

# Inhibition of neutrophil activation by *p*-bromophenacyl bromide and its effects on phospholipase A<sub>2</sub>

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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-[<sup>3</sup>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 sulphhydryl 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<sub>2</sub> activity by pretreatment with 10  $\mu M$  pBPB, although total cellular phospholipase A<sub>2</sub> 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<sub>2</sub> 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  $\beta$ -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<sub>3</sub>-(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  $> 1.5 \text{ mM } Ca^{2+}$ , 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  $\text{Ca}^{2+}$  dependent (Derkens & Cohen, 1975) activation of a cell bound phospholipase A<sub>2</sub> (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<sub>2</sub> 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<sub>2</sub> 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<sub>2</sub> (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  $\text{O}_2 \cdot 10^6 \text{ cells}^{-1} \cdot 60 \text{ min}^{-1}$ . Calculation of the actual amount of nmol of cytochrome c reduced was made utilizing an extinction coefficient of  $21.1 \text{ cm}^{-1} \text{ mM}^{-1}$  for cytochrome c (reduced minus oxidized) at 550 nm. Lysozyme,  $\beta$ -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  $\mu\text{M}$  pBPB from ( $10^{-2} \text{ M}$  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<sup>-1</sup>) 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  $\mu\text{M}$ ) for 10 min at room temperature before assay for NADPH oxidase. The ability of 50  $\mu\text{l}$  of each preparation to produce superoxide dismutase-inhibitable  $\text{O}_2^-$  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  $\text{O}_2^- \text{ mg}^{-1}$  protein.

### *Membrane potential changes*

Membrane potential changes were measured as previously described (Duque *et al.*, 1983). Neutrophils ( $2 \times 10^6$ ) were equilibrated for 3–4 min with  $2 \times 10^{-6} \text{ M}$  diS-C<sub>3</sub>-(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<sub>2</sub>SO) 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 ( $\Delta F$ ). 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<sup>+</sup>]<sub>o</sub> at which there is no change in fluorescence intensity upon the addition of valinomycin ( $2 \times 10^{-6} \text{ M}$ ) in the presence and absence of pBPB (5  $\mu\text{M}$  for 10 min at 37°C).

diS-C<sub>3</sub>-(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 luciferin-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<sub>3</sub>-(5) fluorescence is not significantly affected by anaerobic conditions (Whitin *et al.*, 1980) which would have dramatic impact on mitochondrial 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 pBPP 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 fMet-Leu-[<sup>3</sup>H]-Phe binding

The following protocol was used to assess the effect of pBPP on the time course of N-formylmethionyl-leucyl-[<sup>3</sup>H]-phenylalanine (fMet-Leu-[<sup>3</sup>H]-Phe) binding. Neutrophils ( $2.0 \times 10^7$  cells ml<sup>-1</sup>) 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 pBPP (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<sub>3</sub> 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 fMet-Leu-[<sup>3</sup>H]-Phe in siliconized glass 12 × 75 mm test tubes. After these preliminary studies, binding studies were routinely performed by incubating 10 µM pBPP with cells for 15 min. The cells were washed, resuspended to  $2 \times 10^7$  cells ml<sup>-1</sup> and analyzed for fMet-Leu-[<sup>3</sup>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<sub>2</sub> assay

Phospholipase A<sub>2</sub> assay was performed according to Franson *et al.* (1974) with slight modification. L-α-1-Palmitoyl-2-[palmitoyl-9,10-<sup>3</sup>H]-phosphatidylcholine (30–60 Ci mmol<sup>-1</sup>) was used as substrate. Rat peritoneal neutrophils ( $10^8$ ) were treated with 10<sup>-5</sup> M pBPP at 37°C for 15 min. Control cells were similarly incubated in the absence of pBPP. 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  $2 \times 10^6$  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<sub>2</sub> and 100 nM Tris HCl pH 8.0. After 60 min at 37°C, the entire mixture was extracted with CHCl<sub>3</sub>-CH<sub>3</sub>OH 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<sup>-1</sup> 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<sub>2</sub> 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-formylmethionyl-leucylphenylalanine (fMet Leu Phe), pBPP, phenolphthalein β-glucuronic acid, NADPH, 5,5'-dithiobis (2-nitrobenzoic acid), palmitic acid and porcine pancreatic phospholipase A<sub>2</sub> were obtained from

Sigma Chemical Co. (St. Louis, MO). diS-C<sub>3</sub>-(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-[<sup>3</sup>H]-Phe (47.6 Ci mmol<sup>-1</sup>) and L- $\alpha$ -palmitoyl - 2 - [palmitoyl - 9,10 - <sup>3</sup>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<sub>2</sub> 1.8, MgSO<sub>4</sub> 0.8, Na<sub>2</sub>HPO<sub>4</sub> 0.8, KH<sub>2</sub>PO<sub>4</sub> 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<sup>-1</sup>.

#### 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  $\beta$ -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  $>10 \mu\text{M}$ , 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

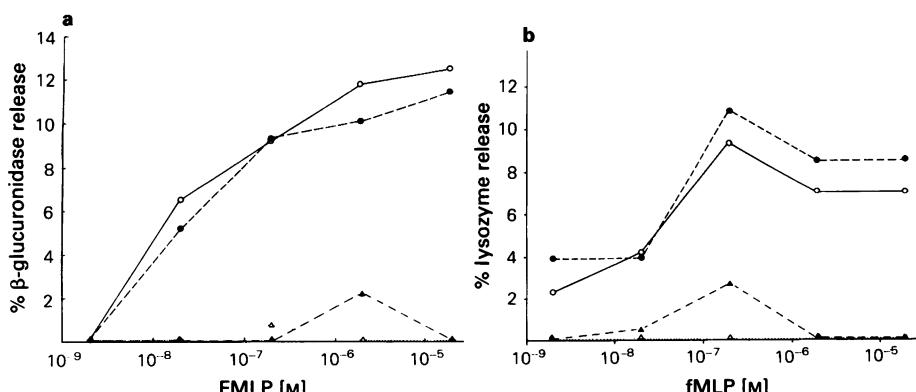
stimulation (data not shown). This inhibition also showed virtually identical kinetics as the inhibition of transmembrane potential changes (shown in Figure 4). pBPB had no effects on the enzyme assays themselves. These doses of pBPB also had no effect on viability as determined by the lack of LDH activity (above baseline control levels) in the cell supernate.

##### Effects on superoxide production

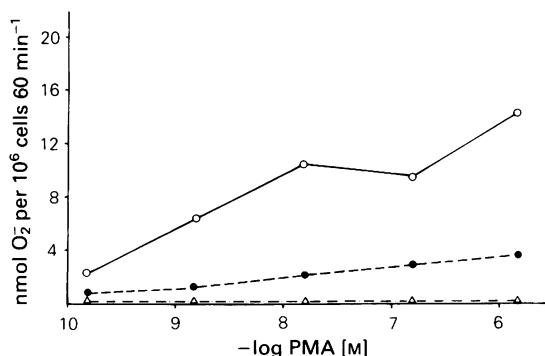
The inhibitory effect of pBPB was also observed in f Met-Leu-Phe stimulation of O<sub>2</sub><sup>-</sup> production. Although this peptide is a weak or suboptimal stimulator of O<sub>2</sub><sup>-</sup> production in rat neutrophils, a clear inhibitory effect by 5  $\mu\text{M}$  pBPB was observed. Thus, 10<sup>-6</sup> M f Met-Leu-Phe caused the production of 5.6  $\pm$  0.27 (s.e., n = 3) mmoles by 6  $\times$  10<sup>6</sup> cells ml<sup>-1</sup> in 15 min, which reduced to 2.3  $\pm$  0.03 (n = 5) in the presence of 5  $\mu\text{M}$  pBPB.

Since rat neutrophils produced relatively small amounts of O<sub>2</sub><sup>-</sup> upon f Met-Leu-Phe stimulation, we have confirmed this inhibitory effect using PMA, which is a more potent stimulant of O<sub>2</sub><sup>-</sup> release. Figure 2 shows a dose-dependent inhibition of O<sub>2</sub><sup>-</sup> production by pBPB. In this case, however, 1  $\mu\text{M}$  was effective in decreasing O<sub>2</sub><sup>-</sup> production by > 50% at all doses of PMA examined, while 5  $\mu\text{M}$  totally abolished O<sub>2</sub><sup>-</sup> 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  $\mu\text{M}$  pBPB inhibited the



**Figure 1** Effect of p-bromophenacyl bromide (pBPB) on (a)  $\beta$ -glucuronidase and (b) lysozyme release following stimulation with N-formylmethionyl-leucylphenylalanine (fMet-Leu-Phe, fMLP). Varying doses of fMet-Leu-Phe were added to 4  $\times$  10<sup>6</sup> neutrophils ml<sup>-1</sup> treated for 10 min with Me<sub>2</sub>SO (0.1% final concentration) (○) or pBPB 1  $\mu\text{M}$  (●), 5  $\mu\text{M}$  (▲) and 10  $\mu\text{M}$  (Δ), 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 phorbol myristate acetate (PMA)-induced superoxide production. Neutrophils ( $10^6 \text{ ml}^{-1}$ ) were incubated with cytochrome c (80  $\mu\text{M}$  final concentration) in the presence (duplicates) and absence (triplicates) of superoxide dismutase (20  $\mu\text{g ml}^{-1}$ ), at 37°C for 60 min. Varying concentrations of PMA were added to cell suspension pre-treated for 10 min at 37°C with ethanol (0.1% final concentration) (O), or pBPB 1  $\mu\text{M}$  (●) and 5  $\mu\text{M}$  (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<sub>3</sub>-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  $> 10 \mu\text{M}$  (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,  $k_{app} = 0.36 \text{ min}^{-1}$  (Figure 4). pBPB inhibition of isolated and purified pancreatic phospholipase A<sub>2</sub> 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

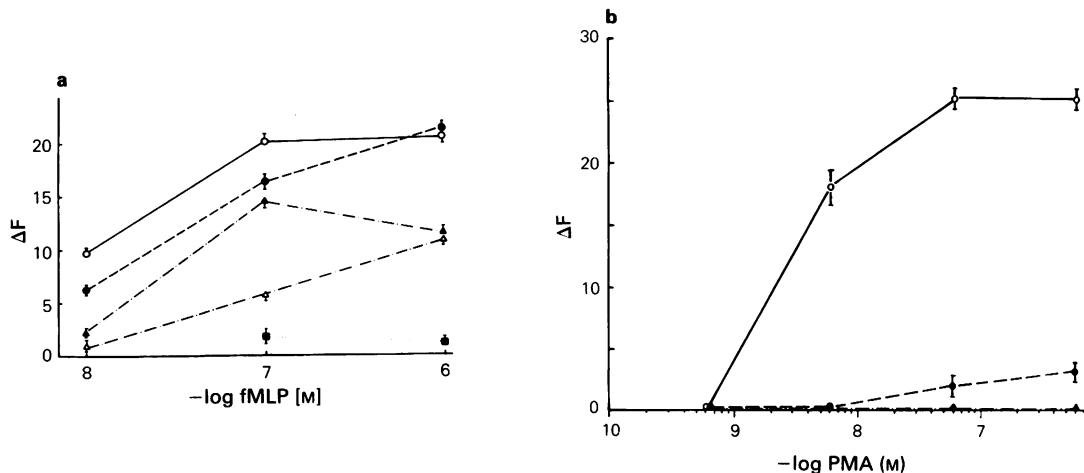
**Table 1** Effect of *p*-bromophenacyl bromide (pBPB) on NADPH oxidase activity of subcellular particles<sup>a</sup>

Whole cell treatment	Subcellular particle treatment	$O_2^-$ ( $\text{nmol mg}^{-1} \text{ protein}$ )
PMA (200 $\text{mg ml}^{-1}$ )	—	$69.1 \pm 2.2$
pBPB (10 $\mu\text{M}$ ) + PMA (200 $\text{mg ml}^{-1}$ )	—	$28.3 \pm 4.5$
PMA (200 $\text{ng ml}^{-1}$ )	pBPB (10 $\mu\text{M}$ )	$61.4 \pm 1.1$

<sup>a</sup>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  $\pm$  s.e. of triplicate determinations.



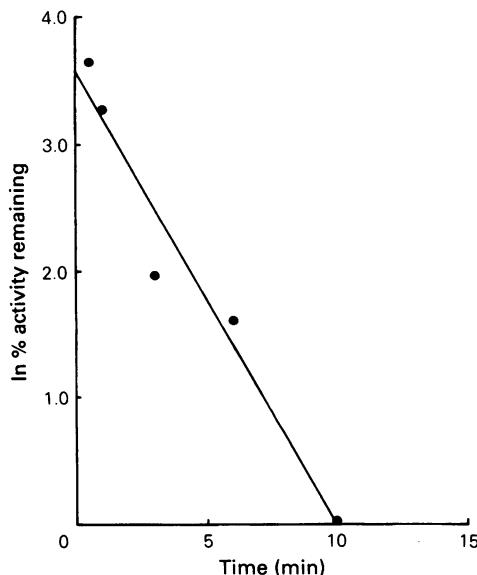
**Figure 3** Effect of *p*-bromophenyl bromide (pBPB) on transmembrane potential. Neutrophils ( $2 \times 10^6 \text{ ml}^{-1}$ ) were allowed to equilibrate with 3,3'-dipropylthiodicarbocyanine iodide ( $2 \times 10^{-6} \text{ M}$ ) at  $37^\circ\text{C}$ . Upon reaching a steady level of fluorescence intensity,  $\text{Me}_2\text{SO}$  (0.1% final concentration) for (a) or ethanol (0.1% final concentration) for (b) (○), pBPB (1  $\mu\text{M}$ ) (●), 2  $\mu\text{M}$  (▲), 5  $\mu\text{M}$  (Δ) and 10  $\mu\text{M}$  (■) 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 ( $\Delta F$ ); vertical lines show s.e.

peptide receptor is dependent on intact free sulphhydryl groups (24,26), this lack of inhibition by pBPB also indicated its lack of effect on accessible free sulphhydryls. This point is an important one since pBPB at high doses ( $> 50 \mu\text{M}$ ) is known to have non-specific effects, such as reducing the number of titratable

sulphhydryls in platelets (Hoffmann *et al.*, 1982). Direct measurement of the number of titratable sulphhydryl groups in whole neutrophils revealed that pBPB at doses of  $< 20 \mu\text{M}$  had no significant effect on this parameter (Table 3). Thus, pBPB inhibition cannot be explained by such non-specific effects.

#### Effects on phospholipase A<sub>2</sub> 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*-bromophenyl bromide (pBPB). Neutrophils ( $2 \times 10^6 \text{ ml}^{-1}$ ) were allowed to equilibrate with 3,3'-dipropylthiodicarbocyanine iodide (diS-C<sub>3</sub>(5)) ( $2 \times 10^{-6} \text{ M}$ ). Upon reaching equilibrium, pBPB (5  $\mu\text{M}$ , final concentration) was added. N-formylmethionyl-leucylphenylalanine ( $1 \times 10^{-6} \text{ M}$  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-[<sup>3</sup>H]-phenylalanine (f met-Leu-[<sup>3</sup>H]-Phe)

Expt. No.	Control		Treatment <sup>a</sup>	
	Blinding sites per cell	K <sub>D</sub>	p-Bromophenacyl bromide Binding sites per cell	K <sub>D</sub>
1	24,310	1.7 × 10 <sup>-8</sup> M	26,731	1.42 × 10 <sup>-8</sup> M
2	28,414	0.74 ± 10 <sup>-8</sup>	31,367	0.68 ± 10 <sup>-8</sup> M

<sup>a</sup>Cells were treated at 37°C for 15 min with 10 μM pBPB, washed and assayed for f Met-Leu-[<sup>3</sup>H]-Phe binding as described (see Methods). Determination of the number of binding sites per cell and the dissociation constant (K<sub>D</sub>) was made using Scatchard analysis of specific f Met-Leu-[<sup>3</sup>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<sub>2</sub> (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<sub>2</sub> was examined.

Assay of whole cell homogenates (from resting and f Met-Leu-Phe-stimulated cells) revealed <20% inhibition of total phospholipase A<sub>2</sub> activity by pBPB. However, when only the subcellular particulate or membranous fraction (27,000 g pellet) was assayed for phospholipase A<sub>2</sub> 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<sub>2</sub> activity, it was very effective in inhibition of the f Met-Leu-Phe stimulatable activity residing in the subcellular particulate fraction containing plasma membrane and other membranous fragments. The ability of f Met-Leu-Phe to stimulate phospholipase A<sub>2</sub> activity in the subcellular particles is in contrast to the lack of such stimulation in the 100,000 g pellet as reported by Lanni & Becker (1983). This difference may be due to the use of H<sub>2</sub>SO<sub>4</sub> 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<sub>2</sub> activity in rabbit neutrophil plasma membrane (Bormann *et al.*, 1984). Their more recent paper also demonstrates the ineffectiveness of pBPB in inhibiting phospholipase A<sub>2</sub> 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 sulphhydryl groups<sup>a</sup>

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

<sup>a</sup>5 × 10<sup>6</sup> neutrophils ml<sup>-1</sup> were incubated with the indicated concentrations of pBPB for 10 min at 37°C. After washing, the cells were assayed for titratable sulphhydryls 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<sub>2</sub><sup>a</sup>

Stimulant	Inhibitor	
	None	pBPB
None (buffer only)	8.2 ± 0.74	9.3 ± 1.11
f Met-Leu-Phe (10 <sup>-6</sup> M)	13.4 ± 1.23	7.3 ± 1.70

<sup>a</sup>Data are expressed as mean ± s.e. in units per 10<sup>7</sup> 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<sup>-5</sup> M pBPB, washed and either incubated in buffer only, or with 10<sup>-6</sup> M N-formylmethionyl-leucylphenylalanine (fMet-Leu-Phe) for 15 min at 37°C. Subcellular particles were then obtained and assayed for phospholipase A<sub>2</sub> 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 sulphhydryl 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<sub>2</sub>-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<sup>2+</sup>-dependent, neutral pH active phospholipase A<sub>2</sub> 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<sub>2</sub> 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<sub>2</sub> 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<sub>2</sub> 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, sulphhydryl 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 sulphhydryl 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 sulphhydryl groups at concentrations of pBPB below 20 μM. Additionally, if pBPB were to alter non-specifically free sulphhydryl 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 μM) 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 concentrations 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<sub>2</sub> 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<sup>2+</sup>-dependent intracellular signalling system based on the metabolism of inositol phospholipids (Berridge, 1984).

We conclude: (a) that pBPB at concentrations of <10 μM 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<sup>−</sup> 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<sub>2</sub> by pBPB would suggest that this enzyme may be crucial to subsequent cell activation. The inability to inhibit unstimulated (or 'resting') phospholipase A<sub>2</sub> activity would suggest that pBPB may inhibit the regulatory step(s) necessary for activation

of this membrane bound phospholipase A<sub>2</sub>, 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<sup>2+</sup>, 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<sub>2</sub> 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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