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Characterization of inositol hexakisphosphate ($InsP_6$)-mediated priming in human neutrophils: lack of extracellular [^{3}H]-$InsP_6$ receptors

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1 Inositol hexakisphosphate ($InsP_6$) is a ubiquitous and abundant cytosolic inositol phosphate that has been reported to prime human neutrophils for enhanced agonist-stimulated superoxide anion generation. This led to the proposal that the release of $InsP_6$ from necrotic cells may augment the functional responsiveness of neutrophils at an inflammatory focus. The aim of this study was to examine whether the functional effects of $InsP_6$ in neutrophils are receptor-mediated and establish the magnitude of this priming effect relative to other better characterized priming agents.

2 Analysis of [^{3}H]-$InsP_6$ binding to human neutrophil membranes in 20 mM Tris, 20 mM NaCl, 100 mM KCl, 5 mM EDTA (pH 7.7) buffer using 0.1 mg ml^{-1} membrane protein and 2.5 nM [^{3}H]-$InsP_6$ (90 min, 4°C), demonstrated specific low affinity [^{3}H]-$InsP_6$ binding that was non-saturable up to a radioligand concentration of 10 nM.

3 [^{3}H]-$InsP_6$ displacement by $InsP_6$ gave a Hill coefficient of 0.55 and best fitted a two-site logistic model (53% K_D 150 nM, 47% K_D 5 μM). [^{3}H]-$InsP_6$ binding also displayed low (3 fold) selectivity for $InsP_6$ over Ins(1,3,4,5,6)P_5.

4 The specific [^{3}H]-$InsP_6$ binding displayed a pH optimum of 8, was abolished by pre-boiling the membranes, and was enhanced by Ca^{2+}, Mg^{2+} and Na^+.

5 In incubations with intact neutrophils, where high levels of specific [^{3}H]-LTB_4 binding was observed, no [^{3}H]-$InsP_6$ binding could be identified.

6 Preincubation of neutrophils with 100 μM $InsP_6$ had no effect on resting cell morphology, but caused a minor and transient (maximal at 30 s) enhancement of (0.1 nM) fMLP-induced shape change (% cells shape changed: fMLP 53±3%, fMLP + $InsP_6$ 66±4%). Similarly, $InsP_6$ (100 μM, 30 s) had no effect on basal superoxide anion generation and, compared to lipopolysaccharide (LPS, 100 ng ml^{-1}, 60 min), tumour necrosis factor-α (TNFα, 200 u ml^{-1}, 30 min) or platelet-activating factor (PAF, 100 nM, 5 min) caused only a small enhancement of 100 nM fMLP-stimulated superoxide anion generation (fold-increase in superoxide anion generation over fMLP alone: $InsP_6$ 1.8±0.3, LPS 6.8±0.6, TNFα 5.2±0.7, PAF 5.8±0.6).

7 While these data support the presence of a specific, albeit low affinity, [^{3}H]-$InsP_6$ binding site in human neutrophil membrane preparations, the lack of binding to intact cells implies that the functional effects of $InsP_6$ (ie. enhanced fMLP-stimulated superoxide anion generation and shape change) are not receptor-mediated.

Keywords: Inflammation; neutrophil priming; inositol hexakisphosphate; superoxide anions; neutrophil shape-change

Introduction

Inositol hexakisphosphate ($InsP_6$) is the most abundant inositol phosphate found in nature (Cosgrove, 1980), being present in mammalian cells at concentrations between 10 μM and 1 mM (Szwergold *et al.*, 1987). It is an intriguing molecule, whose true physiological role has yet to be revealed. Intracellularly, $InsP_6$ has been proposed to function as a general antioxidant (Graf & Eaton, 1990), Ca^{2+} chelator (Luttrell, 1993), inhibitor of iron-catalysed hydroxyl radical formation (Hawkins *et al.*, 1993) and phosphate store (Berridge & Irvine, 1989). It is also a specific inhibitor of a number of the enzymes involved in inositol polyphosphate metabolism, for example the Ins(1,3,4,5)P_4 3-phosphatase (Hughes & Shears, 1990; Höer & Oberdisse, 1991), and can itself be metabolized into a series of more polar inositol polyphosphates termed pyrophosphates (Mennite *et al.*, 1993; Stephens 1993). Investigations into the effects of calcium-mobilizing agonists on cellular $InsP_6$ levels have demonstrated either no effect (Glennon & Shears, 1993), or a rapid, transient increase that parallels Ins(1,4,5)P_3 accumulation (Sasakawa *et al.*, 1993). In addition, quite marked changes in the concentrations of both $InsP_5$ and $InsP_6$ can be seen with progression through the cell cycle or changes in cell phenotype (e.g. during neutrophilic differentiation of HL-60 cells) (French *et al.*, 1991; Guse *et al.*, 1993).

There is growing evidence that $InsP_6$ may also have a number of extracellular actions. Initial interest focused on its ability to suppress the development of colonic cancer in animal models, probably by chelating metal ions and thereby limiting mitogenic iron-catalysed redox reactions (Graf & Eaton, 1993). It has also been shown to lower blood pressure and heart rate in a reversible manner when infused into specific regions of the rat brainstem (Vallejo *et al.*, 1987). At a cellular level, $InsP_6$ has been shown to elicit Ca^{2+} influx and catecholamine release in bovine adrenal chromaffin cells (Regunathan *et al.*, 1992) and to enhance Ca^{2+} influx in cultured neuronal cells (Nicoletti *et al.*, 1989). However, the powerful Ca^{2+} chelation (Cosgrove, 1980) and autofluorescence properties of $InsP_6$ complicate the interpretation of such studies (Sun *et al.*, 1992).

It has recently been reported that $InsP_6$ may also function as a neutrophil priming agent and hence have a pro-inflammatory role (Eggleton *et al.*, 1991). Preincubation of neutrophils with 10–250 μM $InsP_6$ was shown to enhance subsequent agonist-

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induced superoxide anion generation and result in a rapid and sustained assembly of F-actin (Crawford & Eggleton, 1992). This led to the proposal that release of $InsP_6$ from necrotic cells at an inflammatory focus may upregulate, or prime, the functional responsiveness of adjacent neutrophils to secretagogue agonists. Since priming has been shown to be a prerequisite for neutrophil-mediated tissue injury, this event could play a vital role in modulating the extent of inflammation-induced organ damage (Smedley *et al.*, 1986).

In view of recent reports identifying the presence of specific, high affinity [^{3}H]-$InsP_6$ receptors in the rat brain (Hawkins *et al.*, 1990), and their subsequent characterization as the α-subunits of the clathrin assembly protein AP-2 (Volgmaier *et al.*, 1992), we have examined whether the reported functional effects of $InsP_6$ in human neutrophils are mediated by similar receptors. Our findings indicate that while specific [^{3}H]-$InsP_6$ binding sites are present on neutrophil membranes, they do not display the characteristic high affinity and selective $InsP_6$ binding properties reported in other cell types, and more importantly, are not present on intact cells: hence it is unlikely that the functional effects of $InsP_6$ are receptor-mediated. A more complete re-evaluation of the functional effects of $InsP_6$ demonstrates that this molecule has only very modest and transient effects on human neutrophil function compared to more established priming agents.

Methods

Neutrophil preparation

Blood was taken from healthy adult volunteers, anticoagulated with 4 ml 3.8% sodium citrate 40 ml^{-1} blood, and centrifuged (300 *g*) for 20 min. Neutrophils were isolated as detailed by Haslett *et al.* (1985) using dextran sedimentation and discontinuous plasma-Percoll gradients. The purified neutrophils were washed sequentially in platelet-poor plasma, PBS without, and then PBS with Ca^{2+} and Mg^{2+}. All procedures were conducted at 25°C. Cell purity and viability (assessed by trypan blue exclusion), were routinely >95% (<0.5% monocyte contamination) and >99.5% respectively.

[^{3}H]-$InsP_6$ binding to neutrophil membranes

Membranes were prepared as detailed by Hawkins *et al.* (1990). In brief, neutrophils were resuspended at 15×10^6 cells ml^{-1} in 20 mM Tris (pH 7.7), 20 mM NaCl, 100 mM KCl and 5 mM EDTA (4°C), homogenized (Polytron), centrifuged (35,000 *g*, 30 min), and the resulting membranes washed twice before use. Membrane protein concentrations were determined by the Pierce-BCA protein assay with BSA as standard.

[^{3}H]-$InsP_6$ binding was performed according to the method of Hawkins *et al.* (1990). Freshly prepared membranes (0.1 mg ml^{-1}) were incubated at 4°C in 20 mM Tris (pH 7.7), 20 mM NaCl, 100 mM KCl and 5 mM EDTA with 2.5 nM (90,000 d.p.m.) [^{3}H]-$InsP_6$, in a final volume of 1 ml. Separation of bound from free radioligand was achieved by centrifugation (13,000 *g*, 6 min, 4°C), with non-specific binding (NSB) determined in the presence of 100 μM $InsP_6$. Pellets were dissolved overnight in Soluene and their radioactivity determined by liquid scintillation counting. In preliminary experiments, [^{3}H]-$InsP_6$ binding was found to be linear up to a protein concentration of 0.2 mg ml^{-1} with equilibrium between free and bound [^{3}H]-$InsP_6$ achieved by 90 min (data not shown).

To assess whether there was any metabolism of [^{3}H]-$InsP_6$ during these assays, pre- and post-incubation supernatants were analysed by anion exchange h.p.l.c., using a Partisphere 5-SAX column (250 × 4.6 mm) fitted with a Whatman SAX guard cartridge eluted (flow rate 1.25 ml min^{-1}, 0.3 min fractions) with the following gradient: A (H_2O), B (3.5 M ammonium formate, pH adjusted to 3.7 with orthophosphoric acid): 0–5 min 0% B; 10–12 min 21.4% B; 18–23 min 28.5% B; 30 min 40.0% B; 40 min 42.0% B; 60–65 min 100% B.

In competition assays, displacing agents ($InsP_6$, 0.1 nM–0.1 mM; Ins(1,3,4,5,6)P_5, 10 nM–0.1 mM and Ins(1,4,5)P_3, 10 nM–0.1 mM) were added in 100 μl (10× final concentrations) aliquots. The pH-dependency of [^{3}H]-$InsP_6$ binding was examined by resuspending the neutrophil membranes in 20 mM Tris, 20 mM NaCl, 100 mM KCl, 5 mM EDTA buffered over an appropriate pH range with Trizma maleate-HCl (pH 5.5–7.0) or Trizma base-HCl (7.5–9.0). The effect of the cations Mg^{2+} and Ca^{2+} on [^{3}H]-$InsP_6$ binding was investigated using predetermined EDTA, EGTA and $MgCl_2$ additions to the above buffer, as detailed in the results section. The effect of protein denaturation on [^{3}H]-$InsP_6$ binding was assessed by heating the membranes to 100°C for 90 min prior to use.

To examine whether the [^{3}H]-$InsP_6$ binding observed was to an intra- or extracellular site, assays were performed with intact, freshly prepared neutrophils (3×10^6 ml^{-1}, equivalent to 0.1 mg ml^{-1} protein) incubated at 4°C in either PBS containing 25 mM HEPES (pH 7.4) or 20 mM Tris (pH 7.5), 20 mM NaCl, 100 mM KCl and 5 mM EDTA. Cells were layered over 0.4 ml silicone oil, incubated for 90 min on ice and then centrifuged (15,000 *g*, 1 min). Aliquots (200 μl) of the supernatants were removed and transferred to scintillation vials. The remaining supernatant and oil layers were aspirated and discarded, and the cell pellets dissolved in methanol and radioactivity determined. Parallel incubations were performed to assess [^{3}H]-$InsP_6$ binding to neutrophil membranes prepared from the same batch of cells and [^{3}H]-LTB_4 binding to intact cells, as detailed previously (O'Flaherty *et al.*, 1986; 1991).

Neutrophil shape change assay

The effect of $InsP_6$ on fMLP-induced shape-change was assessed by incubating 3×10^6 neutrophils in 500 μl PBS containing 1 mM $MgCl_2$, 1 mM $CaCl_2$ and 25 mM HEPES (pH 7.3) at 37°C, with a pre-determined optimal concentration of $InsP_6$ (100 μM), for 0.5–30 min prior to addition of 0.1 nM fMLP for 5 min. Preliminary concentration-response studies had identified this as the fMLP concentration required to induce submaximal (approx. 50%) shape change (data not shown). Incubations were terminated by the addition of 500 μl 2.5% gluteraldehyde and shape-change was quantified by phase contrast light microscopy as the percentage of neutrophils extruding more than one pseudopodium. Identical incubations were performed with LPS (100 ng ml^{-1}, 60 min), TNFα (200 u ml^{-1}, 30 min) and PAF (100 nM, 5 min).

Superoxide anion generation

Neutrophils were resuspended at 1×10^6 cells ml^{-1} in PBS containing 1 mM $CaCl_2$, 1 mM $MgCl_2$ and 25 mM HEPES (pH 7.3) and preincubated at 37°C with buffer, $InsP_6$ (100 μM, 30 s), LPS (100 ng ml^{-1}, 60 min), TNFα (200 u ml^{-1}, 30 min) or PAF (100 nM, 5 min) in a final volume of 100 μl. These pretreatment periods and agonist concentrations were established in preliminary experiments designed to ascertain optimal priming conditions for each agent. The cells were then stimulated with fMLP (100 nM, 15 min) in the presence of 80 μM cytochrome C, with superoxide dismutase (375 u) added to one tube in each set of quadruplicate incubations. Reactions were terminated by placing the cells on ice followed by centrifugation (15,000 *g*, 5 min, 4°C). The superoxide-dismutase-inhibitable reduction of cytochrome C was determined in each supernatant by measurement of the peak absorbance between 535–565 nm, with a Pye-Unicam scanning spectrophotometer, and expressed as nmol superoxide anion generated per 10^6 cells.

Drugs and chemicals

Inositol hexakisphosphate ($InsP_6$, di-potassium salt), N-formyl-methionyl-leucyl-phenylalanine (fMLP), superoxide dismutase, cytochrome C, platelet-activating factor (PAF), lipopolysaccharide (LPS, E. coli 0111:B4), phosphate-buffered

saline (PBS, with or without $CaCl_2$ and $MgCl_2$), dextran-500 and Percoll were all purchased from Sigma (Poole). Tumour necrosis factor-α (TNFα) was obtained from Genzyme (Cambridge, MA, U.S.A.). Inositol pentakisphosphate ($Ins(1,3,4,5,6)P_5$) was purchased from Calbiochem (Nottingham) and inositol 1,4,5-trisphosphate ($Ins(1,4,5)P_3$) from RBI (St Albans). [^{3}H]-inositol hexakisphosphate (specific activity 15–24 Ci mmol^{-1}) was obtained from DuPont-New England Nuclear (Stevenage, Herts.). Silicone oil F-50 was obtained from Croylek Ltd. (Surrey). All other reagents and chemicals were purchased from Life Technologies (Paisley), BDH (Poole), Phoenix Pharmaceuticals Ltd. (Gloucester) or Packard (Pangbourne, Berks.) and were of the highest grade available.

Statistics

All values are expressed as means ± s.e.mean of (n) separate experiments. Values, where applicable, were compared by ANOVA or Student's t test for paired data, with $P < 0.05$ considered to be significant. Significant differences between groups were determined by the Newman-Keuls procedure.

Results

[^{3}H]-$InsP_6$ binding sites in human neutrophil membranes

Under the assay conditions defined (2.5 nM [^{3}H]-$InsP_6$, 0.1 mg membrane protein, 90 min incubations on ice), total and non-specific [^{3}H]-$InsP_6$ binding represented approximately 3,000 (approximately 200 fmol mg^{-1} protein) and 300 d.p.m. respectively. Analysis of [^{3}H]-$InsP_6$ displacement by $InsP_6$ (Figure 1a) gave a Hill coefficient of 0.55 and a curvilinear bound versus bound × inhibitor plot (Figure 1b), indicating the presence of at least two binding sites. The curve was best-fitted to a two-site logistic model, where 53% of the $InsP_6$ bound to a site with a K_D of 150 nM and the remainder to a 5 μM K_D site. As predicted from these values, [^{3}H]-$InsP_6$ binding failed to saturate fully up to a radioligand concentration of 10 nM and kinetic experiments demonstrated incomplete displacement of steady-state [^{3}H]-$InsP_6$ binding following addition of 100 μM unlabelled $InsP_6$ (60% displacement at 45 min, data not shown). $Ins(1,3,4,5,6)P_5$ and $Ins(1,4,5)P_3$ displaced [^{3}H]-$InsP_6$ binding with IC_{50} values of 430 nM and 30 μM respectively ($n = 8$) (Figure 1a). In the absence of membranes, total [^{3}H]-$InsP_6$ binding was equal to the non-specific binding determined in the presence of membranes. Incubations with pre-boiled membranes reduced specific [^{3}H]-$InsP_6$ binding by > 90% ($n = 8$, data not shown).

The possibility that the multi-site, low affinity [^{3}H]-$InsP_6$ binding observed was due to metabolism of the radioligand was investigated by h.p.l.c. analysis of the post-incubation supernatants, by a method designed to detect inositol hexakisphosphate metabolites ([^{3}H]-$InsP_{1\text{-}5}$) (Hawkins *et al.*, 1990). These experiments demonstrated a start radioligand purity of > 99.9% and no detectable [^{3}H]-$InsP_6$ metabolism during the 90 min incubation period (data not shown).

Effect of pH on [^{3}H]-$InsP_6$ binding in human neutrophil membranes

Specific [^{3}H]-$InsP_6$ binding was markedly enhanced under alkaline conditions, with maximum binding at pH 8.0 (750 fmol mg^{-1} protein) (Figure 2). Non-specific binding was similar at all pH values studied (313 ± 24 d.p.m.).

Modulation of [^{3}H]-$InsP_6$ binding in human neutrophil membranes by mono- and divalent cations

In view of the suggestion that [^{3}H]-$InsP_6$ may associate with membranes through non-protein interactions, in a manner dependent upon trace metals (Poyner *et al.*, 1993), we examined the ability of various mono- and divalent cations to influence total [^{3}H]-$InsP_6$ binding to neutrophil membranes. For each buffer condition, [^{3}H]-$InsP_6$ binding was compared to that obtained in 20 mM Tris (pH 7.7), 20 mM NaCl, 100 mM KCl and 5 mM EDTA, with this value referred to as 100% binding (Figure 3). Omission of 5 mM EDTA increased total binding by 158 ± 20%. Replacement of the EDTA with 5 mM EGTA caused a 76 ± 25% increase in binding, with the further addition of 1 mM Mg^{2+} augmenting the binding by an additional 89 ± 3%. [^{3}H]-$InsP_6$ binding was also influenced by manipulating the concentration of Na^+ and K^+ present, with an increase in binding of 79 ± 18% seen in the absence of KCl and a decrease of 61 ± 6% seen with NaCl exclusion. Thus, the presence of Ca^{2+}, Mg^{2+} and Na^+ all appear to enhance, whereas K^+ inhibits, [^{3}H]-$InsP_6$ binding to human neutrophil membranes. Non-specific binding, determined in the presence of 100 μM $InsP_6$, was similar under all conditions studied

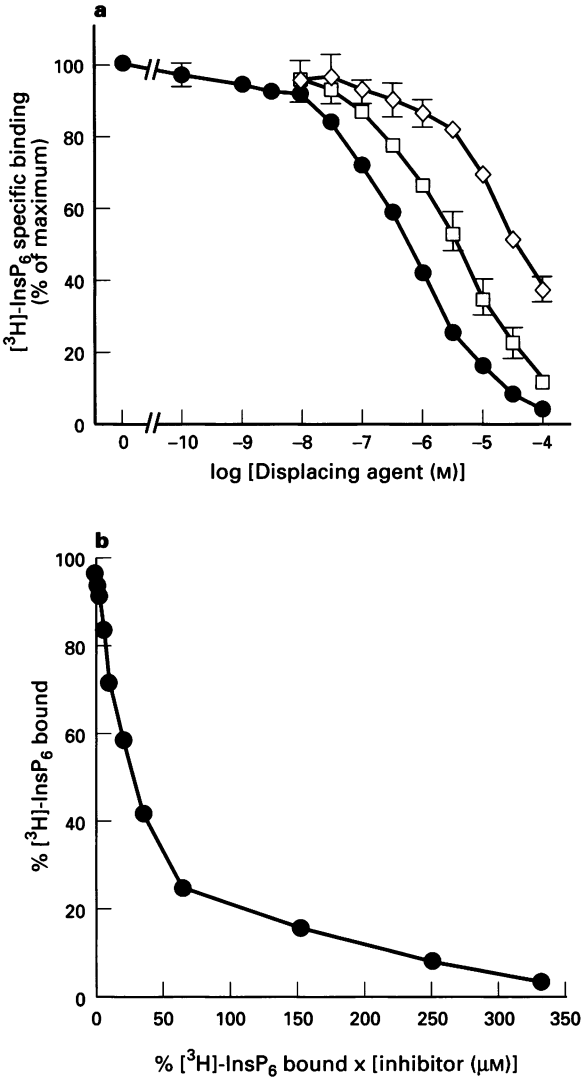


Figure 1 (a) Displacement of [^{3}H]-$InsP_6$ binding to human neutrophil membranes by $InsP_6$, $Ins(1,3,4,5,6)P_5$ and $Ins(1,4,5)P_3$; (b) bound versus bound × inhibitor plot for competition of [^{3}H]-$InsP_6$ binding by $InsP_6$. Assays were performed with 2.5 nM [^{3}H]-$InsP_6$, 0.1 mg of human neutrophil membrane fraction and increasing concentrations of $InsP_6$, (●), $Ins(1,3,4,5,6)P_5$ (□) and $Ins(1,4,5)P_3$ (◇) in 20 mM Tris/HCl/20 mM NaCl/100 mM KCl/5 mM EDTA buffer, pH 7.7 (final volume 1 ml). Incubations were performed for 90 min at 4°C, with separation of bound from free radioligand by centrifugation. Non-specific binding was determined in the presence of 100 μM unlabelled $InsP_6$. Values represent mean ± s.e.mean for 8 experiments each performed in duplicate.

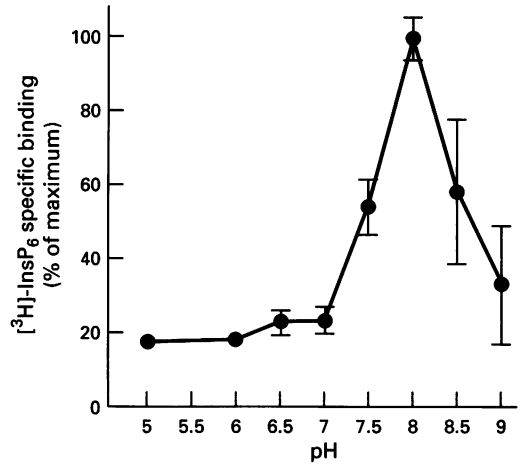


Figure 2 pH-dependence of specific [^{3}H]-$InsP_6$ binding to human neutrophil membranes. [^{3}H]-$InsP_6$ binding to human neutrophil membranes was determined using 2.5 nM [^{3}H]-$InsP_6$ and 0.1 mg membrane protein (as detailed in the legend to Figure 1) in a range of 25 mM Tris (pH 7.5–9) and Tris-maleate (pH 5.5–7) buffers (see Methods). Incubations were performed at 4°C for 90 min and non-specific binding determined in the presence of 100 μM unlabelled $InsP_6$. Values represent mean ± s.e.mean of maximal specific [^{3}H]-$InsP_6$ binding (13,355 ± 743 d.p.m.) for 6 determinations in two separate experiments.

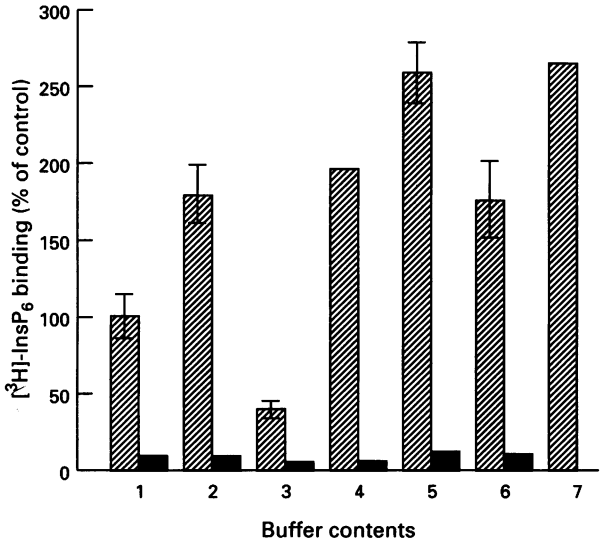
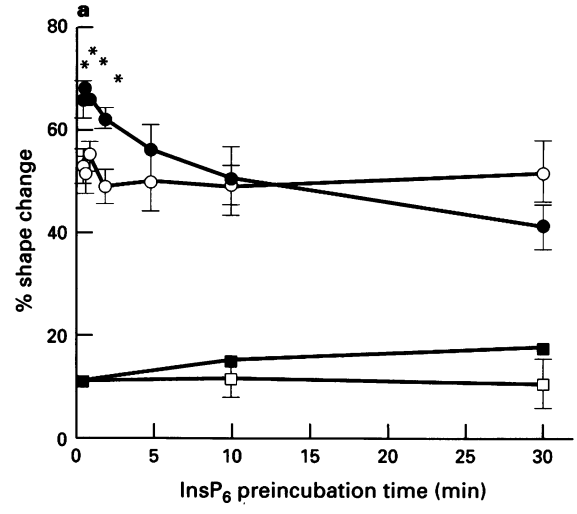


Figure 3 Effects of Mg^{2+}, Ca^{2+}, Na^+ and K^+ on [^{3}H]-$InsP_6$ binding to human neutrophil membranes. Assays were performed as outlined in the legend to Figure 1 except that following isolation, neutrophils were resuspended in a series of 20 mM Tris/HCl buffers (pH 7.7) with varying amounts of EDTA/EGTA/KCl/NaCl/$MgCl_2$ as detailed below. The cells were then homogenized, pelleted and resuspended in the same series of buffers at 0.1 mg protein ml^{-1} and [^{3}H]-$InsP_6$ binding (hatched columns) determined using 2.5 nM [^{3}H]-$InsP_6$ and an incubation period on ice of 90 min. Non-specific binding (solid columns) was determined in the presence of 100 μM unlabelled $InsP_6$. The buffers used were: Column (1), 5 mM EDTA, 100 mM KCl, 20 mM NaCl; Column (2), 5 mM EDTA, 20 mM NaCl; Column (3), 5 mM EDTA, 100 mM KCl; Column (4), 5 mM EDTA; Column (5), 100 mM KCl, 20 mM NaCl; Column (6), 5 mM EGTA, 100 mM KCl, 20 mM NaCl; Column (7), 5 mM EGTA, 100 mM KCl, 20 mM NaCl, 1 mM $MgCl_2$. Values represent mean ± s.e.mean of 3 experiments each performed in duplicate. (Where not shown, s.e.means were < 2% of means and fall within symbols). 100% binding represents 4,958 ± 197 d.p.m. Non-specific binding in buffer 7 was > 70% total [^{3}H]-$InsP_6$ added (see Results).



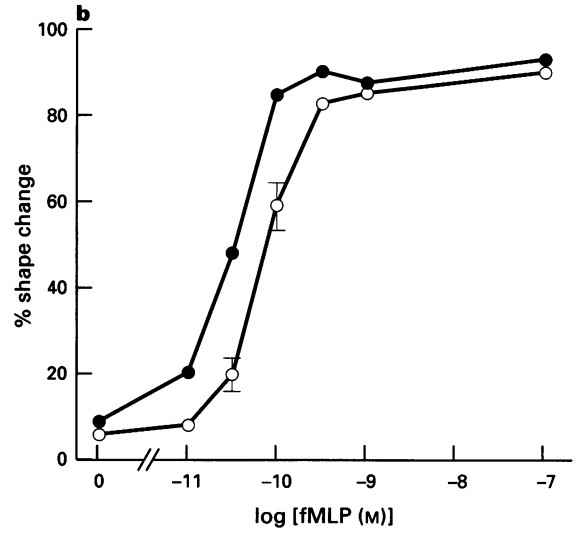


Figure 4 (a) Effect of $InsP_6$ preincubation time on fMLP-induced neutrophil shape change. Purified human neutrophils (3×10^6 ml^{-1}) were preincubated for various periods (0.5–30 min) with either $InsP_6$ (100 μM, closed symbols) or 20 mM HEPES PBS buffer (pH 7.3) (open symbols) prior to 5 min treatment with fMLP (0.1 nM, circles) or buffer (squares). Reactions were terminated, and shape change assessed as detailed in the Methods section. Values represent mean ± s.e.mean of 3 experiments, each performed in duplicate. $^*P < 0.05$, significantly different from fMLP alone (ANOVA). (b) Effect of $InsP_6$ on fMLP concentration-response curve for neutrophil shape change. Neutrophils were preincubated for 30 s with either $InsP_6$ (100 μM, closed symbols) or buffer (open symbols), prior to a 5 min treatment with fMLP. Values represent mean ± s.e.mean of triplicate determinations from a single experiment, with similar results obtained in a further 4 experiments.

(7.3 ± 0.8% of total binding) except that in the presence of 5 mM EGTA plus 1 mM $MgCl_2$ there was a dramatic increase in membrane pellet associated [^{3}H]-$InsP_6$ (52,014 ± 4,362 d.p.m. ie. approximately 70% of the total [^{3}H]-$InsP_6$ added), suggesting precipitation of an $InsP_6$-Mg^{2+} complex similar to that observed with Fe^{3+} concentrations > 10 μM (Poyner *et al.*, 1993).

[^{3}H]-$InsP_6$ binding to intact human neutrophils

A number of methods were used to assess whether the [^{3}H]-$InsP_6$ binding observed in neutrophil membranes represented binding to an intra- or extracellular recognition site. Incubation of freshly prepared neutrophils at 4°C for 90 min with

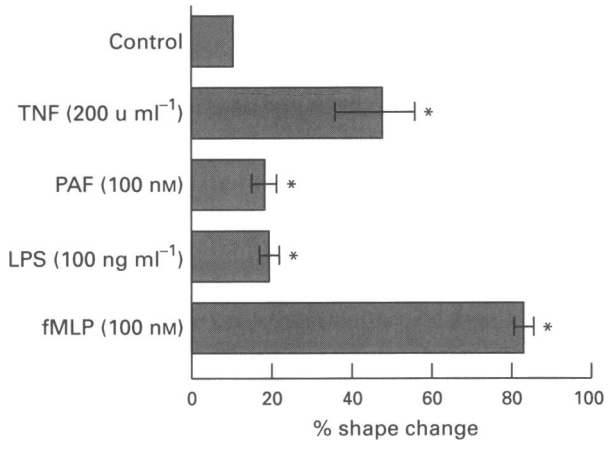


Figure 5 Effect of TNFα, PAF and LPS on neutrophil shape change. Human neutrophils (3×10^6 ml^{-1}) were incubated with TNFα (200 u ml^{-1}, 30 min), PAF (100 nM, 5 min), LPS (100 ng ml^{-1}, 60 min), fMLP (100 nM, 15 min), or 25 mM HEPES PBS buffer (pH 7.3) (control). Reactions were terminated, and shape change assessed as detailed in the Methods section. Values represent mean ± s.e.mean of 3 experiments, each performed in duplicate. Where not shown, s.e.means are <2% of means and fall within symbols. $^*P < 0.05$ significantly different from control (ANOVA).

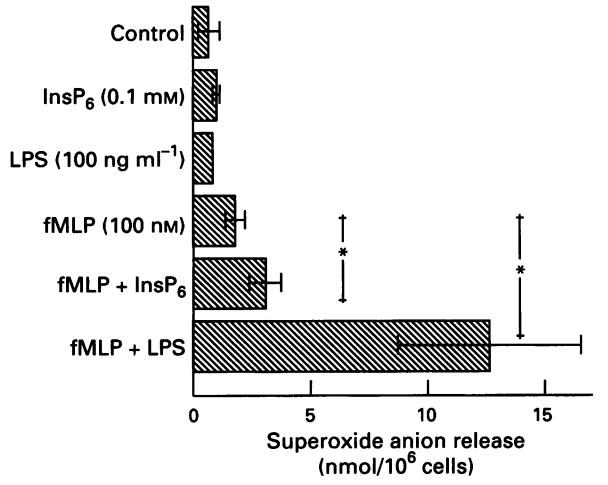


Figure 6 Comparison of the effects of $InsP_6$ and LPS on fMLP-induced superoxide anion generation in human neutrophils. Human neutrophils were suspended in PBS containing 25 mM HEPES as detailed in the Methods section and preincubated with 100 μM $InsP_6$ for 30 s or 100 ng ml^{-1} LPS for 60 min prior to a 15 min challenge with fMLP (100 nM). Superoxide anion release was measured with a spectrophotometric cytochrome C reduction assay and expressed as nmol superoxide anion generated/10^6 cells. Values represent mean ± s.e.mean from 10 experiments each carried out in triplicate. $^*P < 0.005$, significantly different from fMLP alone.

2.5 nM [^{3}H]-$InsP_6$ in either the above intracellular-like binding buffer or in 25 mM HEPES-buffered PBS containing 1 mM $CaCl_2$ and $MgCl_2$ (pH 7.5) produced a marked reduction in specific [^{3}H]-$InsP_6$ binding (76 ± 0.8% and 74 ± 1.2% respectively) compared to that observed in membranes. However, since assessment of cell viability demonstrated that approximately 10% of the pelleted neutrophils were trypan blue positive, an alternative separation method was followed using centrifugation through an inert oil cushion. Using this protocol, <0.03% of the [^{3}H]-$InsP_6$ added was associated with the cell pellet irrespective of the incubation buffer used. Under identical conditions, and in the same experiment, >16% specific [^{3}H]-LTB_4 binding was observed ($n = 2$, data not shown).

Table 1 Effects of TNFα and PAF on unstimulated and fMLP-induced superoxide anion generation in human neutrophils

	Superoxide anion generation (nmol/10^6 cells)	
	Unstimulated	*Stimulated*
Control	0.56 ± 0.08	3.50 ± 0.26
TNFα	0.88 ± 0.05	18.04 ± 2.33
PAF	0.78 ± 0.11	20.42 ± 2.16

Human neutrophils were suspended in PBS containing 25 mM HEPES as detailed in the Methods section, and preincubated with TNFα (200 u ml^{-1}, 30 min) or PAF (100 nM, 5 min) prior to a 15 min treatment with fMLP (100 nM). Superoxide anion release was assessed spectrophotometrically by a cytochrome C reduction assay and expressed as nmol superoxide anion generated/10^6 cells. Values represent mean ± s.e.mean of 3 separate experiments, each performed in triplicate.

Effect of $InsP_6$ on fMLP-stimulated shape change and superoxide anion generation

In view of the above data indicating the absence of true extracellular $InsP_6$ receptors in neutrophils, we sought to re-evaluate the functional effects of $InsP_6$ in these cells using respiratory burst activity and shape change as activation indices. The effect of $InsP_6$ on basal and fMLP-induced shape change was used as a sensitive indicator of potential chemotactic (Qu *et al.*, 1995) and priming (Haslett *et al.*, 1985) activity and also to determine the optimal $InsP_6$ preincubation period required for subsequent superoxide anion-priming experiments. Figure 4a illustrates the effects of incubating unprimed neutrophils with 100 μM $InsP_6$ for 0.5–30 min on basal and submaximal (0.1 nM) fMLP-induced neutrophil shape-change. $InsP_6$ (100 μM), unlike other established priming agents (Figure 5), had no effect on basal shape change (Figure 4a), but did cause a small and transient enhancement (26 ± 1.2% at 30 s) of fMLP-induced shape change (Figure 4a). TNFα, PAF and LPS did not enhance fMLP (100 nM)-induced shape change (data not shown). This pattern of effects (ie. transient enhancement of fMLP-induced shape change, but no effect of $InsP_6$ alone) correlates well with the time course effects of $InsP_6$ on fMLP-induced superoxide anion release reported by Eggleton & colleagues (1991) but is not observed with LPS, TNFα or PAF and hence appears to be unique to this priming agent (Young *et al.*, 1990). $InsP_6$ (100 μM, 30 s) also caused a small leftwards shift in the concentration-response curve for fMLP-induced shape change (fMLP alone, EC_{50} 76 pM; fMLP + $InsP_6$, EC_{50} 33 pM, $P < 0.01$).

The ability of $InsP_6$ to prime human neutrophils for enhanced fMLP-stimulated superoxide anion release was compared to the effects of lipopolysaccharide (100 ng ml^{-1}, 60 min), a well established neutrophil priming agent. $InsP_6$ alone (100 μM, 30 s) had no effect on basal superoxide anion release and caused only a very minor (1.8 ± 0.3 fold, $P < 0.005$, $n = 4$) enhancement of fMLP-stimulated superoxide anion generation compared with LPS (6.8 ± 0.6 fold, $P < 0.005$, $n = 4$) (Figure 6). This degree of priming of the fMLP-stimulated superoxide anion response by $InsP_6$ is very similar to that reported by Eggleton *et al.* (1991). In a separate series of experiments TNFα (200 u ml^{-1}, 30 min) and PAF (100 nM, 5 min) also enhanced fMLP-induced superoxide anion generation to a considerably greater extent than observed formerly with $InsP_6$ (Table 1).

Discussion

Neutrophils play a key role in defending the body against infection. However, the enormous histotoxic capacity of these cells dictates that uncontrolled or inappropriate activation can cause significant host tissue damage. One of the most important control steps involved in regulating respiratory burst activity is the requirement for the neutrophil to be primed before it will respond to a secretagogue challenge. While a wide variety of cell- and bacterial-derived products (eg. granulocyte-macrophage colony stimulating factor, PAF, TNFα and LPS) and physicochemical insults (eg. hypotonic challenge) can prime neutrophils, the specific intracellular mechanisms responsible for this process are yet to be fully defined.

Recently, $InsP_6$, a ubiquitous and abundant cytosolic inositol polyphosphate (Bunce *et al.*, 1993; Stuart *et al.*, 1994), was identified as a novel neutrophil priming agent, being able to facilitate fMLP-induced superoxide anion release without affecting basal superoxide anion generation (Eggleton *et al.*, 1991). In this study, preincubation of human neutrophils with $InsP_6$ (up to 250 μM) had no effect on basal superoxide anion generation but caused a 2 fold enhancement of the response to fMLP (2 μM). This led to the proposal that $InsP_6$, released from dying or effete cells at an inflammatory focus, may serve to augment local neutrophil respiratory burst activity. Our experiments sought to identify whether this effect of $InsP_6$ is receptor-mediated and re-evaluate its priming potential relative to other more established agents. Our data indicate that while specific, low affinity [^{3}H]-$InsP_6$ binding can be detected in neutrophil membranes, intact cells do not bind [^{3}H]-$InsP_6$, and that the absolute priming effect of $InsP_6$ is extremely weak and short-lived in comparison to other priming agents such as LPS and granulocyte macrophage colony stimulating factor, where the priming effect lasts for several hours (Balazovich *et al.*, 1991).

Analysis of [^{3}H]-$InsP_6$ binding to neutrophil membranes demonstrated the presence of at least two low affinity binding sites (K_D values of 0.15 and 5 μM), and displayed only a 3 fold selectivity for $InsP_6$ over Ins(1,3,4,5,6)P_5. These data contrast to the readily saturable, high affinity [^{3}H]-$InsP_6$ binding previously reported in, for example, rat cerebellum (Hawkins *et al.*, 1990), bovine adrenal chromaffin cells (Regunathan *et al.*, 1992) and canine cardiac microsomes (Kijima & Fleischer, 1992), and suggest that $InsP_6$ binding in human neutrophils may not reflect an interaction with any of the currently identified membrane-associated $InsP_6$ binding sites: these include the G-protein receptor regulatory protein arrestin (Regunathan *et al.*, 1992; Palczewski *et al.*, 1991), the IGF-II receptor (Kar *et al.*, 1994), the Golgi K^+ channel coatomer (Fleischer *et al.*, 1994) and the α-subunit of the clathrin assembly protein AP-2, recently identified as the $InsP_6$ receptor in rat cerebellum (Volgmaier *et al.*, 1992). This latter molecule is a 300–350 kDa protein involved in the formation of clathrin-coated vesicles at the plasma membrane, and is comprised of multiple subunits, including two doublets of 115 kDa and 105 kDa, which bind $InsP_6$ with a K_D of 12 nM (Theibert *et al.*, 1992), and two non-binding singlets of 50 and 17 kDa.

The pH-dependency of [^{3}H]-$InsP_6$ binding in neutrophil membranes also differs from that obtained in rat cerebellum (Theibert *et al.*, 1992) and rat cerebral cortex (Nicoletti *et al.*, 1990), where maximal binding occurred at pH 7 and 6, respectively. In addition, a pH optimum of 8, with marked inhibition of [^{3}H]-$InsP_6$ binding observed at more alkaline values, makes a simple charge-based membrane interaction unlikely. The ability of Mg^{2+} to potentiate [^{3}H]-$InsP_6$ binding in neutrophil membranes is qualitatively very similar to findings reported in rat cerebellum, where multivalent cations (Mg^{2+} and trace amounts of contaminating Fe^{3+} and Al^{3+}) augmented specific [^{3}H]-$InsP_6$ binding, possibly by acting as bridges between $InsP_6$ and negatively charged membrane phospholipid phosphates (Poyner *et al.*, 1993).

A variety of potential non-receptor mechanisms may underly the ability of $InsP_6$ to function as a weak priming agent. For example, it has recently been shown that negatively charged agents *per se* potentiate superoxide anion generation (Miyahara *et al.*, 1993) and also that $InsP_6$ can inhibit CD62-L (L-selectin)-mediated adherence of neutrophils to activated endothelial cells (Cecconi *et al.*, 1994). It is uncertain however how relevant this latter observation is to the priming effect of $InsP_6$ since cross-linking of CD62-L has recently been reported to induce rather than inhibit, neutrophil priming (Waddell *et al.*, 1994). It is also clearly possible that the powerful Ca^{2+} chelation properties or other, as yet unidentified, effects of $InsP_6$ may perturb neutrophil homeostasis. It should be noted however, that the studies of Eggleton and co-workers (1991) indicated that a similar priming effect is not observed with the lower inositol polyphosphates including Ins(1,3,4,5,6)P_5.

In summary, this study provides evidence for specific, low affinity, membrane associated [^{3}H]-$InsP_6$ binding in human neutrophils that is pH-dependent, heat-labile, augmented by Mg^{2+}, Ca^{2+} and Na^+ and located intracellularly. $InsP_6$, released from damaged or necrotic cells at an inflammatory focus, may interact with the neutrophil surface in a non-receptor-mediated fashion, to cause priming of NADPH oxidase function and polarization responses, but these effects are modest in comparison to other established priming agents.

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