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Inhibition of neutrophil activation by *p*-bromophenacyl bromide and its effects on phospholipase A_2

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1 In an effort to elucidate the nature of the inhibitory effects of *p*-bromophenacyl bromide (pBPB) on neutrophil stimulation, we have examined its effects on several stages of stimulus-response coupling.

2 Pretreatment of rat neutrophils with pBPB resulted in a dose- and time-dependent irreversible inhibition of both N-formylmethionyl-leucylphenylalanine (f Met-Leu-Phe)-induced lysosomal enzyme release and change in transmembrane potential.

3 Inhibition of the biological responses to the chemotactic peptide f Met-Leu-Phe was not due to receptor inactivation since f Met-Leu-[^{3}H]-Phe binding to the formyl peptide receptor was not significantly altered by pBPB pretreatment.

4 Inhibition by pBPB of phorbol myristate acetate (PMA)-induced changes in transmembrane potential and the generation of superoxide (O_2^-) was also observed.

5 pBPB treatment appeared to inhibit activation of the NADPH oxidase without a direct effect on the oxidase itself.

6 This inhibitory effect was not accompanied by cell death or decrease in cellular titratable sulphydryl groups (at least at doses $< 20\,\mu$M).

7 There was, however, significant inhibition of a membranous fraction of f Met-Leu-Phe-induced phospholipase A_2 activity by pretreatment with $10\,\mu$M pBPB, although total cellular phospholipase A_2 was only minimally ($< 20\%$ inhibition) affected.

8 These data would indicate that pBPB inhibits an early event associated with stimulus-response coupling in rat polymorphonuclear leukocytes (i.e. change in transmembrane potential). The inhibitory effects of pBPB may be secondary to the inhibition of a critical membranous fraction of cell bound phospholipase A_2 activity or its activation, necessary for the initiation of cell activation.

Introduction

Stimulation of polymorphonuclear leukocytes by chemotactic or phagocytic stimuli results in a series of reactions that eventually terminate in chemotaxis and/or phagocytosis with associated generation of oxygen radicals and release of granule bound enzymes such as lysozyme and β-glucuronidase (Weissman *et al.*, 1980). Additionally, oxidation products of arachidonic acid via the cyclo-oxygenase, and lipoxygenase pathways are formed which can in turn modulate the cellular responses (Goetzl, 1980).

The mechanisms involved in the process of stimulus-secretion coupling have been partially elucidated (Weissman *et al.*, 1980). Among these, depolarization of the transmembrane potential as assessed by an increase in the fluorescence intensity of the optical probe of membrane potential 3-3′-dipropylthiadicarbocyanine iodide, [diS-C_3-(5)], is detectable within seconds of exposure of neutrophils to different stimuli (Sklar *et al.*, 1980). This event can be correlated to the functional response phase of neutrophil stimulation (Duque *et al.*, 1983). Moreover, using protease inhibitors, such as L-1-tosylamide-2-phenylethyl chloromethyl ketone (TPCK), we have recently shown that a chymotrypsin-like activity is required for cell stimulation including transmembrane potential changes. This inhibition can be overcome by exposure of TPCK-treated neutrophils to the calcium ionophore A23187 in the presence of > 1.5 mM Ca^{2+}, suggesting that the TPCK inhibitable activity precedes a Ca^{2+} requiring step (Duque *et al.*, 1983). This

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esterase activity may be related to the Ca^{2+} dependent (Derksen & Cohen, 1975) activation of a cell bound phospholipase A_2 (phosphatide 2-acyl-hydrolase, EC 3.1.1.4), which has been identified in plasma membranes of neutrophils (Victor *et al.*, 1981). The subsequent release of unesterified arachidonic acid from cellular phospholipids by the activated phospholipase A_2 is considered to be an initial requirement for the synthesis and secretion of prostaglandins and other arachidonic acid metabolites (Kuehl & Egar, 1980). Arachidonic acid can also be released by the combined action of phospholipase C and diacylglycerol lipase (Bell *et al.*, 1979).

Treatment of neutrophils with *p*-bromophenacyl bromide (pBPB), an 'active site directed' inhibitor of phospholipase A_2 by alkylation of a histidine residue, has been reported to inhibit superoxide generation and enzyme release (Smolen & Weissman, 1980). Although there is currently no evidence to indicate that the neutrophil enzyme contains a histidine residue at its active site, a likely target for this inhibition is the membrane bound phospholipase A_2 (Bormann *et al.*, 1984). Accordingly, we have investigated the effects of *p*-bromophenacyl bromide on several physiological responses associated with stimulus-response coupling in rat neutrophils, and attempted to demonstrate the extent of specificity of this reagent on the observed inhibition.

Methods

Neutrophils

Healthy, adult, male, Long-Evans rats (Charles River Laboratory) (250 to 350 g) were used. Neutrophils were elicited from the peritoneal cavity by use of a solution of 1% oyster glycogen (Becker, 1972). Cell suspensions were >95% neutrophils as assessed by toluidine blue staining. All assays described below were performed at least in duplicate, although most were done in triplicate. Data in the various tables and figures are typical of 3–6 experiments performed on as many different cell preparations.

Superoxide production and enzyme release

Superoxide was measured as the superoxide dismutase inhibitable reduction of cytochrome c as described by McCord & Fridovich (1969). Results are expressed as nmol O_2^- 10^6 cells^{-1} 60 min^{-1}. Calculation of the actual amount of nmol of cytochrome c reduced was made utilizing an extinction coefficient of 21.1 cm^{-1}mM^{-1} for cytochrome c (reduced minus oxidized) at 550 nm. Lysozyme, β-glucuronidase and lactic dehydrogenase activities were measured as described previously (Becker *et al.*, 1977). Results were expressed as percentage of enzyme activity present in lysates of cells treated with 0.1% Triton X-100.

Preparation of subcellular particles and NADPH oxidase assay

In selected experiments the NADPH oxidase activity was determined in the 27,000 *g* particle fraction of sonicated neutrophils as previously described (McPhail *et al.*, 1976). Briefly, rat neutrophils were divided into two aliquots. One was treated with 10 μM pBPB from (10^{-2} M stock solutions made in absolute ethanol) for 10 min at room temperature, washed, and followed by stimulation with phorbol myristate acetate (PMA, 200 mg ml^{-1}) for 10 min at 37°C. A second group was treated with PMA alone. Following sonication and low speed centrifugation to remove intact cells and nuclei, the 27,000 *g* pellet was obtained and designated as subcellular particles. Subcellular particles from PMA-treated cells were also prepared and treated with pBPB (10 μM) for 10 min at room temperature before assay for NADPH oxidase. The ability of 50 μl of each preparation to produce superoxide dismutase-inhibitable O_2^- after incubation for 15 min at room temperature in the presence of NADPH was determined. Particles from non-PMA-stimulated cells routinely produced less than 2 nmol O_2^- mg^{-1} protein.

Membrane potential changes

Membrane potential changes were measured as previously described (Duque *et al.*, 1983). Neutrophils (2×10^6) were equilibrated for 3–4 min with 2×10^{-6} M diS-C_3-(5), in a Varian SF-330 spectrofluorometer at 37°C, with constant stirring. The excitation wavelength was 622 nm and the emission wavelength, 665 nm, with bandwidths of 10 nm. Final concentrations of dimethyl sulphoxide (Me_2SO) and ethanol did not exceed 0.1%. Stimuli were added after equilibration and the fluorescence monitored for 6–7 min using a chart recorder. Data are expressed as the maximal change in fluorescence (ΔF). Exposure of cell suspensions to the dye never exceeded 10 min. The resting membrane potential was calculated by the 'null point' method (Laris *et al.*, 1976), based on the external potassium concentration $[K^+]_o$ at which there is no change in fluorescence intensity upon the addition of valinomycin (2×10^{-6} M) in the presence and absence of pBPB (5 μM for 10 min at 37°C).

diS-C_3-(5) has been reported to exert toxic effects on Ehrlich ascites tumour cells by rapidly depleting them of ATP in glucose-free medium (Smith *et al.*, 1981). Accordingly, we measured ATP in suspensions of neutrophils as assessed by the chemiluminescent response in the luciferrin-luciferase system (Stanley & Williams, 1969). Under the conditions of our experi-

ments (i.e. buffer with 5 mM glucose), we detected ATP values that were within 10% of control values up to 30 min after exposing the cells to the dye. Cell death as assessed by inability to exclude Trypan blue was minimal (>90% viability) under these conditions of incubation.

Since this carbocyanine dye is also expected to partition in mitochondrial membranes, the observed net change in fluorescence may have some contribution from changes in potential across mitochondrial membranes. However, this contribution, if any, is minimal because PMA-induced changes in diS-C_3-(5) fluorescence is not significantly affected by anaerobic conditions (Whitin *et al.*, 1980) which would have dramatic impact on mitochrondrial potential.

Sulphydryl group titration

Titration of free sulphydryl groups was accomplished using 5,5'-dithiobis(2-nitrobenzoic acid) according to Deakin *et al.* (1963). Cells with or without pBPB pretreatment were reacted with this reagent (100 μM) for 30 min at room temperature. The absorbence at 412 nm was determined and used to quantitate the amount of free SH groups (Deakin *et al.*, 1963).

Analysis of f Met-Leu-[^{3}H]-Phe binding

The following protocol was used to assess the effect of pBPB on the time course of N-formylmethionyl-leucyl-[^{3}H]-phenylalanine (f Met-Leu-[^{3}H]-Phe) binding. Neutrophils (2.0×10^7 cells ml^{-1}) were preincubated at 37°C for 15 min in buffer before the start of each experiment. The cells were divided into two tubes, one of which was treated with pBPB (10 μM final concentration): 350 μl aliquots were then removed from each tube at various times, placed into 12 × 75 mm glass test tubes, diluted with 4 ml of ice cold buffer (no dextrose) and centrifuged at 900 *g* for 8 min. The supernatants were discarded, and the cell pellets were resuspended in 350 μl of buffer, to which 10 mM 2-deoxy-D-glucose and 109 mM NaN_3 had been added to minimize receptor internalization during the binding studies (Marasco *et al.*, 1983). Aliquots of cells (100 μl) were then incubated for 25 min at 24°C with 20 nM f Met-Leu-[^{3}H]-Phe in siliconized glass 12 × 75 mm test tubes. After these preliminary studies, binding studies were routinely performed by incubating 10 μM pBPB with cells for 15 min. The cells were washed, resuspended to 2×10^7 cells ml^{-1} and analyzed for f Met-Leu-[^{3}H]-Phe binding under equilibrium conditions as previously described (Marasco *et al.*, 1983). Only specific binding is described in this paper. The cells were harvested by a glass fiber vacuum filtration method and analyzed for cell bound radioactivity. All data points represent the means of triplicate determinations.

Phospholipase A_2 assay

Phospholipase A_2 assay was performed according to Franson *et al.* (1974) with slight modification. L-α-1-Palmitoyl-2-[palmitoyl-9,10-^{3}H]-phosphatidylcholine (30–60 Ci mmol^{-1}) was used as substrate. Rat peritoneal neutrophils (10^8) were treated with 10^{-5} M pBPB at 37°C for 15 min. Control cells were similarly incubated in the absence of pBPB. Cells were then washed once with 0.34 M sucrose in 10 mM Tris HCl pH 7.5, and sonicated on ice at 30 W in three bursts of 10 s each. Cell breakage was better than 90% as monitored by the microscope. After centrifugation at 600 *g* for 20 min, 4°C, the supernatant was further spun at 27,000 *g* for 20 min at 4°C. The supernatant and pellet were collected and used for assay as the soluble and particulate or membranous fractions (subcellular particles) respectively. Fractions of these samples (equivalent to 2×10^6 cells) were incubated with 0.2 μCi substrate diluted with 5 nmol of cold phosphatidylcholine in a final volume of 0.5 ml. The buffer contained 10 mM $CaCl_2$ and 100 nM Tris HCl pH 8.0. After 60 min at 37°C, the entire mixture was extracted with $CHCl_3$-CH_3OH containing 0.5 mg palmitic acid as previously described (Blight & Dyer, 1959). The extracts were dried under vacuum with a Speed Vac (Savant Instruments) concentrator, resuspended in 200 μl of methanol and analysed by reverse phase h.p.l.c. H.p.l.c. was performed using a Varian Instruments Vista 5560 system with an SP-C18 (Varian Instruments, Palo Alto, CA) 3 μm particle size column (4.6 mm × 15 cm). Elution at 0.8 ml min^{-1} was undertaken using isocratic conditions of 90% methanol and 10% water at 40°C. Detection of the effluent was by an on-line radioactive detector (Radiomatic, Tampa, FL). Under these conditions, palmitic acid eluted at 13.5 min after injection, as ascertained by use of pure palmitic acid. For positive control, 20 μg of porcine pancreatic phospholipase A_2 was used, and negative control contained only buffer. The negative control exhibited negligible release of radioactive palmitic acid. Data were expressed as relative integration units (under the palmitic acid peak at 13.5 min), which were the output from the Vista 402 data processing unit. A unit of activity was defined as 10^6 relative integration units of the palmitic acid peak. Normalization was to per 10^7 cells.

Materials

Cytochrome c (type III) from horse heart, superoxide dismutase, Micrococcus lysodeikticus, sodium pyruvate (22 mM), NADH, valinomycin, N-formyl-methionyl-leucylphenylalanine (f Met Leu Phe), pBPB, phenophthalein β-glucuronic acid, NADPH, 5,5'-dithiobis (2-nitrobenzoic acid), palmitic acid and porcine pancreatic phospholipase A_2 were obtained from

Sigma Chemical Co. (St. Louis, MO). diS-C_3-(5) was obtained from Molecular Probes, Inc. (Junction City, OR). Phorbol myristate acetate (PMA) was purchased from Consolidated Midland, Brewster, N.J. f Met-Leu-[^{3}H]-Phe (47.6 Ci mmol^{-1}) and L-α-palmitoyl - 2 - [palmitoyl - 9,10 - ^{3}H] - phosphatidylcholine, were purchased from New England Nuclear (Boston, Mass.). All other chemicals were of analytical reagent grade.

Buffer

Unless otherwise specified, the buffer used for all experiments, consisted of (mM): NaCl 140, KCl 5.4, $CaCl_2$ 1.8, $MgSO_4$ 0.8, Na_2HPO_4 0.8, KH_2PO_4 0.8, Tris 22.5, glucose 5, pH 7.4. In addition, enzyme release assays were performed in the presence of bovine serum albumin 1 mg ml^{-1}.

Results

Effects on lysosomal degranulation

The oligopeptide f Met-Leu-Phe stimulates rat neutrophils to release lysosomal enzymes in a dose-dependent manner. Figures 1a and b show the dose-dependent release of β glucuronidase and lysozyme respectively. Preincubation (10 min at 37°C) of these cells with pBPB before stimulation inhibited this degranulation in a dose-dependent manner, such that at $>10\,\mu$M, there was a virtually total inhibition of enzyme release. This inhibition was irreversible as determined by its persistence after removal of unreacted pBPB by exhaustive washing of the cells prior to stimulation (data not shown). This inhibition also showed virtually identical kinetics as the inhibition of transmembrane potential changes (shown in Figure 4). pBPB had no effects on the enzyme assays themselves. These doses of pBPB also had no effect on viability as determined by the lack of LDH activity (above baseline control levels) in the cell supernate.

Effects on superoxide production

The inhibitory effect of pBPB was also observed in f Met-Leu-Phe stimulation of O^-_2 production. Although this peptide is a weak or suboptimal stimulator of O^-_2 production in rat neutrophils, a clear inhibitory effect by 5 μM pBPB was observed. Thus, 10^{-6} M f Met-Leu-Phe caused the production of 5.6 ± 0.27 (s.e., $n = 3$) mmoles by 6×10^6 cells ml^{-1} in 15 min, which reduced to 2.3 ± 0.03 ($n = 5$) in the presence of 5 μM pBPB.

Since rat neutrophils produced relatively small amounts of O^-_2 upon f Met-Leu-Phe stimulation, we have confirmed this inhibitory effect using PMA, which is a more potent stimulant of O^-_2 release. Figure 2 shows a dose-dependent inhibition of O^-_2 production by pBPB. In this case, however, 1 μM was effective in decreasing O^-_2 production by $>50\%$ at all doses of PMA examined, while 5 μM totally abolished O^-_2 production. This inhibition was also irreversible and time-dependent (data not shown).

These data confirm a previous study with a higher dose of pBPB in human neutrophils (Smolen & Weissman, 1980). This inhibition was not due to direct inhibition of the NADPH oxidase itself as shown by the data in Table 1. These studies revealed that treatment of intact cells with 10 μM pBPB inhibited the

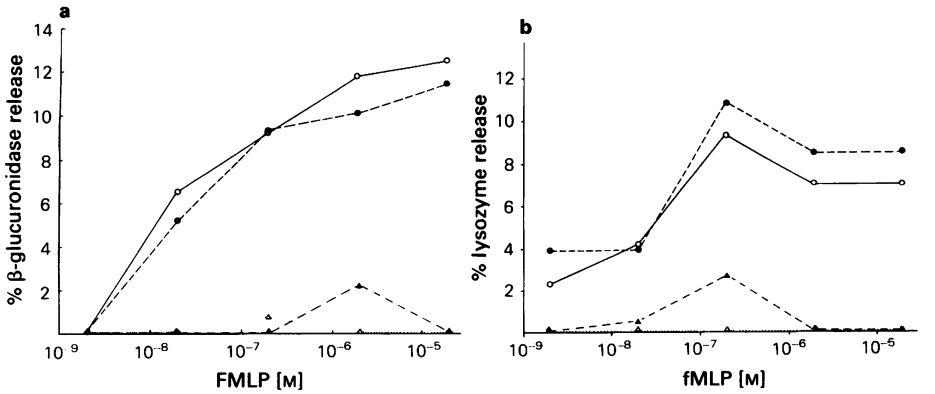

Figure 1 Effect of *p*-bromophenacyl bromide (pBPB) on (a) β-glucuronidase and (b) lysozyme release following stimulation with N-formylmethionyl-leucylphenylalanine (f Met-Leu-Phe, fMLP). Varying doses of f Met-Leu-Phe were added to 4×10^6 neutrophils ml^{-1} treated for 10 min with Me_2SO (0.1% final concentration) (○) or pBPB 1 μM (●), 5 μM (▲) and 10 μM (△), washed and resuspended in buffer. Results are expressed as the means of duplicate determinations of % of total (Triton X-100, 0.1%) releasable activity, with <10% variation.

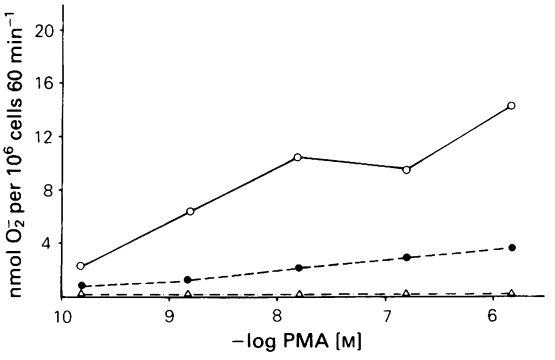

Figure 2 Effect of *p*-bromophenacyl bromide (pBPB) on phorbolmyristate acetate (PMA)-induced superoxide production. Neutrophils (10^6 ml^{-1}) were incubated with cytochrome c (80 μM final concentration) in the presence (duplicates) and absence (triplicates) of superoxide dismutase (20 μg ml^{-1}), at 37°C for 60 min. Varying concentrations of PMA were added to cell suspension pretreated for 10 min at 37°C with ethanol (0.1% final concentration) (O), or pBPB 1 μM (●) and 5 μM (Δ).

PMA-stimulated expression of NADPH oxidase activity in isolated subcellular particles by 59%. On the other hand, direct exposure of active subcellular particle preparations (from PMA stimulated cells) to pBPB failed to inhibit significantly this NADPH oxidase activity (11% inhibition). Consequently pBPB inhibited O_2^- production by preventing the PMA-induced activation or assembly of the NADPH oxidase complex without significantly affecting the structural protein(s) comprising this enzyme complex. To dissect out more closely the activation process susceptible to pBPB inhibition, an earlier step of stimulus-response coupling was examined.

Effects on transmembrane potential changes

Depolarization of the resting cellular transmembrane potential is an early response of neutrophils to f Met-Leu-Phe as well as PMA stimulation (Sklar *et al.*, 1980; Whitin *et al.*, 1980; Duque *et al.*, 1983). Rat neutrophils showed similar dose-dependent responses (Figures 3a and b). These changes as probed using the fluorescent dye diS-C_3-(5) (Laris *et al.*, 1976, Whiting *et al.*, 1980), were also inhibited by pBPB in a dose-dependent manner, with complete inhibition achieved at doses $\geq$ 10 μM (Figures 3a and b). PMA stimulation revealed greater sensitivity to pBPB. At these doses pBPB did not affect either partitioning of this fluorescent dye or the resting cellular transmembrane potential as determined by the 'null point' method (Laris *et al.*, 1976) (data not shown). This inhibition was time-dependent and obeyed pseudo-first order kinetics with a rate constant, $k_{app} = 0.36\,min^{-1}$ (Figure 4). pBPB inhibition of isolated and purified pancreatic phospholipase A_2 also obeys similar kinetics with comparable k_{app} (Roberts *et al.*, 1977).

Effects on ligand-receptor binding

The above data would suggest that pBPB inhibited these diverse neutrophil responses to two different stimuli at an early step of cell activation. This step, however, is not at the level of ligand (f Met-Leu-Phe)-receptor binding as shown in Table 2. In two separate experiments, pBPB pretreatment failed to affect significantly both the number of binding sites per cell and the equilibrium dissociation constant, K_D. As a matter of fact, there was a small increase in binding sites per cell and a slight reduction in K_D, which would be more consistent with increased binding and stimulation rather than the observed inhibition of functional responses. Since the functional integrity of the formyl

Table 1 Effect of *p*-bromophenacyl bromide (pBPB) on NADPH oxidase activity of subcellular particles[a]

Whole cell treatment	*Subcellular particle treatment*	O_2^- (nmol mg^{-1} protein)
PMA (200 mg ml^{-1}	—	69.1 ± 2.2
pBPB (10 μM) + PMA (200 mg ml^{-1})	—	28.3 ± 4.5
PMA (200 ng ml^{-1})	pBPB (10 μM)	61.4 ± 1.1

[a]Neutrophils were treated as indicated (under 'whole cell treatment') and subcellular particle isolated as described in Methods. These particles were then exposed to the treatment as indicated under 'subcellular particle treatment'. They were then assayed for NADPH-dependent O_2^- production as described in Methods.
PMA = phorbol myristate acetate.
Data are expressed as mean values ± s.e. of triplicate determinations.

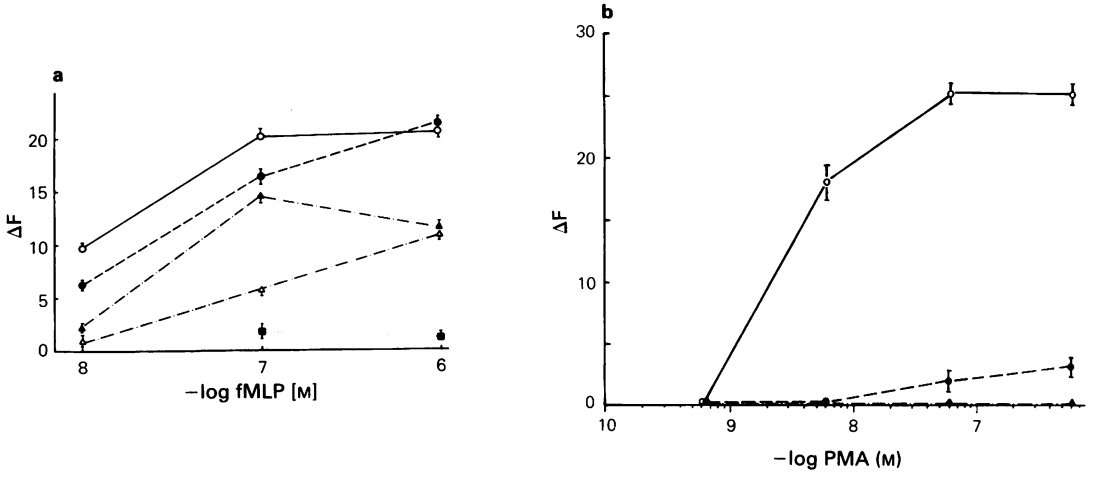

Figure 3 Effect of *p*-bromophenacyl bromide (pBPB) on transmembrane potential. Neutrophils (2×10^6 ml^{-1}) were allowed to equilibrate with 3-3'-dipropylthiodicarbocyanine iodide (2×10^{-6} M) at 37°C. Upon reaching a steady level of fluorescence intensity, Me_2SO (0.1% final concentration) for (a) or ethanol (0.1% final concentration) for (b) (○), pBPB (1 μM) (●), 2 μM (▲), 5 μM (△) and 10 μM (■) were added. After 8–9 min (a) N-formylmethionyl-leucylphenylalanine (fMLP) or (b) phorbol myristate acetate (PMA) was added at the indicated concentrations. The results as expressed as the means of triplicate determinations of the maximal change in fluorescence (ΔF); vertical lines show s.e.

peptide receptor is dependent on intact free sulphydryl groups (24,26), this lack of inhibition by pBPB also indicated its lack of effect on accessible free sulphydryls. This point is an important one since pBPB at high doses (>50 μM) is known to have non-specific effects, such as reducing the number of titratable sulphydryls in platelets (Hoffmann *et al.*, 1982). Direct measurement of the number of titratable sulphydryl groups in whole neutrophils revealed that pBPB at doses of <20 μM had no significant effect on this parameter (Table 3). Thus, pBPB inhibition cannot be explained by such non-specific effects.

Effects on phospholipase A_2 activity

The above studies revealed that pBPB inhibited an early step in the sequence of events leading to neutro-

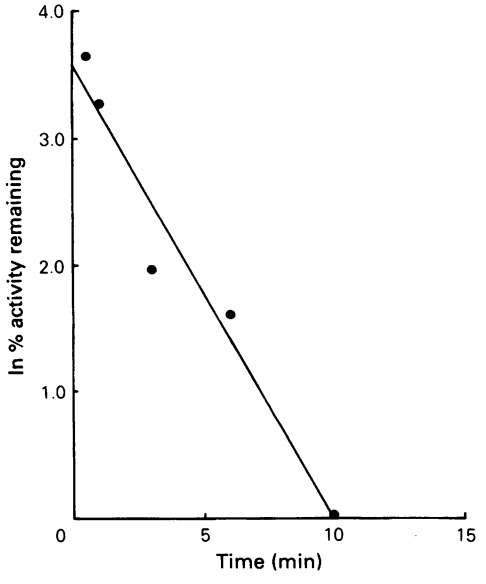

Figure 4 Kinetics of inhibition of transmembrane potential changes by *p*-bromophenacyl bromide (pBPB). Neutrophils (2×10^6 ml^{-1}) were allowed to equilibrate with 3-3'-dipropylthiodicarbocyanine iodide (diS-C_3-(5)) (2×10^{-6} M). Upon reaching equilibrium, pBPB (5 μM, final concentration) was added. N-formylmethionyl-leucylphenylalanine (1×10^{-6} M final concentration) was added after 30 s, 1, 3, 5 and 10 min of incubation with the inhibitor, and the change in fluorescence quantitated. Results are expressed as the mean of duplicate determinations of transmembrane potential changes, with <10% variation. The data are presented as a semi-log plot of remaining activity vs time of incubation with pBPB. The y-axis labels are natural logarithms of the % of activity remaining after addition of the indicated concentrations of pBPB (thus 4.6 = 100% activity); linear regression analysis of the data point results in the drawn line.

Table 2 Effect of *p*-bromophenacyl bromide (pBPB) on binding of N-formylmethionyl-leucyl-[^{3}H]-phenylalanine (f met-Leu-[^{3}H]-Phe)

	Treatment[a]			
	Control		*p-Bromophenacyl bromide*	
Expt. No.	*Blinding sites per cell*	K_D	*Binding sites per cell*	K_D
1	24,310	1.7×10^{-8} M	26,731	1.42×10^{-8} M
2	28,414	0.74 ± 10^{-8}	31,367	0.68 ± 10^{-8} M

[a]Cells were treated at 37°C for 15 min with 10 μM pBPB, washed and assayed for f Met-Leu-[^{3}H]-Phe binding as described (see Methods). Determination of the number of binding sites per cell and the dissociation constant (K_D) was made using Scatchard analysis of specific f Met-Leu-[^{3}H]-Phe binding.

phil activation. Although high doses (>50 μM) of pBPB have a multitude of effects (Hoffmann *et al.*, 1982; Kyger & Franson, 1984), these had not been observed at doses <10 μM (see above). Since this reagent is an active-site directed inhibitor of phospholipase A_2 (Roberts *et al.*, 1977), it seems reasonable to surmise that pBPB may be inhibiting neutrophil activation by inhibiting such an enzyme necessary for the activation process. To test this hypothesis, the effect of pBPB on neutrophil (both resting and stimulated) phospholipase A_2 was examined.

Assay of whole cell homogenates (from resting and f Met-Leu-Phe-stimulated cells) revealed <20% inhibition of total phospholipase A_2 activity by pBPB. However, when only the subcellular particulate or membranous fraction (27,000 *g* pellet) was assayed for phospholipase A_2 pBPB inhibition was more dramatic (Table 4), and of a similar order of magnitude as its inhibition of stimulated cellular functional responses. The data revealed complete inhibition by pBPB of f Met-Leu-Phe stimulatable activity in these subcellular particles without affecting the resting enzymatic activity (Table 4). Thus, although pBPB failed to inhibit significantly (at least to the extent of its inhibition of stimulated cellular functional responses) whole cell phospholipase A_2 activity, it was very effective in inhibition of the f Met-Leu-Phe stimulatable activity residing in the subcellular pariculate fraction containing plasma membrane and other membranous fragments. The ability of f Met-Leu-Phe to stimulate phospholipase A_2 activity in the subcellular particles is in contrast to the lack of such stimulation in the 100,000 *g* pellet as reported by Lanni & Backer (1983). This difference may be due to the use of H_2SO_4 to 'extract' the enzyme before assay, in their study. A subsequent paper from the same laboratory however, supported our observation of an inducible phospholipase A_2 activity in rabbit neutrophil plasma membrane (Bormann *et al.*, 1984). Their more recent paper also demonstrates the ineffectiveness of pBPB in inhibiting phospholipase A_2 added to whole, intact cells (<20%), although this reagent is highly effective in inhibition of enzyme activity in cell sonicates or acid extracts (Lanni & Becker, 1985). In that study however, the effect of f Met-Leu-Phe stimulation is not examined.

Table 3 Effect of *p*-bromophenacyl bromide (pBPB) on cellular titratable sulphydryl groups[a]

pBPB (μM)	*Titratable-SH*
0	(100)
1	93 ± 3.9
5	100 ± 5.7
10	97 ± 5.1
20	67 ± 6.1

[a]5×10^6 neutrophils ml^{-1} were incubated with the indicated concentrations of pBPB for 10 min at 37°C. After washing, the cells were assayed for titratable sulphydryls as described in Methods. Data are means ± s.e. of triplicate determinations. They are expressed as % of the untreated control mean value.

Table 4 Effect of *p*-bromophenacyl bromide (pBPB) on subcellular particulate phospholipase A_2[a]

	Inhibitor	
Stimulant	*None*	*pBPB*
None (buffer only)	8.2 ± 0.74	9.3 ± 1.11
f Met-Leu-Phe (10^{-6} M)	13.4 ± 1.23	7.3 ± 1.70

[a]Data are expressed as mean ± s.e. in units per 10^7 cells ($n = 3$). Units of activity are as defined in Methods.
Cells were treated for 15 min at 37°C in the presence of 10^{-5} M pBPB, washed and either incubated in buffer only, or with 10^{-6} M N-formylmethionyl-leucylphenylalanine (fMet-Leu-Phe) for 15 min at 37°C. Subcellular particles were then obtained and assyed for phospholipase A_2 activity as described in Methods

Discussion

The data presented herein suggest a role for a pBPB inhibitable step(s), in the sequence of events that take place following receptor-ligand binding and prior to the secretion phase. These data extend the previously reported inhibitory effects of pBPB on neutrophil responses (Smolen & Weissman, 1980) by demonstrating that an earlier step (membrane potential changes) is also inhibitable by pBPB. Furthermore, our data demonstrate that this irreversible and time-dependent inhibition is not mediated by altering surface receptor density and ligand-receptor binding kinetics. Nor does it inhibit by modification of free sulphydryl groups or the structural proteins comprising the NADPH oxidase.

The inhibitory effects of pBPB on membrane potential changes suggest a requirement for a pBPB-sensitive activity in the production of a state of altered permeability to ions following stimulation. That a phospholipase A_2-like activity could also be involved is suggested by recent reports (Yorio *et al.*, 1983) showing that phospholipid metabolites can contribute to alteration of membrane permeability in frog skin epithelium and that the increase in sodium transport observed is mediated through the release of arachidonic acid and the synthesis of prostaglandins. Furthermore, a Ca^{2+}-dependent, neutral pH active phospholipase A_2 has been described in rabbit alveolar macrophages (Franson *et al.*, 1973) and granulocytes (Franson *et al.*, 1974).

Recent studies by another laboratory have also reported on a stimulatable phospholipase A_2 activity in rabbit neutrophil plasma membranes (Bormann *et al.*, 1984). The same laboratory also more recently noted, that despite the ability of pBPB to inhibit phospholipase A_2 activity in cell sonicates and acid-extracts, it is only minimally inhibitory ($<20\%$) if introduced to intact cells (Lanni & Becker, 1985). Our data are consistent with these findings, and we have extended them further by reporting inhibition of only the f Met;Leu-Phe stimulatable enzyme activity.

Additional evidence for the role of a cell bound phospholipase A_2 in the release of arachidonate from human neutrophil phospholipids has been presented by Walsh *et al.* (1980; 1981). The ability of some of these arachidonate metabolites to initiate and/or modulate ion fluxes would then have an impact on transmembrane potential changes.

Although localization and identification of the target site(s) for pBPB remain uncertain, our data demonstrate that, with the dose and conditions used, sulphydryl groups are not affected. This conclusion is based partially and indirectly on the lack of inhibition of f Met-Leu-Phe binding, since it is known that intact sulphydryl groups are essential to maintain the integrity of the formyl peptide receptor (Schiffmann *et al.*, 1980; Niedel, 1981). Furthermore, and more directly we could not demonstrate significant reduction of cellular titratable sulphydryl groups at concentrations of pBPB below 20 μM. Additionally, if pBPB were to alter non-specifically free sulphydryl groups, one would expect inhibition of NADPH oxidase activity (McPhail *et al.*, 1976). However, this was not observed and instead pBPB was found to inhibit a regulatory step other than the structural proteins involved in the expression of NADPH oxidase activity. The data would suggest interference with activation steps required for the expression or assembly of NADPH oxidase activity and not on the oxidase itself. In this study pBPB was used at considerably lower concentrations ($<10\,\mu$M) than was the case in studies by other investigators who have shown non-specific inhibitor effects on other enzymes (Hofmann *et al.*, 1982; Kyger & Franson, 1984). In one of these studies (Hofmann *et al.*, 1982), 30 μM pBPB caused a 63% inhibition of phospholipase C activity after 15 min incubation. Diglyceride lipase was inhibited by only 56% after 30 min incubation with 500 μM pBPB. These concentratons are from 3–50 times the maximal dose used in this study, which could account for the differences between that study and our data. Another study purporting to show inhibition of acid proteases by pBPB (Ackerman *et al.*, 1983) to explain its neutrophil inhibitory effects, failed to realize that pBPB does not inhibit acid proteases at $pH > 5.0$ (Gross & Morell, 1966). In view of these considerations, the effects of pBPB exposure described in this paper are likely to be due to inhibition of one or more early regulatory activities, of which a critical membranous fraction of stimulatable cellular phospholipase A_2 appears to be a candidate. However, other sites of inhibition, as yet unidentified cannot be ruled out at this time especially in view of the hydrophobic nature of pBPB and its ability to partition at high local concentrations in membranes. A possible and as yet unexplored target is the Ca^{2+}-dependent intracellular signalling system based on the metabolism of inositol phospholipids (Berridge, 1984).

We conclude: (a) that pBPB at concentrations of $<10\,\mu$M inhibit neutrophil functional responses to several stimuli; (b) the inhibitable step(s) involved in the stimulus-response process precede the activation or assembly of the NADPH oxidase required for the generation of O^-_2 and (c) the inhibitory effects include 'early' events such as the ligand-induced change in transmembrane potential. Finally, the inhibition of a critical membranous fraction of cell bound phospholipase A_2 by pBPB would suggest that this enzyme may be crucial to subsequent cell activation. The inability to inhibit unstimulated (or 'resting') phospholipase A_2 activity would suggest that pBPB may inhibit the regulatory step(s) necessary for activation

of this membrane bound phospholipase A_2, rather than alkylating the activatable or activated enzyme itself. This possibility is also suggested by the ability of pBPB to inhibit the stimulatable enzyme activity in the presence of physiological concentrations of Ca^{2+}, which is known to make the enzyme less susceptible to alkylation by pBPB (Roberts *et al.*, 1977). There is no conclusive evidence at this time that the neutrophil phospholipase A_2 contains a histidine at its active site, although its susceptibility to pBPB inhibition (Lanni & Lecker, 1985) makes it a likely possibility. Finally, the available data would not allow any conclusions to be reached concerning the commonness of the mechanism by which fMet-Leu-Phe and PMA stimulate neutrophils.

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