

Cell Surface Dynamics of Neutrophils Stimulated with Phorbol Esters or Retinoids

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Abstract. Neutrophils undergo rapid morphological changes as well as metabolic perturbations when stimulated with certain phorbol esters. Stimulated cells initially exhibit pronounced projections emanating from the cell bodies, followed by rounding of the cells, reduction in granule number, and the appearance of intracellular vesicles. We show these vesicles to be derived, at least in part, from the plasmalemma. The experimental approach involved labeling stimulated and unstimulated cells with native ferritin and cationized ferritin, along with the cytochemical localization of ecto-5'-nucleotidase. The labeling patterns of the vesicles indicate that these structures are involved in both phorbol ester-stimulated adsorptive and fluid-phase endocytosis.

Neutrophils stimulated with 12-O-tetradecanoyl-phorbol-13-acetate (TPA) exhibit two distinct rates of superoxide release in which the second, prolonged level is ~50% of the initial rate. All-trans-retinal, which we have recently shown to stimulate O_2^- release but not granule exocytosis or cell vesiculation, induces a single prolonged rate of maximal O_2^- release. Neutrophils treated with both all-trans-retinal and TPA exhibit only a single sustained rate of maximal O_2^- release similar to that observed with all-trans-retinal alone. Moreover, treatment of cells with all-trans-retinal blocks the vesiculation of neutrophils induced by TPA in a dose-dependent manner. This observation provides a possible explanation for the differences in the kinetics of superoxide release.

NEUTROPHILS undergo remarkable changes in their metabolism and morphology when stimulated. They display enhanced oxygen consumption with concomitant production of superoxide (O_2^-)¹ and other oxygen-derived products. These products constitute key components of the oxygen-dependent antimicrobial mechanisms of phagocytic leukocytes (1, 2).

A variety of agents can stimulate neutrophils either by binding to specific receptors on these cells (e.g., phorbol esters, chemotactic peptides [19, 24, 42]) or by partitioning into and disordering the plasmalemma (e.g., *cis*-unsaturated fatty acids [4], retinoids [6, 25]). Changes in cell shape, some quite dramatic, occur with all stimuli (e.g., 4, 21). The release of enzymes and other proteins from intracellular granules through exocytotic processes (degranulation) frequently occurs as well. Retinoids, however, induce O_2^- release without causing degranulation (6).

Neutrophils and macrophages exhibit numerous vesicles, some much larger than pinosomes, after treatment with certain phorbol esters (5, 7, 35, 43). The origin of these vesicles is of topical interest since treatment of phagocytic leukocytes and other cells with 12-O-tetradecanoyl-phorbol-13-acetate (TPA) can diminish the number of a variety of receptors on

the cell surface (e.g., 8, 12, 23, 26), diminish the subsequent response of the cell to a second stimulus (e.g., 28), and increase pinocytosis (38).

In this study, we describe some novel and very dramatic shape changes rapidly undergone by neutrophils in suspension upon stimulation with TPA. These have not been previously reported. We further show directly by cytochemical procedures that the intracellular vesicles subsequently observed are derived, at least in part, from invaginations of the plasmalemma. Evidence is provided which indicates that these vesicles are involved in TPA-stimulated adsorptive and fluid-phase endocytosis. Further, we report that all-trans-retinal inhibits this vesiculation in a dose-dependent manner and modifies the progress curve for O_2^- release induced with TPA. Time-dependent differences in cell morphology and the release of O_2^- are correlated.

Materials and Methods

Materials

Ferricytochrome *c* (type VI), superoxide dismutase from bovine erythrocytes, Triton X-100, AMP (type II), all-trans-retinal (type XVI), TPA, agarose (type VII), and calcium ionophore A23187 were purchased from Sigma Chemical Co., St. Louis, MO. Ferritin (twice crystallized) and cationized ferritin were obtained from Miles Scientific, Naperville, IL. Cerium chloride was purchased from Alfa Products, Danvers, MA. Sodium caseinate was obtained from Fisher Scientific Co., Fairlawn, NJ. All other

1. *Abbreviations used in this paper:* 5'-NT, 5'-nucleotidase; O_2^- , superoxide; TPA, 12-O-tetradecanoyl-phorbol-13-acetate.

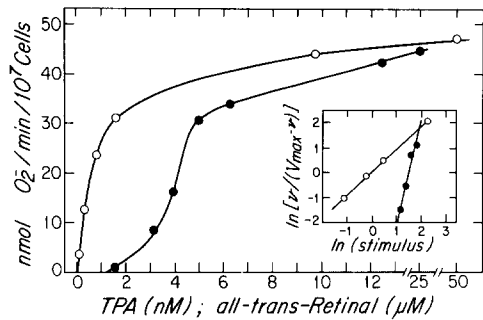


Figure 1. Ability of different concentrations of TPA and all-trans-retinal to stimulate superoxide release by guinea pig neutrophils. Superoxide release was monitored after stimulation of the cells ($1.0 \times 10^6/\text{ml}$) with different concentrations of TPA (○) or all-trans-retinal (●) as described under Materials and Methods. The inset shows the Hill plots of these data.

chemicals were of the highest available grade. Male Hartley guinea pigs² were obtained from Elm Hill Breeding Laboratories (Chelmsford, MA).

Preparation of Cells

Peritoneal guinea pig neutrophils were elicited and prepared as described previously (14). Human neutrophils were purified from fresh whole blood by a procedure outlined in detail elsewhere (3). Cell preparations from guinea pigs and humans contained ≥ 90 and 98% neutrophils, respectively, with viabilities always $\geq 90\%$.

Superoxide Production

O_2^- release was monitored as previously described (34, 35).

Ultrastructural and Cytochemical Studies

Morphology. The morphology of neutrophils was determined at different time intervals after the addition of a stimulus. The medium used in these studies was the same as that used in the superoxide assay (i.e., 138 mM NaCl, 2.7 mM KCl, 16.2 mM Na_2HPO_4 , 1.47 mM KH_2PO_4 , 0.90 mM CaCl_2 , 0.5 mM MgCl_2 , 7.5 mM D-glucose, pH = 7.35) except that ferricytochrome *c* and superoxide dismutase were omitted. Cells (1×10^6 cells/ml; 10 ml total volume) were incubated at 37°C for 3 min before addition of the stimulus. After stimulation, they were fixed by the addition of 0.85 ml of 25% glutaraldehyde; cells remained in fixative for 1 h before washing in medium (three to four times). These were postfixed in osmium (2% OsO_4 -0.1 M phosphate buffer, pH 7.2, reduced with 3% potassium ferrocyanide) (22, 36) for 45–60 min at room temperature, then washed three times in 0.1 M phosphate buffer, pH 7.2. Washed cells were resuspended in low temperature gelling agarose (2%) at 37°C and then pelleted in microcentrifuge tubes. The agarose was gelled by transferring to ice. Portions of the agarose pellet were cut into small pieces, dehydrated, infiltrated, and embedded in Epon by standard methods. Semithin sections (1 μm) were cut with glass knives, mounted on slides, stained with toluidine blue, and examined with a Zeiss UEM light microscope. Photomicrographs

2. Animals used in this study were maintained in accordance with the guidelines of the Committee on Animals of the Harvard Medical School and those prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council (DHEW publication No. 78-23, revised 1978).

Figure 3. Time course of TPA-induced morphological changes in human neutrophils. (A) Control cells which were incubated in suspension for 5 min at 37°C with DMSO (0.25% vol/vol) before fixation. Note that the cells have a generally rounded appearance with some short surface projections, numerous intracellular granules, and few vesicular structures. (B) Cells that were stimulated in suspension with 12 nM TPA for 1 min at 37°C before fixation. During this early phase of stimulation, the cells have lost their rounded appearance and display dramatic shape changes. (C) Cells that were stimulated for 3 min before fixation. During this phase of stimulation, the cells are in the process of returning to a more rounded appearance and display increased numbers of intracellular vesicles (arrowheads). (D) Cells that were stimulated for 5 min before fixation. During this phase of stimulation, the cells become progressively more rounded and display a greater number of vesicles (arrowheads). Bar, 1 μm .

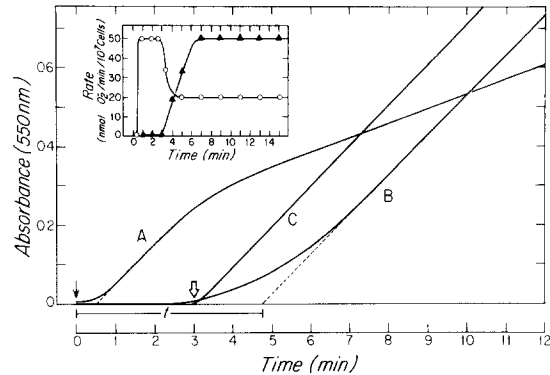
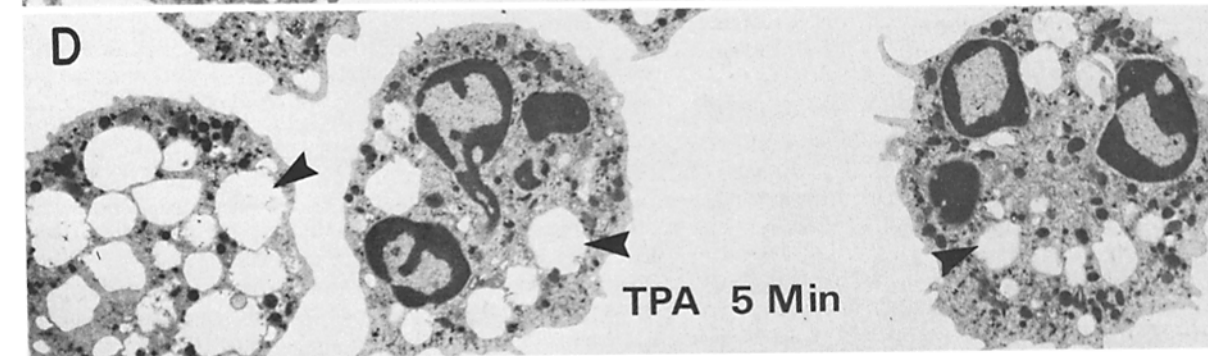
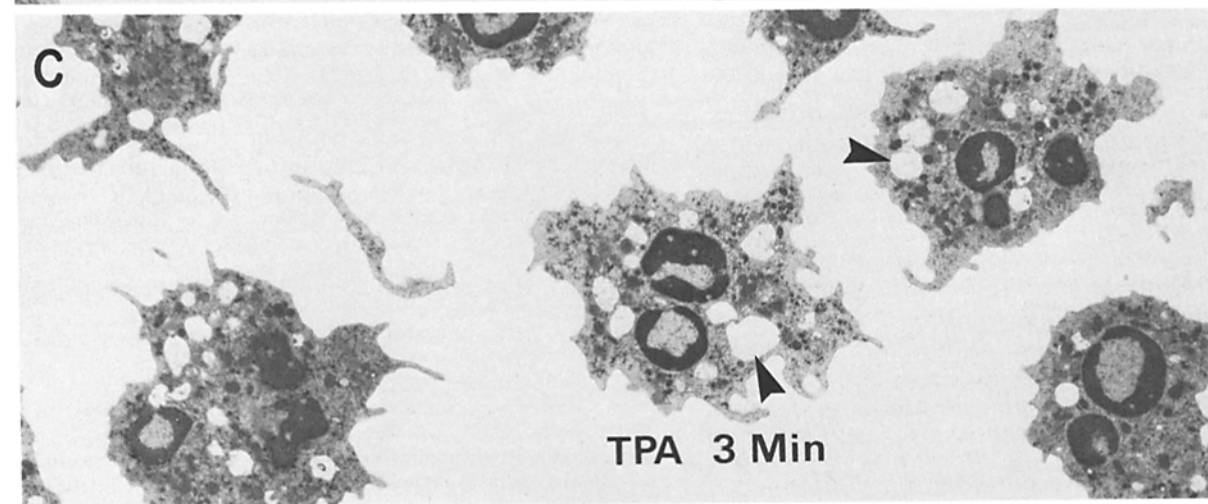
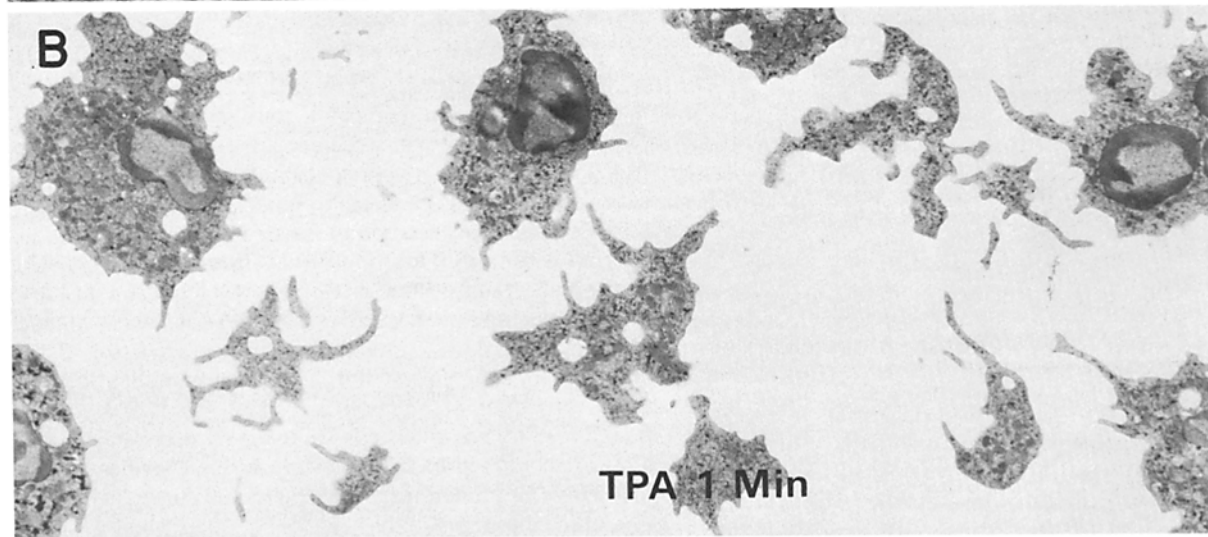
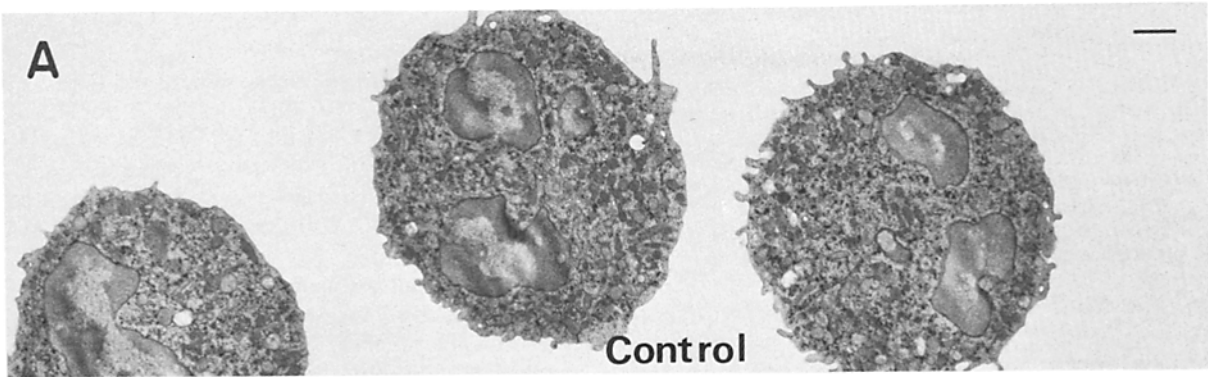


Figure 2. Time course of superoxide release by guinea pig neutrophils stimulated with TPA and all-trans-retinal. The figure shows portions of the progress curves for superoxide release from neutrophils (1.0×10^6 cells/ml) stimulated with 10 nM TPA (A) and 25 μM all-trans-retinal (B) which were added last to the assay mixture to initiate the reactions (arrow). The time (*t*) required to reach maximal activity was approximated by back-extrapolation to zero absorbance change (i.e., dashed lines). Also shown is the progress curve (C) for superoxide release from neutrophils incubated with all-trans-retinal (12.5 μM) for 3 min followed by addition of 12 nM TPA (stippled arrow). Note the single, linear rate of O_2^- release. The inset shows the derivatives of curves A (open circles) and B (solid triangles).

were made on Kodak Panatomic-X film. Thin sections were cut on a diamond knife, stained with lead citrate and uranyl acetate, and examined in a Philips 200 EM.

Labeling with ferritin and cationized ferritin. Native ferritin and cationized ferritin were used as exogenous markers to follow plasma membrane movement during neutrophil stimulation. Cationized ferritin binds to the plasma membrane through electrostatic interactions (e.g., 16–18) and thus provides an ultrastructural probe for membrane movement. Native ferritin does not bind to the plasmalemma, and thus provides a probe to follow fluid-phase uptake by cells (e.g., 16, 17). Neutrophils (1×10^6 cells/ml; 10 ml total volume) were treated as in the morphological experiments (described above). Native (final concentration, 2.0 mg/ml) or cationized (final concentration, 0.11 mg/ml) ferritin was added simultaneously with TPA. Control cells were incubated with the solvent for TPA (DMSO; 0.25% vol/vol) along with native or cationized ferritin. Glutaraldehyde was added directly to the incubation mixture at various times. Samples were processed for electron microscopy as above. In some studies, cells were first stimulated with TPA for varying times, fixed, washed, and then labeled with cationized ferritin (10 times more cationized ferritin was used with fixed cells than with unfixed; final concentration, 1.1 mg/ml).

Cytochemical localization of ecto-5'-nucleotidase. Cytochemical localization of ecto-5'-nucleotidase (5'-NT) was used to follow an intrinsic plasma membrane enzyme during neutrophil stimulation. This activity is restricted almost entirely to the external surface of plasmalemma in guinea pig neutrophils and is thus a convenient marker for that organelle (14). Activity of 5'-NT was detected with cerium as the capture agent as described (32); in some preparations a low level of Triton X-100 (0.0001%) was included in the cytochemical medium to detect intracellular sites of activity (i.e., vesicles) (31). Cytochemical staining of 5'-NT activity is very sensitive to the type of buffer and the pH used during fixation (40). The medium used for stimulating cells with TPA in these studies was the same as that used for the O_2^- measurements and other ultrastructural studies, except that Tris (20 mM) was substituted for phosphate. It was determined that cells stimu-



lated with TPA in the Tris-buffered medium released O_2^- at the same rate and with the same kinetics as in the standard assay medium (data not shown).

Cells ($10^6/ml$; 10 ml total volume) were stimulated with TPA for varying times, chilled in an ice-water bath, and quickly pelleted. The pellets were resuspended in ice cold 1% glutaraldehyde-0.1 M Tris-maleate buffer, pH 6.0, with 5% sucrose and fixed on ice for 15–20 min (32). The cells were washed once in 0.1 M Tris-maleate, pH 6.0, and then twice in 0.1 M Tris-maleate, pH 7.4 (both buffer solutions contained 5% sucrose). The cytochemical reactions were carried out on fixed cells in 5 ml of the cerium-containing medium for a total of 1 h at 37°C with gentle agitation (the cells were pelleted and resuspended in fresh cytochemical medium after 30 min) (32). They were subsequently processed for electron microscopy as described above, except that cacodylate buffer (0.1 M, pH 7.2) was used in place of phosphate buffer during osmication. Regular osmium was used in some preparations (i.e., not reduced with potassium ferrocyanide).

Analysis of data. All biochemical and ultrastructural observations were confirmed on at least three different preparations of cells. For qualitative ultrastructural studies (e.g., shape changes, 5'-NT staining), at least 100 cells from each preparation were examined. Virtually all of the cells in each preparation responded similarly. The time course of native and cationized ferritin uptake was determined by counting labeled vesicles directly from micrographs. Only cell profiles containing two or three lobes of the nucleus were analyzed in the native and cationized ferritin experiments in order to sample approximately the same amount of cytoplasm in each case.

Results

Kinetics of O_2^- Release by Neutrophils Stimulated with TPA or All-Trans-Retinal

Dose-Response curves. Neutrophils release O_2^- in a dose-dependent manner upon stimulation with TPA or all-trans-retinal (Fig. 1). TPA was more effective than all-trans-retinal at low concentrations; half-maximal activity with TPA occurred at ~ 1 nM (0.99 ± 0.15 nM [SD, $n = 3$]). Half-maximal activity with all-trans-retinal occurred at ~ 4 μ M (4.1 ± 0.6 μ M [SD, $n = 3$]). All of the rates shown in Fig. 1 were obtained from the steepest, linear portion of the progress curves (see below). The maximal rates of O_2^- release for guinea pig neutrophils stimulated with an optimal amount of TPA (12 nM) or all-trans-retinal (25 μ M) at 37°C were the same, 49.9 ± 10.6 (SD, $n = 6$), and 52.6 ± 5.0 (SD, $n = 6$) nmol O_2^-/min per 10^7 cells, respectively. The dose-response curve with TPA was hyperbolic (Hill coefficient = 0.94 ± 0.1 [SD, $n = 3$]), whereas that for all-trans-retinal was cooperative (Hill coefficient = 3.8 ± 0.2 [SD, $n = 3$]) (Fig. 1, inset). Neutrophils stimulated with TPA (30 ng/ml) at 4°C for up to 1 h did not release any O_2^- .

Progress curves. The progress curves for O_2^- release from guinea pig neutrophils stimulated with an optimal amount of TPA (12 nM) or all-trans-retinal (25 μ M) are shown in Fig. 2. Stimulation with TPA leads to maximal release of O_2^- after a short lag period (~ 30 s). The maximal rate of O_2^- release stimulated by TPA is linear for ~ 2 min, after which the progress curve exhibits a transition phase followed by a second linear period of O_2^- release. The second linear period of O_2^- release lasts several minutes (Fig. 2) and exhibits a rate of $\sim 50\%$ of that of the initial, maximal response (Fig. 2, inset). In contrast, the progress curve for O_2^- release from neutrophils stimulated with an optimal amount of all-trans-retinal exhibits a longer lag period (~ 4.5 min) followed by a sustained period (>10 min) of maximal O_2^- release. The progress curves for O_2^- release from human neutrophils stimulated with TPA or all-trans-retinal were similar to those presented for the guinea pig cells (data not shown).

If cells are exposed to all-trans-retinal for 3 min before the

addition of TPA, the resulting progress curve exhibited a single sustained rate of maximal O_2^- release (Fig. 2). The maximal rate of O_2^- release achieved was the same as that observed with either stimulus alone. This indicates that a single oxidase system was probably being affected by these agents. If all-trans-retinal was added to neutrophils 7 min after stimulation by TPA, the second prolonged level of O_2^- release was increased by, at best, 25% (i.e., the rate did not return to the initial maximal response).

Cells exposed simultaneously to the Ca^{++} ionophore A23187 (0.1 and 1.0 μ M) (or pretreated with ionophore for 2 min) and TPA (12 nM) exhibit progress curves for O_2^- release identical to those observed upon stimulation of cells with TPA (12 nM) alone (data not shown). This indicates that the second linear period of O_2^- release does not result from a change in the intracellular concentration of Ca^{++} , since the ionophore should maintain a constant intracellular level of that cation. The low concentration of ionophore A23187 (0.1 μ M) used here is sufficient to effect a Ca^{++} -dependent synergistic stimulation of neutrophils when combined with a suboptimal concentration of TPA (34).

Stimulus-induced morphological changes. Unstimulated neutrophils in suspension are essentially spherical with a few short projections at the cell surface. These cells contain numerous cytoplasmic granules and few vesicles (Fig. 3 A). Dramatic time-dependent morphological alterations occur in these cells after stimulation with TPA (12 nM) (Figs. 3, B–D). Within 1–2 min, cells stimulated with this agent exhibit numerous projections from the cell bodies which markedly distort their normal shape (Fig. 3 B). At later times (≥ 3 min), these projections are less dramatic, and the cells appear to be regaining their rounded appearance, but now exhibit numerous cytoplasmic vesicles and a decreased content of granules (Fig. 3, C and D). The initial TPA-induced changes (i.e., evagination) in morphology occurred in both human and guinea pig cells, but were most dramatic in human neutrophils. The later stage of TPA stimulation (formation of vesicles) appears similar in the two cell types. In contrast, human and guinea pig neutrophils stimulated with all-trans-retinal for up to 20 min did not display similar cytoplasmic vesicles nor do they exhibit an apparent diminution in the number of intracellular granules (6).

If cells are treated with all-trans-retinal (i.e., 3.1–25 μ M) for 3 min, followed by TPA (12 nM), their morphology appeared similar to that of cells exposed only to all-trans-retinal (i.e., cells displayed little vesiculation) when examined by electron microscopy (data not shown) or light microscopy (Fig. 4). Thus, pretreatment of neutrophils with all-trans-retinal blocks the vesiculation induced by TPA in a dose-dependent manner. The concentrations of all-trans-retinal that inhibited vesiculation (Fig. 4) were that which initiated O_2^- release (Fig. 1).

TPA-induced Adsorptive and Fluid-phase Endocytosis

The nature of the TPA-induced vesiculation was examined in guinea pig neutrophils using markers for the cell surface which are visible at the electron microscope level. Neutrophils exposed simultaneously to cationized ferritin and TPA (12 nM) show a time-dependent increase in the uptake of this marker, which binds electrostatically to components of the cell surface (Table I, Fig. 5 A). There was a lag period of ~ 2 –3 min before the internalization of cationized ferritin was evi-

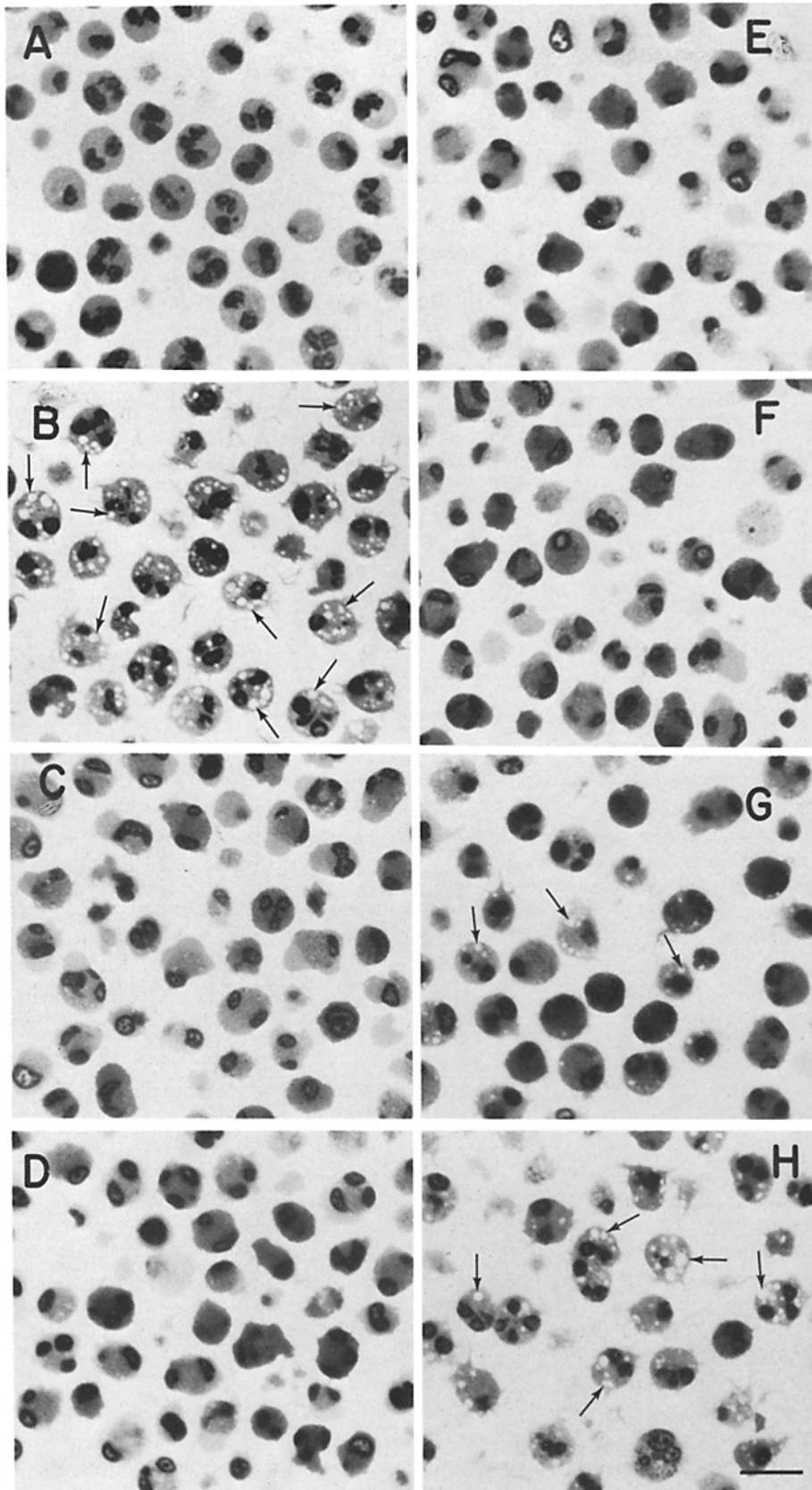


Figure 4. Light micrographs of human neutrophils illustrating the effects of various concentrations of all-trans-retinal- on TPA-induced vesiculation. Cells were incubated in suspension at 37°C for the indicated times and then fixed by the addition of glutaraldehyde. (A) Control cells incubated with DMSO (0.5% vol/vol) for 10 min. This is the maximum concentration of solvent used in these experiments. Note that the cells are generally spherical and do not have intracellular vesicles. (B) Cells incubated with TPA (12 nM) for 10 min. Note that the cells have numerous intracellular vesicles (*arrows*). (C-H) Cells incubated with all-trans-retinal for 3 min and then with TPA (12 nM) for 10 min. The concentrations for all-trans-retinal were 25 μM in C, 12.5 μM in D, 6.25 μM in E, 3.1 μM in F, 1.5 μM in G, and 0.75 μM in H. Note the virtual absence of intracellular vesicles at all-trans-retinal concentrations of 3.1 μM and higher. With all-trans-retinal at 1.5 μM , there is still an apparent reduction in TPA-induced vesicles, while at 0.75 μM the blocking effect of all-trans-retinal has diminished. Bar, 8 μM .

dent. All of the cationized ferritin observed in cells was present in vesicles. Cells incubated with cationized ferritin in the absence of TPA take up little, if any, of this marker, suggesting that binding of cationized ferritin per se does not induce

significant membrane internalization during the time course of these experiments (up to 5 min) (Fig. 5 B).

Neutrophils simultaneously exposed to native ferritin and TPA also show a time-dependent uptake of native ferritin in

Table I. Time Course of TPA-induced Internalization of Cationic Ferritin by Guinea Pig Neutrophils

	TPA (12 nM)					No TPA	
	1 min*	2 min	3 min	4 min	5 min	1 min	5 min
Exp. 1	7.2 ± 2.3‡	18.0 ± 4.1	32.0 ± 8.5	43.3 ± 3.4	45.9 ± 7.6	4.2 ± 2.7	7.6 ± 3.5
Exp. 2	5.3 ± 1.6	11.6 ± 4.5	24.9 ± 3.7	30.1 ± 6.4	36.3 ± 5.8	3.7 ± 1.9	5.7 ± 2.4
Exp. 3	6.4 ± 3.1	15.6 ± 7.3	39.0 ± 6.8	44.6 ± 7.4	52.1 ± 8.3	5.6 ± 3.1	8.7 ± 2.9

* Minutes of incubation with cationized ferritin before fixation.

‡ Average number (± SD) of cationized ferritin-containing vesicles per cell profile; micrographs of 10 cell profiles examined for each time point. See Materials and Methods for details.

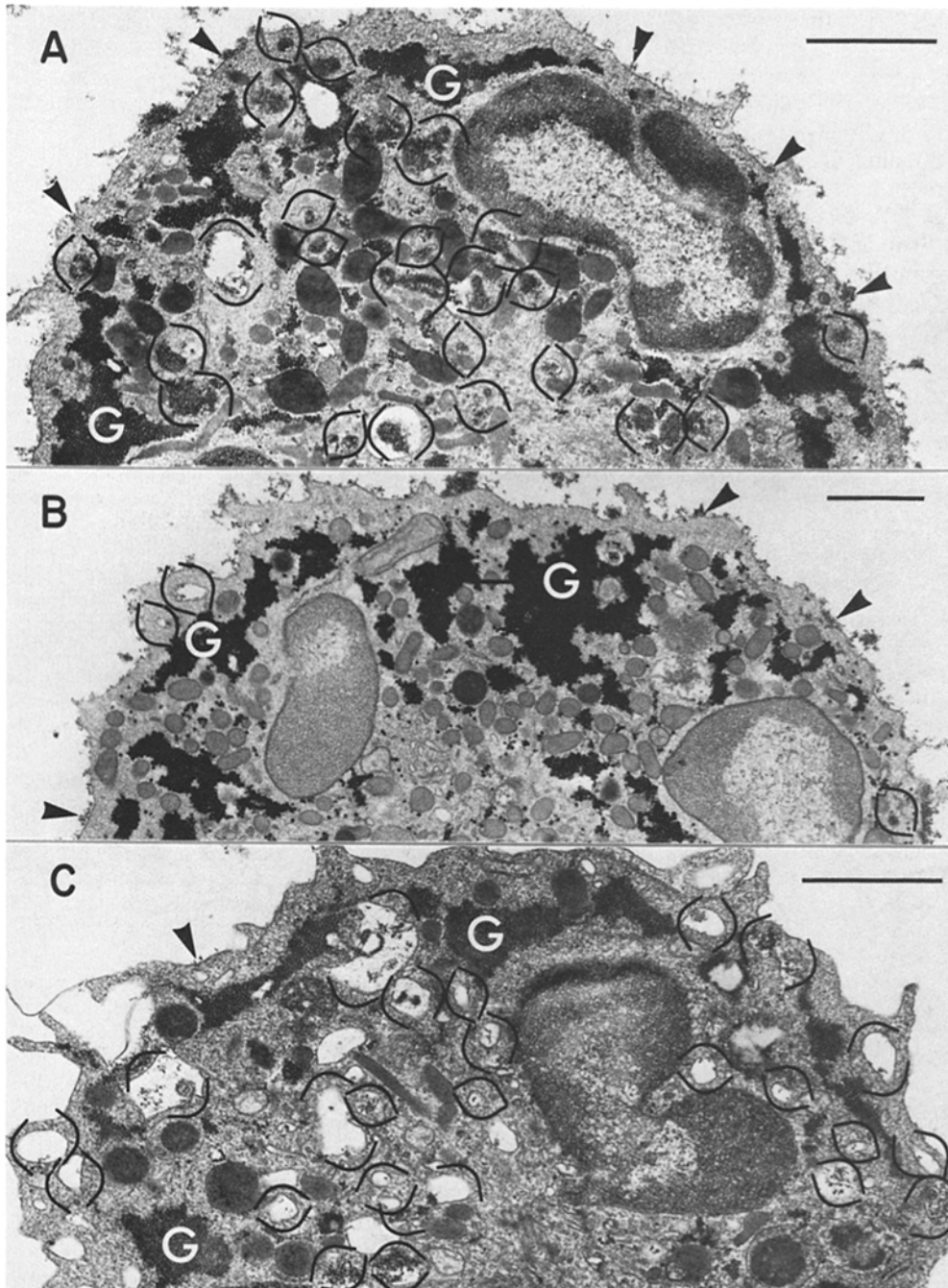


Figure 5. (A) Electron micrograph of a guinea pig neutrophil which was incubated in suspension with 12 nM TPA and 0.11 mg/ml cationized ferritin at 37°C for 5 min before the addition of fixative. The cell has numerous vesicles of varying sizes which are positively labeled with cationized ferritin (denoted with brackets). The cell surface is also positively labeled with this marker (arrowheads). While some cationized ferritin-positive vesicles are near the cell periphery, many of these vesicles are nearer the cytocenter at this time. Glycogen deposits (G) are also evident in this cell profile. (B) Electron micrograph of guinea pig neutrophil which was incubated in suspension with 0.11 mg/ml cationized ferritin alone at 37°C for 5 min before the addition of fixative. This cell has few intracellular vesicles. There are some cationized ferritin-positive vesicles (denoted with brackets) which are restricted to the periphery of the cell. The cell surface is also positively labeled with cationized ferritin (arrowheads). Glycogen (G) deposits are evident. (C) Electron micrograph of a guinea pig neutrophil which was incubated in suspension with 12 nM TPA and native ferritin (2.0 mg/ml) at 37°C for 5 min before the addition of fixative. Under these conditions, there is little if any labeling of the cell surface (arrowhead); however, there are numerous TPA-induced vesicles which are positively labeled with native ferritin (denoted with brackets). Glycogen (G) deposits are evident. Bars, 1 μM.

vesicles (Fig. 5 C). As was the case with cationized ferritin, there is a lag period of ~ 2 min before native ferritin appears in intracellular vesicles. No native ferritin was observed bound to the plasma membrane. Cells incubated with native ferritin in the absence of TPA take up little, if any, of this marker (results not shown), again suggesting that the marker itself does not induce membrane internalization during the time course of these experiments (up to 5 min). The amount of native ferritin usually appeared less than the amount of cationized ferritin per vesicle, even though the concentration of native ferritin added was ~ 20 -fold greater than that of cationized ferritin. This is consistent with the inability of native ferritin to bind to the cell surface. It should be noted that both native and cationized ferritin remained in the TPA-induced vesicles of neutrophils and that there was no apparent difference in the intracellular fate of these markers under the conditions used (at least up to 5 min). This point is of significance since studies using other cell types have shown that native and cationized ferritin are processed differently and can be delivered to different intracellular sites during endocytosis in the absence of TPA (16, 17). These observations were made over periods of 15 to 60 min (16) or 5 to 45 min (17) in contrast to our own (≤ 5 min).

When cells were stimulated with TPA, fixed, and then incubated with cationized ferritin, there is little intracellular labeling, even though the marker binds to glutaraldehyde-fixed membranes (18) (Fig. 6). This result suggests that the majority of the TPA-induced vesicles are closed, at least to the extent that molecules the size of cationized ferritin are excluded in fixed cells.

TPA-induced Internalization of the Cell Surface Enzyme 5'-Nucleotidase

The cell surface enzyme 5'-NT was localized cytochemically in unstimulated and TPA-stimulated guinea pig neutrophils with AMP as the substrate. Reaction product (cerium phosphate) was almost always present on the external surface of the plasmalemma in unstimulated cells, with only occasional staining in small vesicles (Fig. 7 A). After stimulation with TPA, there is an increase in intracellular staining for 5'-NT. Reaction product was observed on the inner face of the TPA-induced vesicles as well as the cell surface (Fig. 7 B). Staining of internal membranes was not observed unless the cells were greatly permeabilized (see Materials and Methods) during the cytochemical reaction. This again indicates that these vesicles are sealed in fixed cells, at least to the extent that they exclude either the substrate (AMP) (molecular weight, 347), cerium ions (atomic wt = 140), or both.

Discussion

The ability of retinoids to stimulate O_2^- release from neutrophils is exceptional in that these substances generally inhibit rather than mimic an effect of phorbol esters on cells (e.g., 20, 39, 41). Protein kinase C is the cellular receptor for TPA (11, 29). Diglyceride, formed by a phospholipase C-catalyzed hydrolysis of phosphoinositides, is the physiological activator of this kinase (11). Certain phorbol esters can substitute for diglyceride in activating protein kinase C (11). We have recently reported that all-trans-retinal (25 μ M) stimulates phospholipase C activity in neutrophils (25). Although



Figure 6. Electron micrograph of a guinea pig neutrophil which was stimulated in suspension with 12 nM TPA for 5 min at 37°C before fixation. The fixed cell was then labeled with cationized ferritin (1.1 mg/ml). Under these conditions cationized ferritin binds to membranes which are in contact with the extracellular medium. The plasma membrane has uniform cationized ferritin labeling (single arrows) as do occasional vesicular structures at the cell periphery (double arrows). These structures are probably open to the exterior and are not true vesicles. Most vesicular structures remain unlabeled under these conditions (arrowheads). Bar, 1 μ M.

protein kinase C from some sources is reportedly inhibited by all-trans-retinal (e.g., 20, 39), the enzyme from neutrophils is largely insensitive to this substance (25). Thus, the pathway of stimulation of O_2^- release by neutrophils treated with TPA or all-trans-retinal appears to be the same in that both are likely to involve activation of protein kinase C. Addition of synthetic diglycerides to neutrophils stimulates O_2^- production and competes with phorbol esters for specific binding sites on these cells (13, 30). All-trans-retinal does not initiate degranulation in neutrophils (6), as does TPA (43),

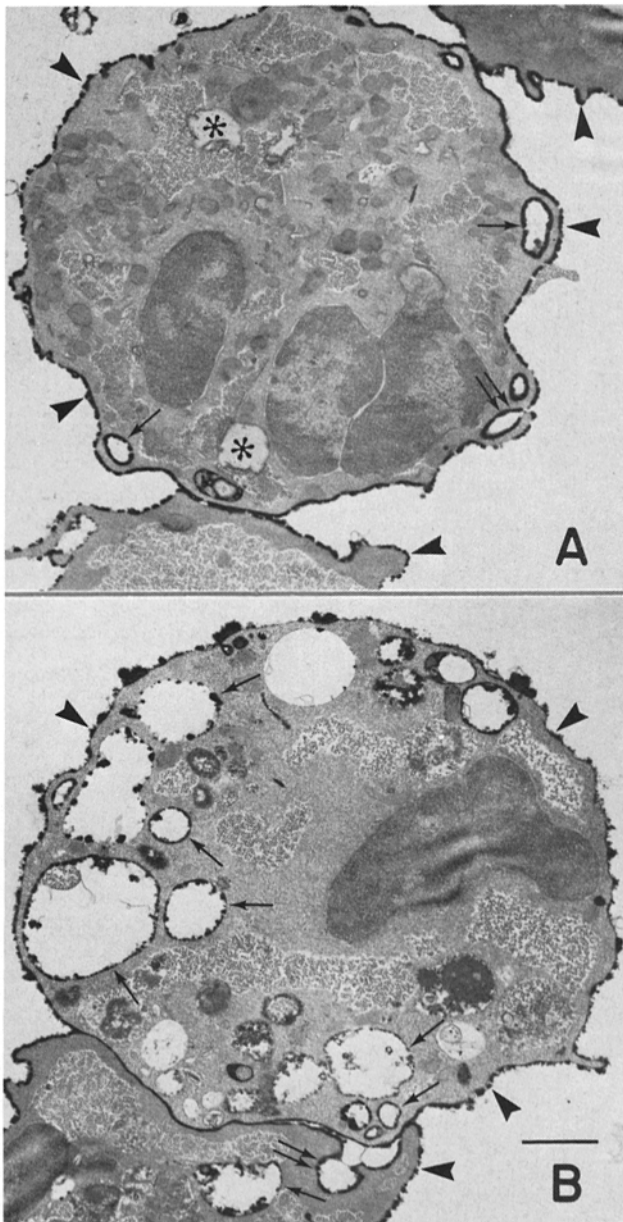


Figure 7. (A) Electron micrograph of unstimulated guinea pig neutrophils that were incubated for the cytochemical detection of ecto-5'-nucleotidase activity. Reaction product is restricted to the cell surface (arrowheads) and a few small vesicular structures at the periphery (arrow). These positive vesicular structures may be continuous with the cell surface (double arrows) and not true intracellular vesicles. Lipid droplets are also indicated (*). (B) Electron micrograph of guinea pig neutrophils that were stimulated with TPA (12 nM) for 5 min at 37°C and then fixed. The cell was subsequently incubated for the cytochemical detection of ecto-5'-nucleotidase activity. Reaction product is present on the cell surface (arrowheads) as well as on TPA-induced vesicles (arrows). Continuity of the cell surface and invaginating surface membrane is also observed (double arrows). Bar, 1 μ M.

and blocks the formation of TPA-induced intracellular vesicles in these cells (Fig. 4). All-trans-retinal appears, therefore, to have secondary inhibitory effects on neutrophils subsequent to the activation of protein kinase C and stimulation of O_2^- release.

Experiments were carried out with the guinea pig neutrophil to test whether the intracellular vesicles observed in TPA-treated cells were derived, at least in part, from internalized plasma membrane. Our results show that TPA-treated neutrophils do internalize plasma membrane by both enhanced adsorptive endocytosis and fluid-phase endocytosis, as demonstrated by the stimulated uptake of cationized (Table I, Fig. 5 A) and native (Fig. 5 C) ferritin, respectively. In addition, we have shown cytochemically that 5'-NT, which in unstimulated guinea pig neutrophils is known to be restricted to the cell surface (14, 32), is present in TPA-induced vesicles (Fig. 7). Thus, it appears that while these vesicles may be enlarged by fusion with granules they are derived, in part, from plasma membrane. TPA induces the internalization of C3b receptors on human neutrophils (12) and increases the rate of fluid-phase pinocytosis in mouse macrophages (38). The data reported here are consistent with those reports. We have previously shown that this vesiculation is also induced by other phorbol esters which activate protein kinase C and can be blocked by antagonists of Ca^{++} -binding proteins (35).

Cells stimulated with TPA exhibit two distinct rates of O_2^- release, an initial rapid rate followed by a prolonged, slower rate. With all-trans-retinal as the stimulatory agent, the cells exhibit a maximal rate of O_2^- release which lasts for several minutes (Fig. 2). A possible explanation which may account for these differences in the progress curves for O_2^- release with these stimuli is suggested by the morphological appearance of the cells after stimulation. It is known from biochemical (15) and cytochemical (5, 9) experiments that the O_2^-/H_2O_2 -producing oxidase system is a component of the neutrophil plasma membrane. It may be that the vesiculation observed after stimulation with TPA results in the removal of a fraction of the oxidase molecules from the cell surface. If many of the vesicles are sealed, as suggested by the present cytochemical results, one would expect to see a decrease in the amount of O_2^- released into the medium. These vesicles have been shown to stain positively for both O_2^- and H_2O_2 (5, 7, 9, 10). The ability to stain for H_2O_2 in these structures suggests that O_2^- dismutates to H_2O_2 in these vesicles rather than being released into the medium. The time course for the uptake of the cytochemical markers cationized ferritin (Table I) and native ferritin closely parallels the time at which cells cease to exhibit the maximal phase of O_2^- release (Fig. 2). This explanation is further supported by the single sustained rate of O_2^- release observed in cells stimulated with all-trans-retinal (Fig. 2). These cells do not become vesiculated even after prolonged exposure to this stimulus (6). In addition, cells treated with all-trans-retinal before exposure to TPA did not vesiculate (Fig. 4), and they exhibited only a single linear rate of O_2^- release (Fig. 2). Alternatively, both the vesiculation and diminution in the rate of O_2^- release might not be causally related but result from another independent event. If this latter explanation is correct, all-trans-retinal would therefore be capable of blocking or counteracting that event.

This study shows that TPA induces dramatic morphological changes in neutrophils. Two distinct steps may be envisaged: an initial rapid shape change in which cytoplasmic projections emanate from the cell body and a later event in which the cells return to a more rounded shape and become vesiculated. These morphological changes may be somewhat analogous to the formation of pseudopods and phagosomes

during phagocytosis. Concomitant with these morphological changes is the biphasic release of O_2^- . The intracellular vesicles are shown to be derived, at least in part, from plasmalemma and to be involved in TPA-stimulated adsorptive and fluid-phase endocytosis. All-trans-retinal blocks this vesiculation in a dose-dependent manner. These rapid shape changes may also be explained, in part, by the addition of membrane from an intracellular pool (i.e., granules) to the cell surface. TPA induces a rapid and reversible redistribution of actin and vinculin (37) in epithelial cells and the reorganization of α -actinin and vinculin in living BSC-1 cells (27). An analogous situation may pertain to neutrophils. Preliminary work shows that there is a dramatic rearrangement in the distribution of actin in neutrophils after stimulation with all-trans-retinal (33). At present, it is not known if or to what extent the stimulation of the O_2^- -generating system, shape changes, degranulation, or membrane internalization are related. The results reported here suggest that retinoids are valuable probes for dissecting these processes.

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