Release of Superoxide and Change in Morphology by Neutrophils in Response to Phorbol Esters: Antagonism by Inhibitors of Calcium-binding Proteins

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ABSTRACT The ability of phorbol derivatives to function as stimulating agents for superoxide (O_2^-) release by guinea pig neutrophils has been evaluated and compared to the known ability of each compound to activate protein kinase C. Those that activate the kinase also stimulate O_2^- release, while those that are inactive with respect to the kinase have no effect on O_2^- release. The same correlation was observed with respect to the ability of phorbol esters to induce morphological changes in neutrophils, i.e., vesiculation and reduction in granule content. Certain phenothiazines and naphthalene sulfonamides that are known antagonists of calcium-binding proteins blocked both phorbol ester–induced O_2^- release and morphological changes in these cells.

Upon stimulation, neutrophils undergo a dramatic series of biochemical and morphological changes. They display enhanced oxygen consumption that is used in the production of superoxide (O_2^-)[1] (for reviews see references 3 and 4). Concomitant with this increase in oxidative metabolism is the release of enzymes and other proteins from intracellular granules through exocytotic processes and alterations in cellular morphology (e.g., 7, 16, 23).

A number of soluble and particulate agents stimulate neutrophils to varying degrees. Particulate stimuli (e.g., opsonized zymosan particles) induce production of O_2^- and morphological alterations after binding and internalization (e.g., 14). Soluble or 'quasi'-soluble stimuli include substances that exert their effects either by partitioning into and disordering membranes (e.g., digitonin, cis-unsaturated fatty acids) (e.g. 7, 11) or by binding to specific receptors in these cells, (e.g., immune complexes, chemotactic peptides) (e.g., 8, 24). The latter group includes 4-β-phorbol 12-myristate 13-acetate (PMA) (37). The major binding site for PMA in all cells examined thus far is the Ca^{2+}-activated, phospholipid-dependent protein kinase (protein kinase C) (9, 35, 45). High levels of activity for protein kinase C are found in neutrophils (21), and specific binding of PMA to these cells has been demonstrated (20, 28, 44). We have previously shown that suboptimal concentrations of PMA and Ca^{2+}-ionophore A23187 stimulate neutrophils synergistically (39).

To characterize further the interaction of phorbol esters with neutrophils, we have compared the ability of a spectrum of phorbol derivatives to stimulate both morphological alterations and O_2^- production in these cells. Comparative studies on the ability of phorbol esters to effect morphological changes in neutrophils are lacking. In addition, the list of phorbol esters tested as stimulating agents for O_2^- release has been expanded. Phenothiazines and naphthalene sulfonamides inhibit both responses. The inhibition of O_2^- release by the drugs is reversible, in contrast to an earlier study (12). This is the first report, to our knowledge, that antagonists of Ca^{++}-binding proteins block morphological changes in neutrophils.

MATERIALS AND METHODS

Reagents: Trifluoperazine (TFP), chlorpromazine (CPZ), promethazine (PMZ), ferricytochyrome *c* (type VI), xanthine oxidase, Triton X-100, and all of the phorbol derivatives of Table I were purchased from Sigma Chemical Co., St. Louis, MO. Trifluoperazine sulfoxide (TFP-SO) and chlorpromazine

[1] *Abbreviations used in this paper*: CPZ, chlorpromazine; CPZ-SO, chlorpromazine sulfoxide; Me_2SO, dimethylsulfoxide; O_2^-, superoxide; PMA, 4-β-phorbol 12-myristate 13-acetate; PMZ, promethazine; TFP, trifluoperazine; TFP-SO, trifluoperazine sulfoxide; W_5, *N*-(6-aminohexyl)-1-naphthalene sulfonamide; W_7, *N*-(6-aminohexyl)-5-chloro-1-naphthalene sulfonamide.

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sulfoxide (CPZ-SO) were gifts from Smith Kline & French Laboratories, Philadelphia, PA. *N*-(6-aminohexyl)-5-chloro-1-naphthalene sulfonamide (W_7) and *N*-(6-aminohexyl)-1-naphthalene sulfonamide (W_5) were obtained from Rikaken Co., Ltd., Nagoya, Japan. Calmidazolium (compound No. R24571) was obtained from Janssen Pharamaceutica, Beerse, Belgium. Glutaraldehyde was obtained from Polysciences, Inc., Warrington, PA and from Alfa Products, Danvers, MA. Osmium tetroxide was purchased from Alfa Products. Tannic acid (No. 1764) was obtained from Mallinckrodt, Inc., St. Louis, MO. Epon 812 was purchased from Balzers Union, Hudson, NH. Sodium caseinate was obtained from Fisher Scientific Co., Pittsburg, PA. All other chemicals were of the highest available grade. Distilled-deionized water was supplied by a Barnstead ultrapure filtration system (Barnstead Co., Boston, MA).

Preparation of Cells: Neutrophils were harvested from the peritonea of guinea pigs (600–1,000 g) (Rockland, Inc., Gilbertville, PA) 18 h after the injection of sterile sodium caseinate as previously described (17). Cell viability as measured by trypan blue exclusion was always >93%. Cell number was determined by direct microscope counting using a hemocytometer.

O_2^- Production: O_2^- release was monitored at 37°C by the continuous spectrophotometric measurement of superoxide dismutase-inhibitable reduction of ferricytochrome *c* at 550 nm (11). The standard assay mixture (1.0 ml) consisted of Dulbecco's modified Eagle's medium that was altered (138 mM NaCl, 2.7 mM KCl, 16.2 mM Na_2HPO_4, 1.47 mM KH_2PO_4, 0.90 mM $CaCl_2$, 0.50 mM $MgCl_2$, 7.5 mM D-glucose, pH 7.35) (18) containing 0.075 mM ferricytochrome *c* and 10^6 cells/ml. The blank contained all of the components listed above plus 20 μg/ml superoxide dismutase to correct for ferricytochrome *c* reduction by agents other than O_2^-. The extinction coefficient for reduced minus oxidized cytochrome *c* at 550 nm was 20,000 M^{-1} cm^{-1} (30).

Ultrastructural Analysis: Morphological experiments were performed at a cell concentration of 1×10^6 cells/ml in 10 ml of the altered Dulbecco's modified Eagle's medium described above except ferricytochrome *c* was omitted. Cells were fixed 5 min after stimulation by rapidly adding 1.0 ml of 25% glutaraldehyde. In some preparations, the fixative was modified to contain tannic acid as described by Maupin and Pollard (29). Cells were postfixed in 2% OsO_4 in 0.1 M phosphate buffer (pH 7.3) and then embedded in Epon as a pellet. Thin sections were cut on a diamond knife and stained with uranyl acetate and lead citrate before they were examined in a Philips 200 electron microscope (Philips Electronic Instruments, Inc., Mahwah, NJ) operated at 60 kV. At least 100 cells from each preparation were examined, and all observations were confirmed on at least three different preparations of cells.

RESULTS

Ability of Different Phorbol Derivatives to Stimulate Release of O_2^-

Casein-elicited guinea pig neutrophils release O_2^- at a rate of 51.4 ± 9.9 (mean ± SD) nmol O_2^-/min per 10^7 cells (n = 24) when stimulated with an optimal concentration (30 ng/ml) of PMA. The structural specificity of the stimulus was assessed by testing various analogues of PMA (Table I). Only β-isomers with both the 12- and 13- positions in ester linkage were active. The nature of the carboxylic acid (e.g., myristate, acetate, benzoate) in these linkages was not crucial in the range examined.

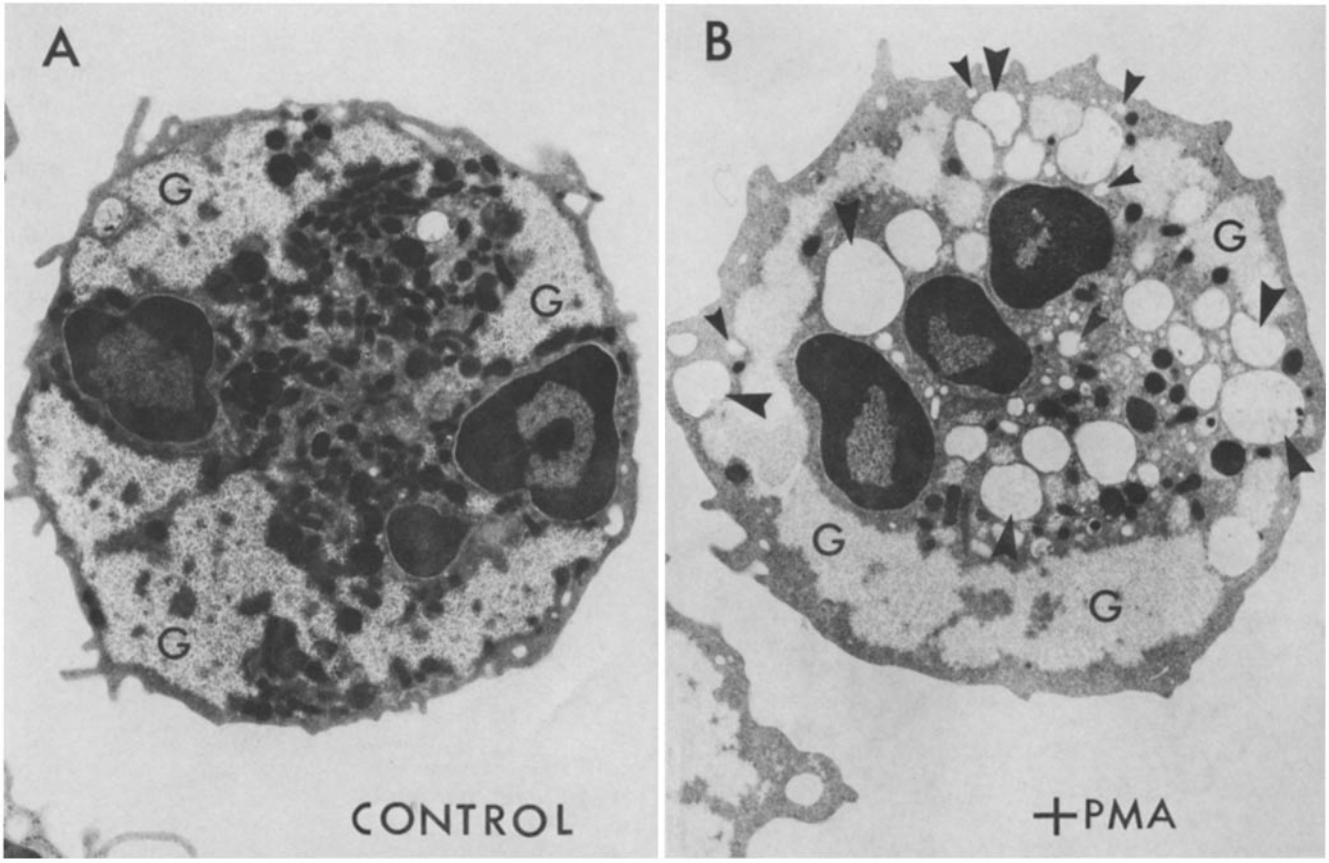

FIGURE 1 Electron micrographs of thin sections of guinea pig neutrophils in suspension . (*A*) Representative control cell that was incubated in medium for 8 min at 37°C before fixation. This cell is typical of elicited guinea pig neutrophils in that it has large accumulations of glycogen (*G*) (38) and numerous electron dense intracellular granules. Note that there are few intracellular vesicles. × 9,750. (*B*) Cell that was preincubated for 3 min in medium at 37°C and then another 5 min after addition of PMA before fixation. This cell is typical of PMA-treated cells in that there are numerous cytoplasmic vesicles (arrowheads). Most of the glycogen (*G*) appears to be retained, but there is an apparent reduction in the number of intracellular granules. × 9,700. PMA was dissolved in Me_2SO such that the final concentration of solvent was 0.25% (vol/vol) in all cases. This concentration of Me_2SO did not affect the morphology of control cells.

TABLE I. *Effects of Various Phorbol Derivatives in Stimulating O_2^- Release and Morphological Alterations in Neutrophils**

Compound	O_2^- release‡	Degree of morphological alteration§
	% activity	
4-β-Phorbol 12-myristate 13-acetate	100	++++ (8)
4-β-Phorbol 12, 13-didecanoate	55 ± 2.65 (3)	++ (3)
4-β-Phorbol 12, 13-dibenzoate	57 ± 12.12 (3)	++ (3)
4-β-Phorbol 12, 13-dibutyrate	40 ± 7.94 (3)	++ (3)
4-β-Phorbol 12-monomyristate	0 ± 0 (3)	− (3)
4-β-Phorbol 13-monomyristate	0 ± 0 (3)	− (3)
4-β-Phorbol	0 ± 0 (3)	− (3)
4-α-Phorbol 12, 13-didecanoate	0 ± 0 (3)	− (3)

* Guinea pig neutrophils were subjected to the various phorbol derivatives (30 ng/ml), and superoxide release and morphological alterations were determined as described. Phorbol derivatives were dissolved in dimethyl sulfoxide such that the final concentration of this solvent was 0.25% (vol/vol) in all cases.

‡ The data for O_2^- release are expressed as the mean ± SD relative to the rate observed with PMA. The number (*n*) of different cell preparations used is given in parentheses.

§ The degree of morphological alteration was determined on cell preparations that were fixed 5 min after addition of the phorbol derivative under study. The qualitative scale is: ++++, greatest degree of vesiculation and granule loss; ++, intermediate level of vesiculation and granule loss; −, no difference from untreated control cells with regard to vesiculation and granule number.

Ultrastructural Analysis of Neutrophils Treated with Phorbol Derivatives

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. 1*A*). Neutrophils treated in suspension with certain phorbol derivatives exhibit a dramatic increase in intracellular vesicles and a decrease in cytoplasmic granules (Fig. 1*B*). The specificity of the phorbol derivatives with respect to these changes in cellular morphology paralleled the potency of these compounds to stimulate O_2^- release (Table I). All of the observations reported here are restricted to 5 min after exposure to the stimulus; at this time, the cells had responded maximally in terms of the rate of O_2^- release (e.g., see Fig. 2*C*). Virtually all of the cells examined in each preparation responded similarly when exposed to a given stimulus.

Effects of Phenothiazines and Naphthalene Sulfonamides on O_2^- Release

Certain phenothiazines and naphthalene sulfonamides that inhibit a variety of Ca^{2+}-dependent proteins (e.g., calmodulin [46], protein kinase C [32]) were tested for their effects on O_2^- release from neutrophils stimulated with PMA. TFP or CPZ incubated with cells 3 min before stimulation inhibited O_2^- release in a cooperative manner (Hill coefficients = 3.2 and 2.1, respectively). Half-maximal inhibition occurred at ~5 μM TFP or 10 μM CPZ (Fig. 2, *A* and *B*). Addition of TFP to cells after stimulation also caused an abrupt cessation of O_2^- release (Fig. 2*C*). Other phenothiazines were tested (Table II). PMZ at 10 μM was less inhibitory than either TFP or CPZ. TFP-SO and CPZ-SO which are not antagonists of Ca^{++}-binding proteins were inactive. Incubation of cells with the naphthalene sulfonamide W_7 for 3 min before stimulation inhibited O_2^- release in a dose-dependent fashion. Half-maximal inhibition with W_7 occurred at ~20 μM. W_5 (which is known not to interfere with Ca^{++}-binding), the unchlorinated analogue of W_7, did not affect O_2^- release (Fig. 3). The inhibitory effects of TFP, CPZ, and W_7 were completely reversed by washing the cells twice in drug-free medium (Table II). The degree of inhibition with these phenothiazines and naphthalene sulfonamides was not affected by increasing the cell concentration from 1.0×10^6 to 2.5×10^6/ml. Therefore, the results obtained with both cell concentrations were analyzed together in Table II. The potent calmodulin inhibitor Calmidozolium (compound No. R24571) was found to be extremely toxic to neutrophils over the concentration range in which it inhibits calmodulin (0.25–2.0 μM).

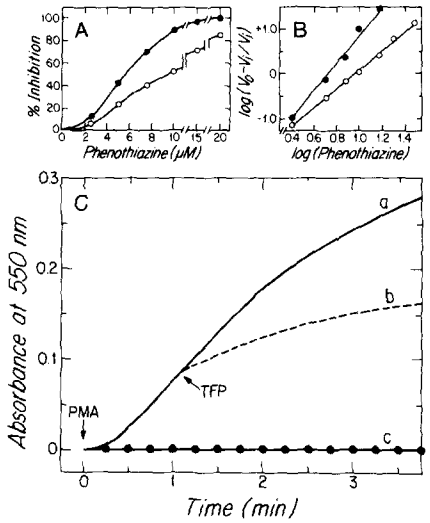

FIGURE 2 Effects of phenothiazines on superoxide release by PMA stimulated guinea pig neutrophils. (*A*) Dose-response curve for inhibition of O_2^- release by TFP (●) and CPZ (○). Cells (1×10^6/ml) were pretreated with these drugs for 3 min in the assay mixture before stimulation with PMA (30 ng/ml). The percent inhibition was calculated from the equation $(V_o - V_i/V_o) \times 100$, where V_o is the activity without inhibitor, and V_i is the activity with inhibitor. (*B*) The Hill plot for the data in *A* (TFP, ●; CPZ, ○). This form of the Hill equation is derived elsewhere by Taketa and Pogel (43). (*C*) Reaction progress curves (continuous tracings) demonstrating the effects of TFP on the O_2^--dependent reduction of ferricytochrome c by neutrophils stimulated with PMA, which was added last to initiate the reactions. Curve *a* shows the initial portion of the progress curve for 10^6 cells/ml. TFP (20 μM) inhibited O_2^- release when it was added to the assay system 3 min before stimulation (curve *c*) or to cells already stimulated (curve *b*). PMA was dissolved in Me_2SO such that the final concentration of solvent was 0.25% (vol/vol) in all cases. This concentration of Me_2SO did not affect O_2^- release.

Effects of Phenothiazines and Naphthalene Sulfonamides on the Ultrastructure of Stimulated Neutrophils

Addition of TFP (10 μM) to neutrophils 3 min before stimulation with PMA blocks the ultrastructural changes noted above. Cells so treated appear similar to unstimulated cells in that they contain few vesicles and a normal complement of granules (Fig. 4). In contrast, similar treatment of cells with 10 μM TFP-SO did not block the PMA-induced alterations (Fig. 4*B*). The naphthalene sulfonamide W_7 (50 μM) also inhibited the ultrastructural changes induced with PMA (Fig. 5*A*), whereas W_5 (50 μM) did not (Fig. 5*B*).

TABLE II. *Effects of Phenothiazines and Naphthalene Sulfonamides on O_2^- Release by Neutrophils Stimulated with PMA: Ability to Reverse the Effects by Washing the Cells*

Drug additions	% Activity	
	Without washing	With washing
None	100	108 ± 19.4
TFP (10 μM)	18.5 ± 6.4	139 ± 28
TFP-SO (10 μM)	102.3 ± 11.3	ND
CPZ (10 μM)	48.9 ± 5.9	125.3 ± 27.7
CPZ-SO (10 μM)	100.9 ± 1.2	ND
PMZ (10 μM)	79.3 ± 8.4	ND
W_7 (50 μM)	8.1 ± 5.6	115.7 ± 16.5
W_5 (50 μM)	101.5 ± 14.5	ND

Neutrophils were incubated in the standard assay mixture for 3 min at 37°C with the compounds listed before stimulation with PMA. For experiments on reversibility, 5 ml of cells (2.5×10^6 cells/ml) were suspended in Dulbecco's modified Eagle's medium and incubated with the drugs for 3 min at 37°C. The cells were then washed twice in ice-cold medium (10 ml), suspended in the assay cuvette at a concentration of 2.5×10^6 cells/ml, and stimulated with PMA. Superoxide was measured as described in Materials and Methods. Data are expressed as the mean ± SD for three to eight different preparations of cells. 100% activity is that observed in the absence of drugs. None of the drugs utilized affected cell viability under the conditions employed or inhibited O_2^- detection in an O_2^--generating system (i.e., xanthine oxidase plus purine). ND, not determined. Three pairs of analogues were compared, as well as PMZ, which was only moderately effective. One member of each pair serves as a control, i.e., is known not to interfere with Ca^{++}-binding proteins.

Treatment of cells with 10 μM CPZ or PMZ resulted in partially inhibiting the morphological alterations induced by PMA, while treatment with CPZ-SO was ineffective in this regard. None of these drugs appeared to affect the ultrastructure of unstimulated cells in suspension (data not shown).

In agreement with these ultrastructural observations, phe-

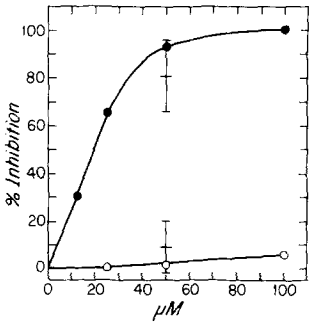

FIGURE 3 Effects of naphthalene sulfonamides on O_2^- release from PMA-stimulated guinea pig neutrophils. Neutrophils (10^6 cells/ml) were incubated with the indicated concentrations of W_7 (●) or W_5 (○) in the standard assay mixture for 3 min at 37°C before stimulation of O_2^- release with PMA. The mean inhibition of O_2^- release ± SD effected by these compounds at 50 μM is given by the error bars (n = 3). The dose response curves are those of a single representative experiment. Compounds W_7 and W_5 were dissolved in Me_2SO such that the final concentration of this solvent in the assay was 0.25% (vol/vol) in all cases. Thus, the total concentration of Me_2SO was 0.5% (drug plus PMA). This concentration of solvent did not affect O_2^- release.

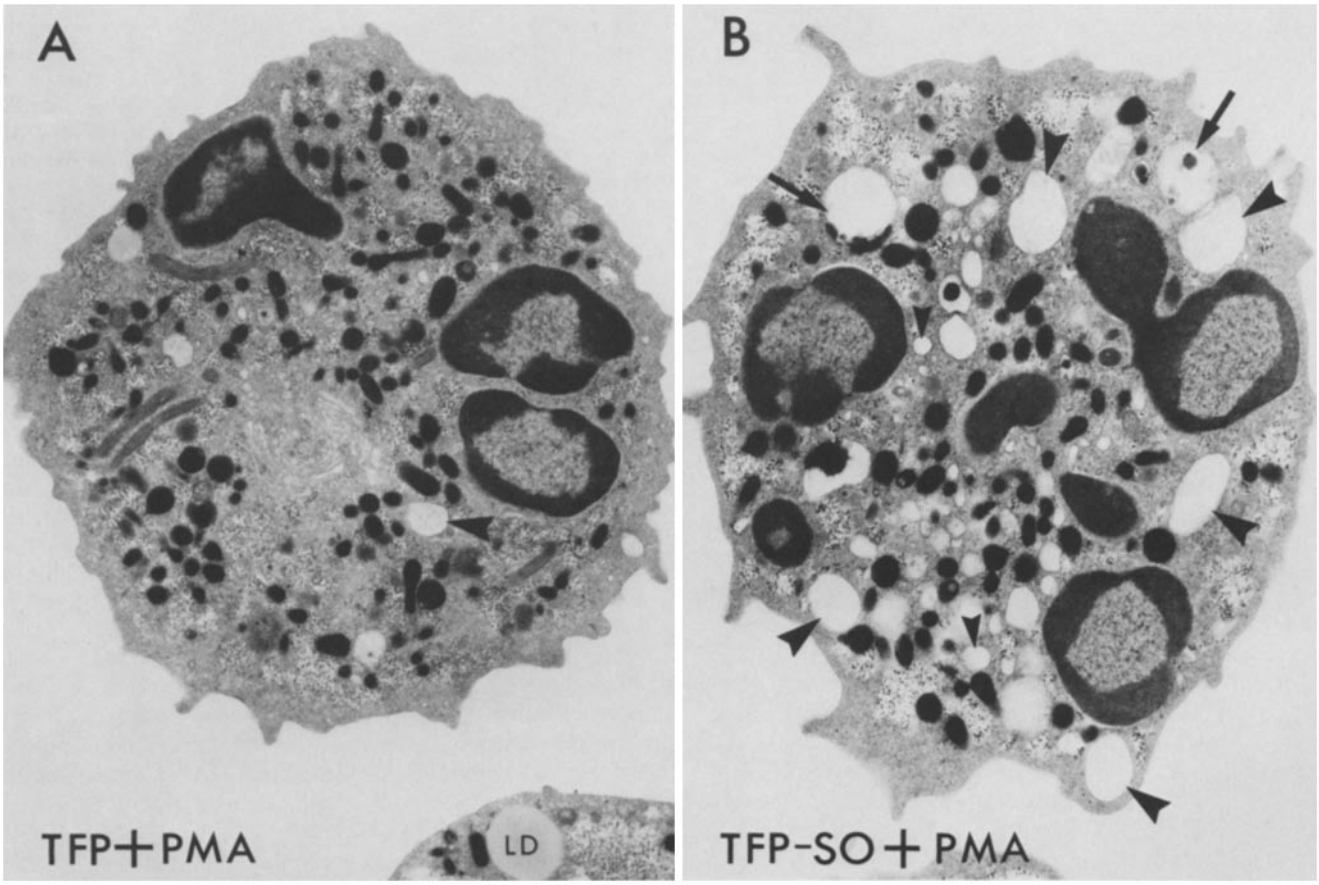

FIGURE 4 Effects of TFP and TFP-SO on PMA-induced ultrastructural changes in guinea pig neutrophils. (*A*) Cell that was incubated in suspension for 3 min in medium containing 10 μM TFP at 37°C and then another 5 min after addition of PMA before fixation. Note that the cell has very few cytoplasmic vesicles (arrowheads). An adjacent cell has a prominent lipid droplet (*LD*). × 10,200. (*B*) Cell that was preincubated for 3 min in medium containing 10 μM TFP-SO at 37°C and then another 5 min after addition of PMA before fixation. Note the presence of numerous cytoplasmic vesicles (arrowheads). Some vesicles contain electron dense material (arrows), which is presumed to be part of the contents of granules that have discharged into these vesicles. × 10,500.

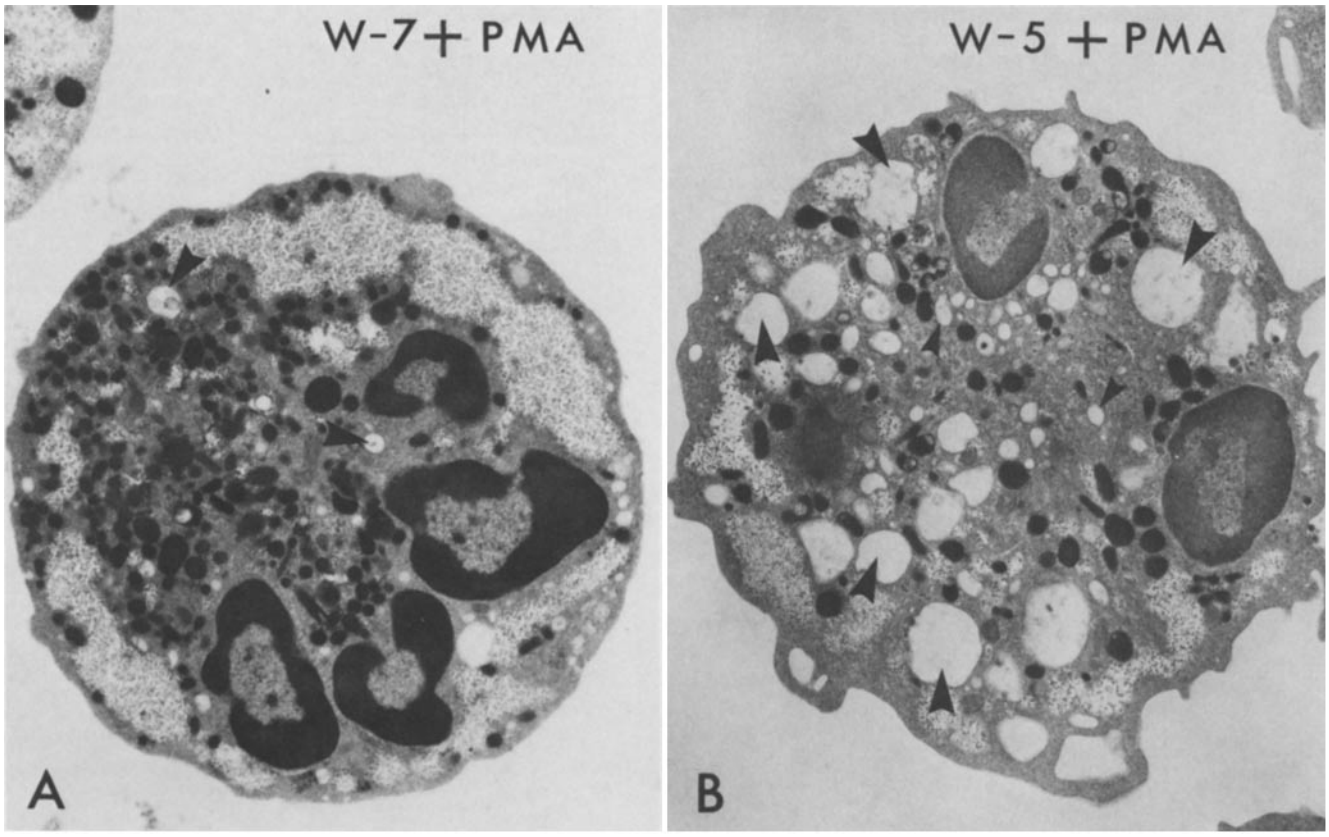

FIGURE 5 Effects of naphthalene sulfonamides on PMA-induced ultrastructural changes in guinea pig neutrophils. (*A*) Cell that was incubated in suspension for 3 min in medium containing 50 μM W_7 at 37°C and then another 5 min after addition of PMA before fixation. Note that the cell is quite round in appearance, has numerous granules, and has few cytoplasmic vesicles (arrowheads). × 10,000. (*B*) Cell that was preincubated for 3 min in medium containing 50 μM W_5 at 37°C and then another 5 min after addition of PMA before fixation. Note the presence of numerous cytoplasmic vesicles (arrowheads) and a reduction in the number of granules. × 10,400.

nothiazines inhibited the release of lysozyme from guinea pig neutrophils stimulated with PMA with the following order of potency: TFP>CPZ>PMZ> >TFP-SO (data not shown). The ability of TFP and W_7 to inhibit release of granule enzymes from neutrophils has been noted previously (e.g., 1, 33, 41, 42, 47).

DISCUSSION

Certain phorbol esters can intercalate into cellular membranes and directly activate protein kinase C (e.g., 9, 45). In the present study, we show that the same phorbol esters reported to activate protein kinase C stimulate release of O_2^- and changes in morphology of guinea pig neutrophils, while those that do not activate kinase C did not affect neutrophils in the ways indicated (Table I). A role for protein kinase C in neutrophil stimulation has been intimated previously on the basis of stimulation of these cells by PMA and/or diacylglycerol (e.g., 15, 19, 31). Diacylglycerol is thought to be the physiological activator of protein kinase C (26). Stimulation of neutrophils with PMA produces rapid alterations in the phosphoproteins of these cells, the identity of which remain to be determined (e.g., 2, 40, 47).

The intracellular vesicles formed upon stimulation of neutrophils with phorbol esters are of interest. These structures were first reported in human neutrophils stimulated with PMA (37) and were then shown to occur in neutrophils from guinea pigs and mice (5) and in murine macrophages (6). Cytochemical studies on these cells have shown that these vesicles and the plasmalemma stain for hydrogen peroxide (5, 6). It was speculated that these vesicles were derived, at least in part, from the plasmalemma and may be somewhat analogous to phagosomes.

As noted above, certain phenothiazines (i.e., TFP, CPZ) and naphthalene sulfonamides (i.e., W_7) inhibit a variety of Ca^{2+}-binding proteins including protein kinase C (e.g. 21, 32) and calmodulin (e.g., 46). Calmodulin modulates many of the second messenger functions of Ca^{2+} by interacting with various enzymes and certain elements of the cytoskeleton (for reviews, see references 10 and 36). Both protein kinase C and calmodulin are present in neutrophils (21, 25). The inhibitory effects of phenothiazines and naphthalene sulfonamides described here exhibit properties very similar to those reported for purified calmodulin-dependent enzymes. One may point in this regard to the order of effectiveness of the phenothiazines (i.e., TFP>CPZ>PMZ> > >TFP-SO, CPZ-SO) (Table II) (34, 46) and naphthalene sulfonamides (W_7> >W_5) (Table II and Fig. 3) (22), the reversibility of the effects of these drugs (Table II) (46), and the Hill coefficient for TFP (n_H = 3.2, Fig. 2*B*) (46). Similar data on reversibility and cooperativity of these drugs with purified protein kinase C are not presently available. The concentrations of TFP, CPZ, and W_7 required for half-maximal inhibition of O_2^- release (i.e., ~5, 10, and 20 μM, respectively) (Figs. 2*A* and 3) are strikingly similar to

those observed for calmodulin-dependent processes (for review, see references 42 and 46) and considerably less than those required to inhibit protein kinase C (13, 21, 27). We have recently reported similar results with these inhibitors using human neutrophils stimulated with arachidonate (15). However, caution is necessary when comparing kinetic constants obtained with purified proteins to those obtained for cellular phenomena since parameters may be affected by differences in protein or lipid concentrations (e.g., 32), assay procedures (e.g., 34), and synergistic effects between the relevant components (39). A previous study on neutrophils obtained from guinea pig peritonea reported that inhibition of O_2^- release by phenothiazines was not reversible (12). The reason for the discrepancy with our results is unknown.

Addition of purified calmodulin to membrane fragments from stimulated neutrophils enhanced the activity of the O_2^--producing oxidase; the ability of phenothiazines to inhibit this oxidase in vitro was also reported (25). White et al. (47) have examined the inhibitory effects of TFP on PMA-induced degranulation and protein phosphorylation patterns. Interestingly, their dose response curve for inhibition of degranulation and for phosphorylation of a 50,000-mol-wt protein is essentially identical to that which we present for inhibition of O_2^- release (Fig. 2*A*).

Thus, the abilities of several phorbol derivatives in stimulating neutrophils to release O_2^- and undergo morphological changes has been compared, and the effectiveness of several phenothiazines and naphthalene sulfonamides in blocking these processes have been determined. Additional work will be required to elucidate the precise mechanisms by which these compounds affect neutrophil function.

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