent from experiment to experiment and within each experiment with section thickness. The error introduced by this variability can be minimized by obtaining counts from multiple sections from each of three experiments. The significance of differences between means was determined by Student's *t* test, and the results were used only to demonstrate treatment effects on microtubule numbers relative to controls. Previous work has shown that counting pericentriolar microtubules, counting numbers of microtubules per square micrometer of peripheral cytoplasm, and point-counting stereology of microtubule volume per unit cytoplasm volume give concordant results when assaying treatment effects on microtubule numbers in PMN (16).

RESULTS

Preliminary experiments on the time-course of calcium and ionophore-induced β -glucuronidase release from PMN (see below) showed that enzyme release was minimal at 2 min and was almost maximal by 5 min. Ionophore-induced release from cytochalasin B-treated PMN occurred much more rapidly and was virtually complete by 2 min. Therefore, unless otherwise indicated, cytochalasin B-treated cells were fixed for electron microscopy 1 min after exposure to the ionophore, and cells without cytochalasin B were fixed 5 min after exposure, whereas enzyme release was measured after 30 min.

Enzyme Release

PMN incubated for 30 min. in 10^{-5} M A23187 in the absence of extracellular calcium released 4–6% of their total content of the cytoplasmic

TABLE I
Effect of Colchicine on Calcium-Induced β -Glucuronidase Release from A23187-Treated Human PMN*

Treatment	β -Glucuronidase release \pm SEM \dagger
None	3.7 ± 1.3
A23187 (10^{-5} M)	3.7 ± 0.7
A23187 + 1 mM Ca^{++}	19.8 ± 1.2
A23187 + 1 mM Ca^{++} + colchicine	18.7 ± 1.6
A23187 + 2 mM Ca^{++}	27.3 ± 3.2
A23187 + 2 mM Ca^{++} + colchicine	29.3 ± 3.6
Cytochalasin B	3.4 ± 1.2
Cytochalasin B + A23187	10.7 ± 1.9
Cytochalasin B + A23187 + 1 mM Ca^{++}	35.9 ± 3.5
Cytochalasin B + A23187 + 1 mM Ca^{++} + colchicine	33.5 ± 2.5
Cytochalasin B + A23187 + 2 mM Ca^{++}	38.9 ± 6.0
Cytochalasin B + A23187 + 2 mM Ca^{++} + colchicine	39.8 ± 4.4

* Cells were incubated with agents for 30 min in HEPES-NaCl buffer at 37°C . Colchicine-treated cells were preincubated with 10^{-5} colchicine for 60 min before the addition of ionophore plus calcium. Cytochalasin B ($5 \mu\text{g}/\text{ml}$) was added for the last 10 min before the addition of ionophore plus calcium. Results are means of four experiments.

\dagger Expressed as percent of total activity released from duplicate tubes by Triton X-100.

enzyme LDH and 0–5% of their content of the azurophilic granule enzyme β -glucuronidase. Addition of calcium to the medium resulted in enhancement of β -glucuronidase but not of LDH release. Pretreatment with cytochalasin B made the cells more sensitive to the effects of the ionophore and calcium. A23187 provoked release of 9–12% of total β -glucuronidase from such cells. Inclusion of calcium (1 mM) in the incubation medium with A23187 caused release of $35.9 \pm 3.5\%$ of total β -glucuronidase from cytochalasin B-treated PMN but only $19.8 \pm 1.2\%$ from PMN without cytochalasin B. Release of β -glucuronidase, induced by A23187 and calcium, was not inhibited by colchicine either in cytochalasin B-treated or in untreated PMN (Table I); nor was release enhanced by cGMP.

Electron Microscopy, Ionophore Alone

An electron-opaque band of filaments was seen in the pericortical cytoplasm of many of these cells

(Fig. 1). The band was variable in length and appeared to represent a contracted region of the cytoplasm, since the plasma membrane above the band was gathered into a series of folds and blebs but remained smooth over other parts of the cell. Views of the band at higher magnification showed that it consisted mainly of thin (4–6 nm) filaments with some thick (10–12 nm) filaments. The filaments were oriented tangential to the surface and were most clearly seen in sections taken in that plane (Fig. 2).

Ionophore and Calcium

The morphological response to A23187 was more dramatic when calcium (0.1–2 mM) was included in the incubation medium. The membrane folding, or crenulation, above the filamentous bands was more extensive than in the absence of calcium. With external calcium concentrations of 1–2 mM, organelles were sometimes trapped between the membrane and the subjacent fila-

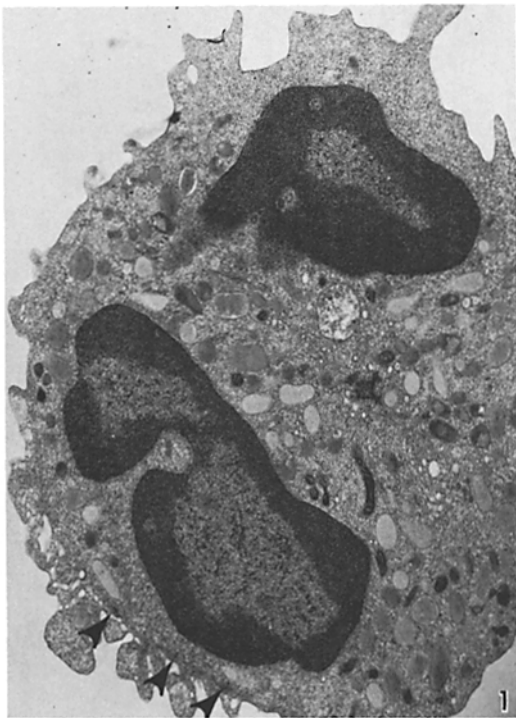


FIGURE 1 Low magnification view of a PMN exposed to A23187 in a calcium-free medium. A filamentous web is visible in the pericortical cytoplasm in the lower left of the figure (arrowheads). The overlying cytoplasm is gathered up into blebs and folds although the remainder of the cell appears normal. $\times 10,500$.

ments (Fig. 3). Vacuoles were consistently seen in the cytocenter of PMN exposed to A23187 in the presence of calcium but not in its absence (Fig. 4). These vacuoles were often irregular in profile, as if a crenulated portion of the plasma membrane had been internalized (Fig. 5). The cytocenter also appeared constricted so that granules were closer to each other and to the centrioles than in untreated cells (Fig. 6).

Ionophore-treated PMN were capable of interacting with and internalizing opsonized zymosan (Fig. 7).¹ Furthermore, membrane folds and blebs were no longer seen in PMN that had internalized zymosan after exposure to ionophore and calcium. The subplasmalemmal web of filaments was less conspicuous after phagocytosis, and filaments were visible in the cytoplasm immediately subjacent to the vacuolar membranes (Fig. 8).

Microtubules retained their normal morphology in cells treated with either the ionophore or calcium, but their appearance was radically altered in PMN exposed simultaneously to both agents. Both diameter and staining intensity were increased. Their apparent diameters now ranged from 30 to 50 nm (Figs. 9 and 10). The increase appeared to result from association of fibrillar material with the external surface of microtubules (Fig. 9). Electron-opaque material projecting from the "decorated" microtubules gave each microtubule, when seen in cross section, the appearance of a star, usually six- but sometimes five-pointed. In some sections the rays of such a star could be observed to contact the outer leaflet of the limiting membrane of a granule. Longitudinal sections of decorated microtubules showed that the fibrillar material had a periodicity along the microtubule of about 25 nm (Fig. 10). Connections between granules and microtubules via several of these fibrils could be demonstrated in some sections.

The appearance of these decorated microtubules was not affected by preincubation with cGMP (10^{-5} M, 2 min). Cells fixed 30 min after exposure to A23187 and calcium still showed blebs and the unusual decorated microtubules. Although preincubation with colchicine (10^{-5} M, 30 min) before the addition of A23187 and calcium decreased the total number of microtu-

¹ This capacity differs from that of diamide-treated PMN which also have a remarkably altered profile in this section and in which a band of apparently contracted microfilaments is evident (24).

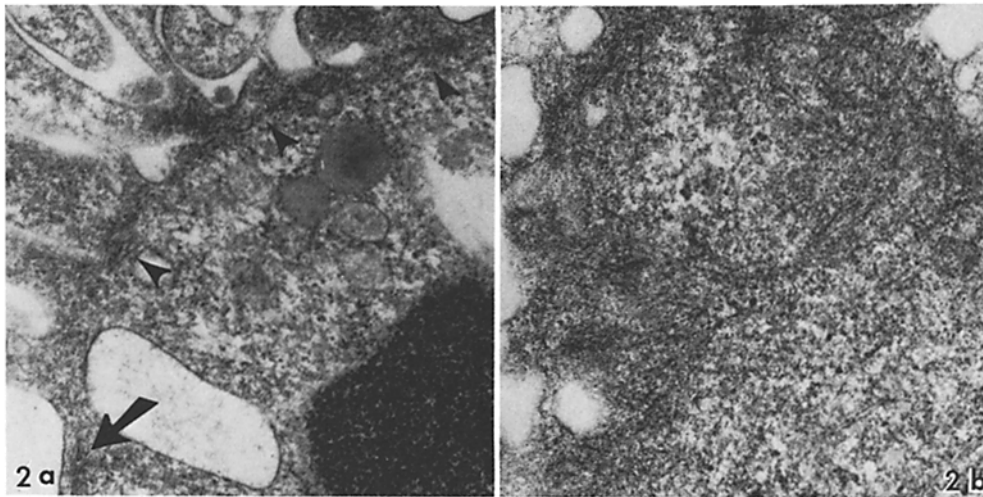


FIGURE 2 (a) A higher magnification view of a portion of a cell treated as in Fig. 1. The pericortical web is again indicated with arrowheads. At the lower left of the figure is a microtubule extending outward from the cytocenter to end in the pericortical web (arrow). $\times 32,000$. A section tangential to and just under the blebbed portion of a similar PMN. The filaments are seen more distinctly from this angle, and it is possible to determine that the web is composed of thick and thin filaments. $\times 41,000$.

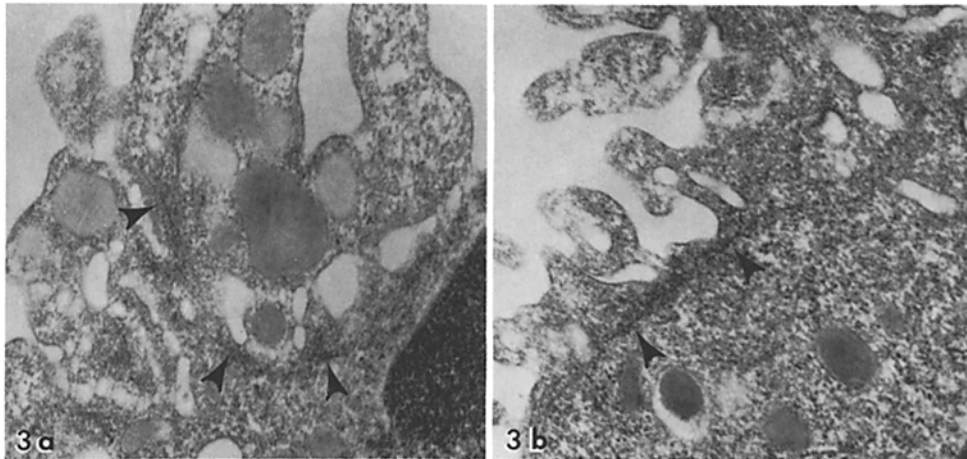


FIGURE 3 (a and b) Portions of the blebbed regions of two PMN treated with A23187 and calcium. Blebbing is extensive, and many granules are caught up in the blebs. $\times 32,000$.

bules, those few that were seen (0-2 per centriolar profile) were decorated.

Cytochalasin B

Cytochalasin B-treated PMN also underwent a profound alteration in morphology upon exposure to the ionophore and calcium. Control cells or cells exposed to exogenous calcium alone were rounded with small cytoplasmic projections from

the cell surface (Fig. 11a). One minute after addition of the ionophore, the cells showed irregular vacuoles and deep invaginations of the plasma membrane in which the discharged content of granules was occasionally seen (Fig. 11b). These clefts and vacuoles were clearly oriented towards the centrioles. The cytoplasm of cytochalasin B-treated PMN contained dense filaments arranged tangential to the plasma membrane and vacuoles (Fig. 12), which were not seen in unstimulated

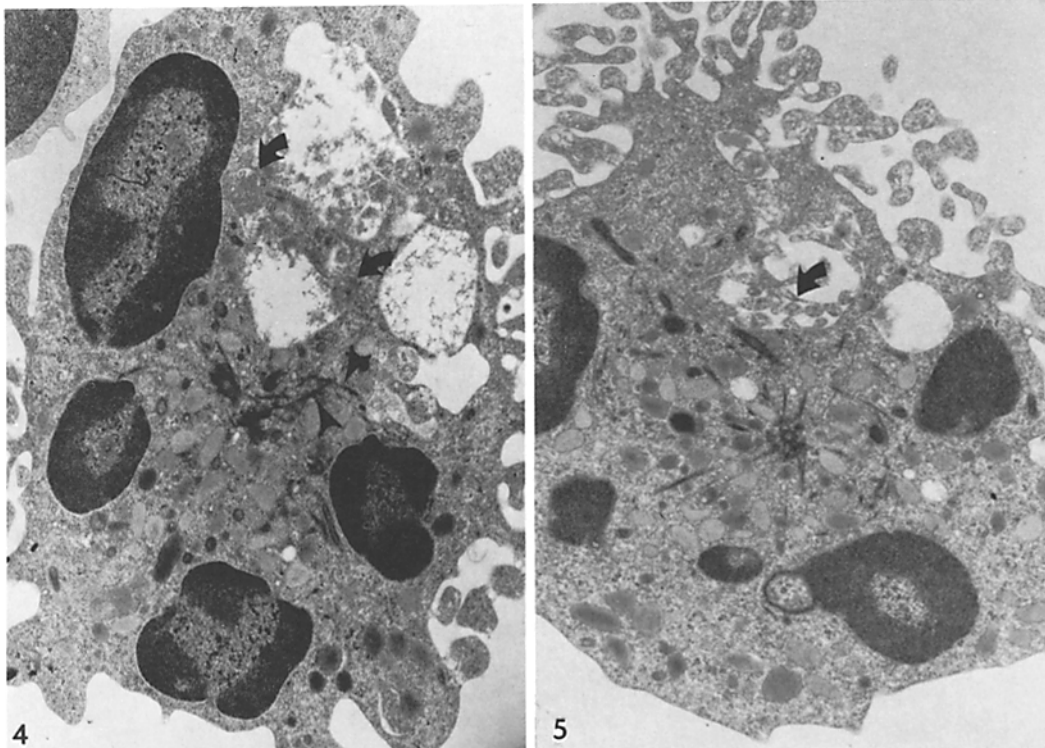


FIGURE 4 A PMN that had been treated with A23187 and 1 mM calcium. The cell margin shows numerous blebs and folds, and several vacuoles containing evidence of recent degranulation (arrow) are visible in the interior. Decorated microtubules (arrowheads) radiate from the centriole, and granules are closer together in the cytocenter than in Fig. 1. $\times 10,500$.

FIGURE 5 Another cell treated as in Fig. 4, showing blebs (arrow) in one of the vacuoles. The cell is otherwise similar with decorated microtubules radiating from the centriole and granules close together in the cytocenter. $\times 17,000$.

control cells. The filaments were 10 nm thick, ca. 300–400 nm long and tapered at both ends.

Two minutes after adding the ionophore and calcium, most of the cells were almost completely degranulated, although a few still contained many granules. Whereas the cell population as a whole released more than one-third of its β -glucuronidase, the appearance of the cells by electron microscopy suggests that not all PMN were equally affected by the treatment. PMN with, and without, residual granules appeared otherwise intact, with centrally located centrioles and microtubules still present. The deep invaginations seen commonly at 1 min were seen less frequently at 2 min, and the long, thin protrusions were now rounded and blunt (Fig. 13).

Although most PMN were indistinguishable from control cells when A23187 was added to

cytochalasin B-treated cells prepared in a calcium-free medium, a small proportion of the cells responded as if calcium were present. Some cells showed an increase in the number of vacuoles, and some had a few invaginations. Preparations in which EDTA or ethylene glycol-bis (β -aminoethyl ether) N,N,N',N' -tetraacetate (EGTA) (3 mM) was included in the incubation medium with A23187 were identical in appearance to preparations with A23187 alone.

Microtubule Numbers

The mean number of microtubules visible in PMN was unaffected by a 5-min incubation with either the ionophore or ionophore plus 1 mM calcium. Microtubules in cytochalasin B-treated PMN were, however, affected by the ionophore. The mean number of microtubules seen in the

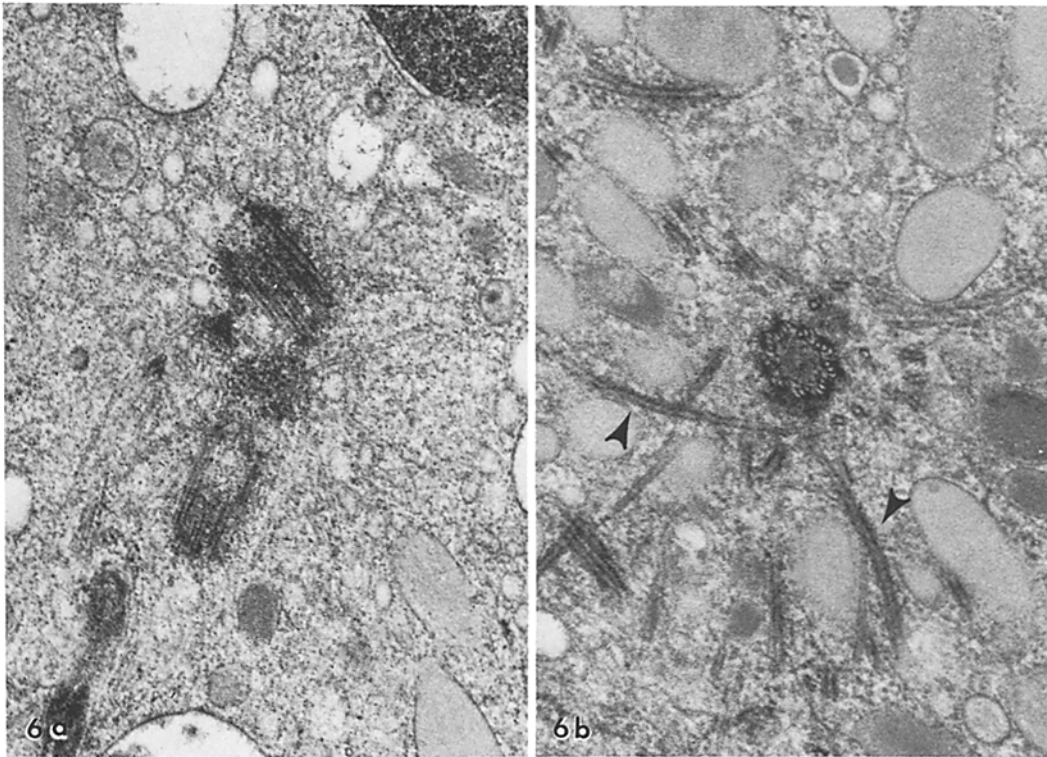


FIGURE 6 The centriolar region of (a) control PMN and (b) PMN treated as in Figs. 4 and 5. Most of the pericentriolar microtubules in Fig. 6b are decorated, and in some places (arrowheads) the periodic nature of the decorations is apparent. Far more granules are also evident in the pericentriolar cytoplasm than in the control, Fig. 6a. $\times 48,000$.

centriolar region decreased from 22.3 ± 1.5 in control cells to 13.9 ± 0.9 in ionophore-treated cells. The inclusion of either calcium (1 mM) or EGTA (3 mM) in the incubation medium with the ionophore had no effect upon the ionophore-induced decrease in microtubule numbers. Prior incubation (2 min) with cGMP (10^{-5} M) did, however, prevent the ionophore-induced decrease in microtubule numbers (Table II).

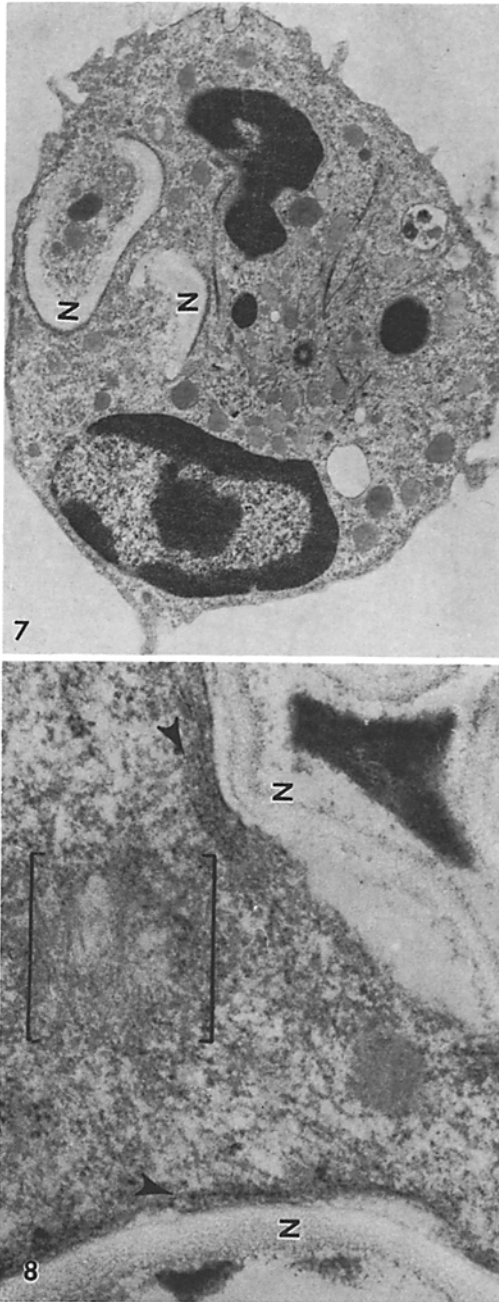
DISCUSSION

Human PMN respond to particulate or immune stimuli with both an increased calcium flux (1, 3, 8, 19) and lysosomal enzyme discharge. Calcium fluxes induced by the ionophore A23187 also result in enzyme discharge (7, 12, 26, 27). Since contractile events in nonmuscle as well as muscle cells appear to be mediated by changes in the cytosol of ionic calcium (6, 15, 23), it was of interest to examine the ultrastructural correlates of calcium ionophore-induced secretion and to

compare these with secretion induced by physiological stimuli.

The data do not permit estimates of the intracellular concentrations of calcium induced by A23187 or indicate how these vary in response to extracellular calcium. Our results do show that enzyme release varies with the extracellular calcium concentration (at a constant ionophore concentration). Estensen et al. (7) have shown that enzyme release from PMN varied with the concentration of ionophore (at a constant calcium concentration). Since micromolar calcium is sufficient to induce contraction of membrane-free cytoplasmic extracts (6), it is likely that in our experiments intracellular calcium approximated micromolar levels, but we have no evidence that it equilibrated with the external calcium concentration.

The results obtained in this study indicate that calcium-mediated enzyme release from ionophore-treated PMN does not depend upon microtubule assembly. Unlike the situation in PMN



exposed to immune stimuli, microtubule numbers were not increased in PMN that were stimulated to secrete by A23187 and calcium. Indeed, microtubules actually decreased in number in cytochalasin B-treated PMN from which lysosomal enzyme release was massive. Furthermore, colchicine, at a concentration that virtually eliminated microtubules (16), had no effect upon enzyme release induced by ionophore.

Two morphological responses were consistently seen in PMN treated with ionophore. One of these responses was the formation of pronounced plasma membrane invaginations, which occurred under all experimental conditions in which significant enzyme release was obtained. The invaginations were rounded, resembling spherical vacuoles, in normal PMN but were deep and narrow in cytochalasin B-treated PMN. The membrane invaginations induced by A23187 and Ca^{++} were similar in ultrastructural appearance to those previously described in PMN induced to discharge granule-bound enzymes by particulate or soluble immune stimuli (9, 16-18). In each of these circumstances, release of granule enzymes is associated with vacuole formation and/or membrane invagination. These results suggest that lysosomal enzyme secretion from PMN is a consequence of movement of stimulated portions of the plasma membrane toward the granule-rich cytocenter rather than movement of lysosomes toward the plasma membrane.

A second response, the formation of a subplasmalemmal, filamentous meshwork, was observed in normal PMN exposed to A23187. This meshwork was more extensive and involved more of the cell surface when cells were exposed to ionophore and calcium. Since the overlying plasma membrane was gathered up into folds and blebs, it is likely that the meshwork was contracted in the plane of the membrane. Calcium-induced for-

FIGURE 7 A PMN exposed to zymosan 5 min after treatment with A23187 and calcium. Zymosan (Z) was ingested, and membrane blebbing is no longer evident. $\times 10,500$.

FIGURE 8 A portion of a PMN with ingested zymosan (Z) showing that filaments (arrowheads) are now subjacent to phagocytic vacuoles. A glancing section of a phagocytic vacuole (brackets) shows that filaments are oriented tangential to the vacuolar membrane. $\times 48,000$.

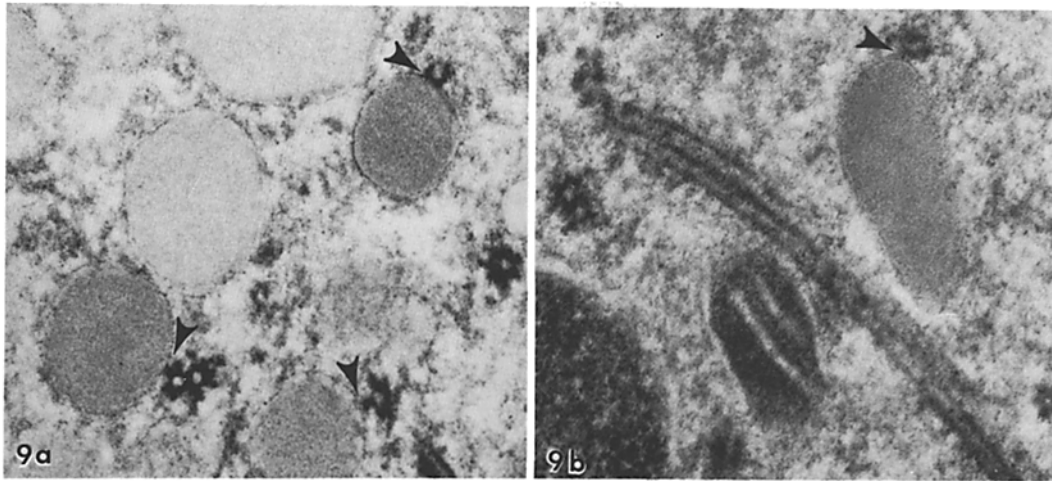


FIGURE 9 Decorated microtubules seen in cross section. The material projecting from the microtubules is wider than it is thick, and in some instances (arrowheads) makes contact with granule membranes. $\times 64,000$.

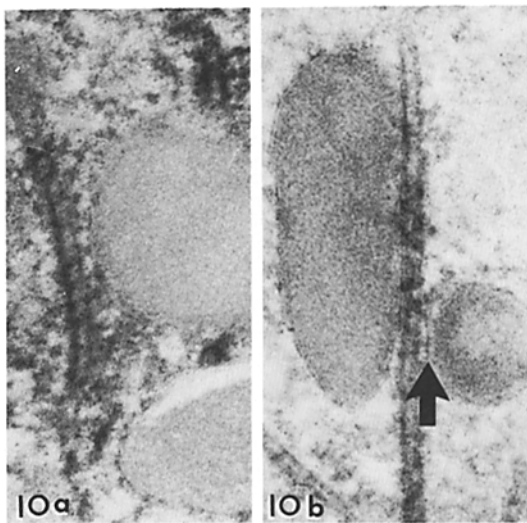


FIGURE 10 Longitudinal views of the interaction of decorated microtubules with granules. The contacts are repeated periodically, and in Fig. 10b a granule membrane is slightly distorted (arrow) suggesting that the structures are somehow adherent to one another. $\times 64,000$.

mation and contraction of the subplasmalemmal meshwork in PMN was reversible, since the excess membrane folds and subjacent filaments were redistributed to the phagocytic vacuoles when the cells were subsequently exposed to opsonized zymosan. Unlike the phenomenon of membrane invagination, which correlated with enzyme secretion, membrane blebbing resulting from contrac-

tion of subplasmalemmal filaments was unrelated to secretion since it was observed under experimental conditions (ionophore alone) in which granule discharge did not occur. The observation that membrane blebbing was never seen in cytochalasin B-treated PMN exposed to A23187 suggests that the interaction between the circumferential band of filaments and the plasma membrane is sensitive to cytochalasin B. In normal cells, this circumferential contraction would be coupled via an actin network and actin-binding protein to the plasma membrane, whereas in cytochalasin B-treated cells the network does not form (14, 35) and the actomyosin contractions occur in isolation.

Two cytoskeletal elements were observed in this study that may provide a structural explanation for some of the observations. One of these is a bipolar, thick filament that resembles leukocyte myosin (31). Such filaments are 10 nm thick, ca. 300–400 nm long and tapered at both ends. They appear as isolated structures parallel to the plasma membrane in stimulated, cytochalasin B-treated PMN and as embedded in a network of other, thinner, filaments in untreated, stimulated PMN. Such filaments were previously reported in PMN after exposure to *C5a*, a chemotactically active split product of complement activation (10, 16) that induces a calcium influx (3). Although these filaments resemble myosin, and appear at sites where one would expect myosin to be, they have not yet been identified as such by biochemical criteria.

Another novel structure was observed in these

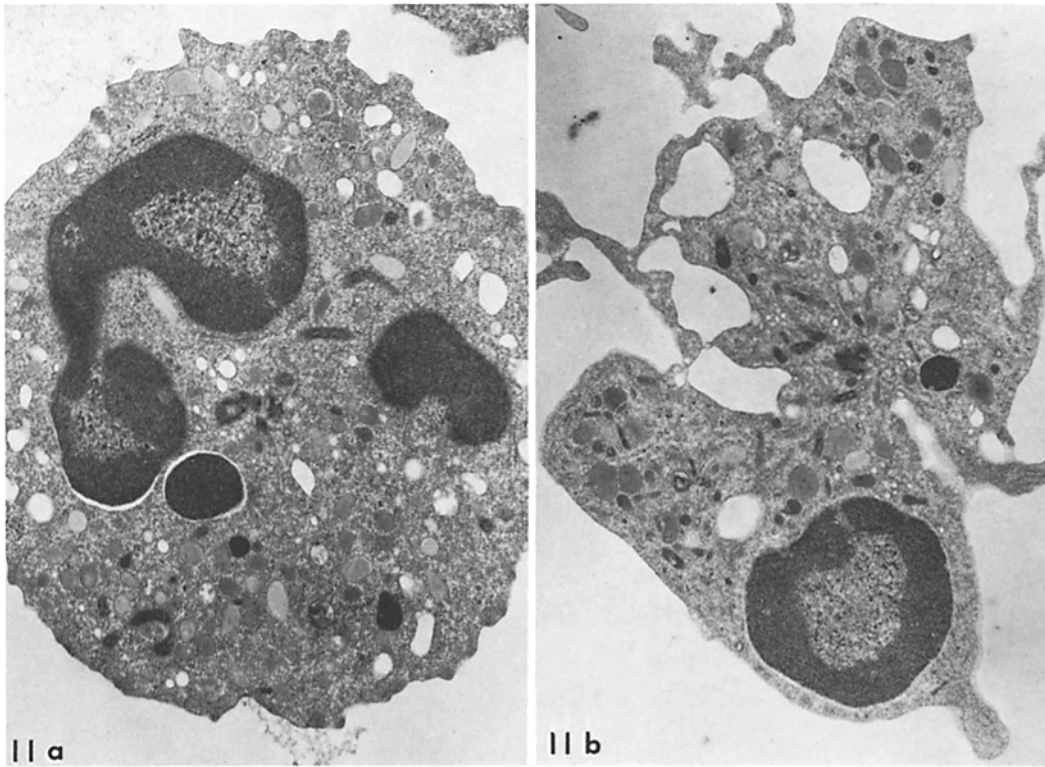


FIGURE 11 (a) A control cytochalasin B-treated PMN. (b) A similar cell exposed to A23187 and calcium (1 mM) for 1 min. Compared to the control cell, the treated cell is deeply invaginated but blebbing has not occurred. $\times 10,500$.

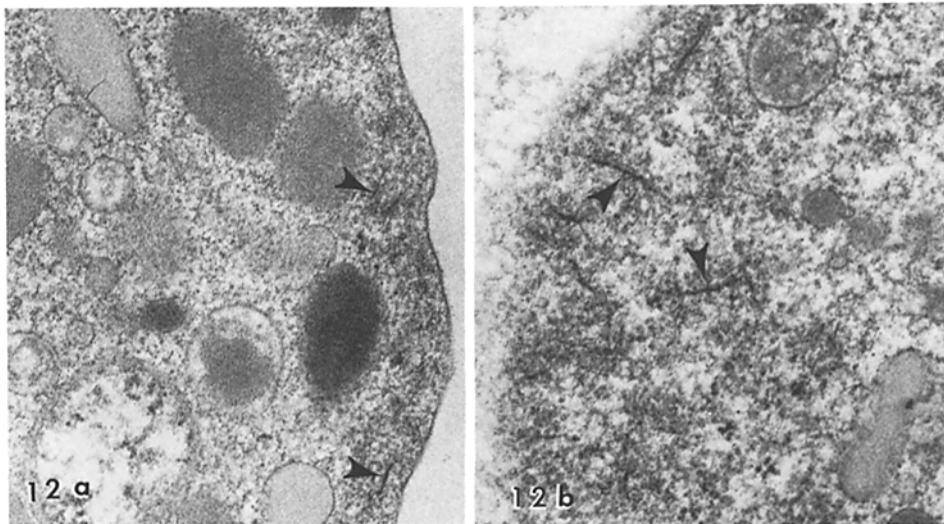
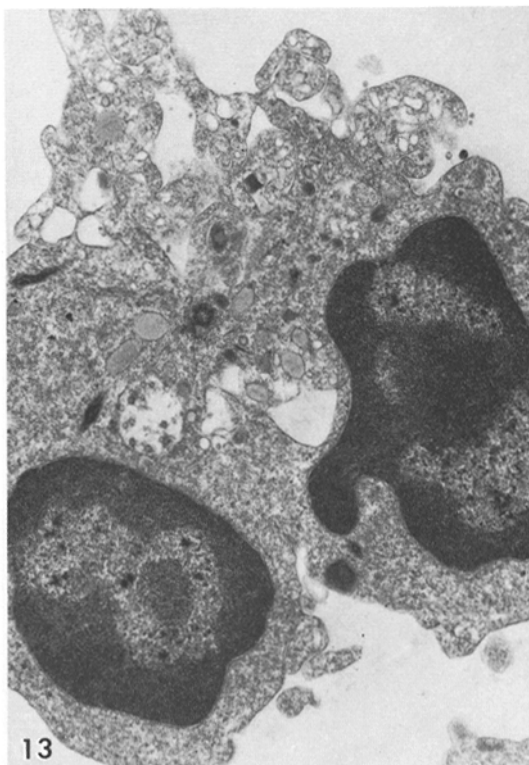


FIGURE 12 Subplasmalemmal filaments in cytochalasin B-treated cells exposed to A23187 and 1 mM calcium for 1 min. In Fig. 12a, the section is approx. at right angles to the membrane, and the filaments (arrowheads) oriented parallel to the membrane and at right angles to the section appear foreshortened. In Fig. 12b, the section is approx. parallel to the membrane, and thick filaments associated with a few thin filaments are in the plane of the section. $\times 48,000$.



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FIGURE 13 A cytochalasin B-treated PMN 2 min after exposure to A23187 and calcium (1 mM). Few granules are left, and the cell is now rounded up. The excess membrane, added during degranulation, shows as a convoluted cell margin at the upper right. $\times 10,500$.

cells. Microtubules of PMN in which a calcium influx was induced by A23187 were decorated at regular intervals with an electron-opaque material, which projected outward from the microtubule to, in some instances, the outer leaflet of the limiting membrane of granules. This microtubule-associated material may be part of the microtubular lattice described by Wolosewick and Porter (37), and may function to stabilize the internal arrangement of the cell. Another possibility is that this material may be responsible for centripetal movement of granules and vacuoles within PMN since this occurs along pathways defined by microtubules.

Contractions both parallel and perpendicular to the plane of the plasma membrane may be important in phagocytosis. Contact between a PMN and a phagocytosable particle leads to a localized contraction just underneath the point of contact and at right angles to the plasmalemma, resulting in a cup-shaped depression into which the particle

fits. Subsequently, the margin of the depression moves inward to enclose the particles in a complete phagocytic vacuole. It is this lateral movement of the plasma membrane that is inhibited by cytochalasin B. Continued movement of the vacuole into the granule-rich cytocenter leads to sufficient proximity between vacuole membrane and granules for fusion to occur. During vacuole formation, differential movements of membrane proteins and lipids have been described (21, 33, 2), suggesting a physical connection between portions of the membrane and subplasmalemmal structures involved in motility. Depending on the location of the forming vacuole with respect to the granule-rich cytocenter, degranulation may begin while the vacuole is still open, a morphological result which has as its biochemical correlate the release of measurable amounts of lysosomal enzymes into the extracellular milieu (16). Inhibition of closure by cytochalasin B increases the quantity of lysosomal enzymes released into the medium (38).

These data and the experiments reported here suggest the existence of at least two sets of contractile proteins with different locations and control mechanisms. One set, sensitive to cytochalasin B and sensitive also to low levels of calcium, is located in the pericortical cytoplasm, and functions mainly in horizontal movement of the plasma membrane. The other set is not cytochalasin B-

TABLE II
Effect of A23187 and Calcium on Microtubule Assembly in Human PMN*

Treatment	Mean microtubule nos.†	P vs. Control
None	22.3 \pm 2.0	Control
A23187	22.3 \pm 1.1	NS
A23187 + 1 mM Ca ⁺⁺	24.9 \pm 1.5	NS
Cytochalasin B	22.3 \pm 1.5	NS
Cytochalasin B + A23187	13.9 \pm 0.9	0.001
Cytochalasin B + A23187 + 1 mM Ca ⁺⁺	13.6 \pm 1.2	0.001
Cytochalasin B + A23187 + 3 mM EGTA	10.3 \pm 1.3	0.001
Cytochalasin B + A23187 + 10 ⁻⁵ cGMP	23.6 \pm 1.8	NS

* Cells were incubated with A23187 10⁻⁵ M and calcium 1 mM at 37°C for 5 min before fixation for electron microscopy. Cells pretreated with cytochalasin B (5 g/ml, 10 min) were incubated with A23187 and calcium for 1 min before fixation.

† Microtubules within a 2 μ m \times 2 μ m square area centered on a centriole. Mean SEM (n = 10-13).

sensitive, requires higher levels of calcium to be activated, and is located mainly in the cytocenter. This set appears to function in centripetal movement of organelles. Moreover, these proteins may be oriented or anchored in some fashion by microtubules since these two are mainly observed in the granule-rich cytocenter. The data indicate that granule discharge in human PMN is a consequence of stimulus-induced retraction of plasma membrane to the granule-rich cytocenter rather than centrifugal movement of granules to the plasma membrane. Microtubules appear not to be critical for the coupling of secretion to stimulation but may guide granules and vacuoles toward the cytocenter. Such guidance may, in some circumstances, be necessary for optimum granule discharge.

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