Dynamic changes in neutrophil cytoskeleton during priming and subsequent surface stimulated functions

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Summary

The naturally occurring inositol polyphosphate, inositol hexakisphosphate (InsP₆), primate human neutrophils for enhanced responses to stimulatory agonists. Unlike other primers InsP₆, has no effect at high doses (up to 500 μM) on the neutrophil's basal rate of superoxide production (O₂⁻). Pretreatment of the cells for 2 min with InsP₆ (100 μM) substantially enhances FMLP (10⁻⁷ M)-induced respiratory burst. In investigations of the dynamic aspect of neutrophil actin during priming and stimulation with FMLP, the content of F-actin has been measured with and NBD (nitrobenz-2-oxa-1,3-diazole)-phallacidin assay. The induced assembly of F-actin is rapid (peaks at ~45 s) followed by depolymerization. In contrast, priming with InsP₆, with or without FMLP stimulation, results in a sustained assembly of F-actin as visualized with a rhodamine-phalloidin staining technique. These changes in macromolecular status and distribution of F-actin as visualized with a rhodamine-phalloidin staining technique. These changes in macromolecular status and distribution of F-actin during InsP₆ priming occur in the absence of any other demonstrable functional responses and thus InsP₆ may be a useful tool to follow early events in neutrophil functions or to monitor the presence of unprimed and preprimed subpopulations in the circulation. Since InsP₆ is present in high concentra-
tions in neutrophils (up to 100 \( \mu m \)), its release from damaged or effete cells may have a modulatory role on neutrophil functions.

Introduction

Cytoskeletal proteins, and particularly the microfilament protein actin, play a key role in regulating certain neutrophil functions. In addition to exerting effects upon surface membrane-receptor expression for activating ligands, and on membrane-associated enzymes involved in signal transduction and the stimulated respiratory burst, changes in the level of organization and disposition of the microfilament protein actin regulate a number of membrane-mediated motile activities, such as locomotion, chemotaxis, phagocytosis and secretion. In resting blood neutrophils, the proportion of total cell actin in the polymeric form is around 20–30%, and most of this is present in a filamentous network lying subadjacent to the cytoplasmic face of the plasma membrane and interacting with it via integral membrane proteins [1]. On stimulation of neutrophils with agonists such as the N-formyl methionyl peptides (e.g. FMLP) or particles for phagocytosis (e.g. bacteria, Zymosan, etc.), there is a rapid assembly of actin polymer (F-actin) from the large monomer pool in the cytoplasm [2], and although some of these subunits are sequestered as a profilactin complex, the pool of free actin monomer is generally sufficiently greater than the critical concentration for polymer assembly such that F-actin can form rapidly and regionally on stimulation without subunit diffusion delay. From DNAase-1 inhibition assays for actin (an assay which measures both monomeric and filamentous forms), and from labelling and assay techniques with fluorescent phalloidin derivatives, some knowledge has accrued about the magnitude of the dynamic shifts in assembly and disassembly of actin filaments in neutrophils in response to stimulation. In a careful study of the kinetics of FMLP-stimulated sequential polymerization and depolymerization of neutrophil actin, Howard & Oresajo [3] reported that the steady-state cell content of F-actin measured after 45 s and after 10 min exposure to FMLP was dose-dependent, but the rate of polymerization was not.

In contrast, the rate of actin depolymerization showed FMLP dose dependency. They suggested that the FMLP-induced polymer assembly and disassembly may be regulated by different intracellular mechanisms. One possible explanation for these differences may lie in the observations of Korchak et al. [4], who reported an FMLP dose-dependent increase in cytosolic \( \text{Ca}^{2+} \) during neutrophil stimulation. Since a \( \text{Ca}^{2+} \)-dependent capping protein, such as gelsolin, would terminate polymerization at the actin filament barbed ends, this could lead to subsequent filament depolymerization. However, the rapid formation and dissociation of the membrane-associated three-dimensional filament networks, the cross-linking actions of actin-binding proteins, such as filamin, the interactions of actin filaments with myosin in producing contractile forces, and the capping and destabilizing and severing mechanisms involved in filament disassembly, are just some of the features for which we at present lack the ‘small print’ molecular detail required to explain properly the multifaceted aspect of cytoskeletal regulation during stimulation. There is another aspect of neutrophil-stimulated functions, however, which is of equal physiological importance and to which little attention has been turned in relation to the membrane/cytoskeletal axis, i.e. the action of ‘priming agents’. It is now well established that the response of neutrophils to a stimulus can be greatly enhanced if the cells are first pretreated for a short period with a ‘priming agent’ before exposure to the stimulant or agonist. Since there is evidence of considerable functional heterogeneity in the neutrophil circulating pool [5, 6], possibly owing to the presence of both primed and unprimed subpopulations, priming may be a prerequisite for early margination, adhesion to vessel walls and ultimately their extravasation to sites of inflammatory foci elsewhere in the body. Our present understanding is that most physiologically relevant priming agents, such as FMLP, platelet-activating factor (PAF) and leukotriene B4 (LTB4), have little effect alone on the cells at low concentrations, but are themselves stimulants at higher concentrations [7]. Although stimulating concentrations are generally well above the threshold for optimum priming effects, the wide variation in dose–responses for stimulants between neutrophils from different normal donors together with the known heterogeneity profile of neutrophils in the circulating pool, due to either differences in receptor status or the presence of primed and unprimed subpopulations, makes for many difficulties in identifying and assessing normal and inflammatory states in human subjects from neutrophil studies. Particularly if priming agents which are themselves activators at higher concentrations, are used.

In this paper we present our findings with a newly discovered [8], physiologically occurring priming agent for human neutrophils, InsP3, and describe its effect on intracellular actin polymerization. Unlike other primers, InsP3 at high concentra-
Measurements of O2 production

Neutrophils incubated for 2 min in the presence or absence of a priming agent (InsP3) were assayed for the FMLP-stimulated production of O2 by the cytochrome c-reductase procedure [13] performed in the presence and absence of superoxide dismutase. In this assay the stimulus was 10−8 M FMLP and the dismutase-inhibitable reduction of the ferric cytochrome c was monitored at 550 nm and expressed as nmol/min per 107 cells. Basal values for O2 production of resting unstimulated neutrophils were generally below the sensitivity limits of the assay.

Total cell F-actin content

This assay was based upon that described in [3]. Neutrophil suspensions (0.75 ml; 107 cells/ml) were preincubated at 37°C for 5 min and exposed to primer, stimulant or control HBSS buffer for timed periods according to the experimental protocol. For experiments with FMLP, the concentration of carrier vehicle (dimethylsulphoxide) in solutions of 10−6 M-FMLP was <0.01% (v/v) and without effect upon the basal F-actin content in control studies. The cells were then fixed by adding an equal volume of formalin (3.7%, v/v) at 25°C. After 10 min the fixed cells were washed with buffer and permeabilized and stained with 2 mg of lysophosphatidylcholine/ml containing 330 nM-NBD-phallacidin (Molecular Probes, Eugene, Oregon). After incubation at room temperature in the dark, the NBD-phallacidin-stained cells were sedimented and extracted with methanol overnight at 4°C with frequent vortexing. After centrifugation the supernatant was removed. The relative fluorescent intensity measured in an Aminco Bowman spectrofluorimeter (excitation 465 nm and emission 535 nm). Non-specific phalloidin binding (generally <5%) was determined from parallel experiments in which the NBD-phallacidin binding was blocked with a 100-fold molar excess of non-fluorescent phallacidin.

Morphological studies

For these studies we used the rhodamine-phalloidin-binding assay developed by Cassimeris et al. [14]. Briefly, finger-prick drops of blood were allowed to clot on a glass coverslip sealed in a moist chamber in an incubator at 37°C for 45 min. The clot was rinsed away with HBSS leaving a monolayer of mainly neutrophils on the glass. These attached cells were incubated with primer, stimulant or buffer for periods of 45 s, 2 min and 10 min. The coverslips were then washed by immersion in HBSS and fixed in 3.7% (v/v) formalin for 10 min. After fixation the adherent cells were permeabilized with lysophosphatidylcholine (2 mg/ml) and stained with rhodamine-phalloidin (330 nM in HBSS) for 1 h. The coverslips were then mounted on slides and the cells examined with the u.v. microscope and photographed at 400 × magnification.
**Results**

In preliminary investigations we confirmed our earlier observations [8] that the preincubation of human neutrophils with InsP₄ (in the range 25-500 μM) for periods up to 5 min at 37°C had no measurable effect upon the basal rate of O₂ production as determined by the spectrophotometric cytochrome c-reduction assay.

Fig. 1 shows a dose-dependent priming effect when neutrophils from three normal subjects were preincubated with InsP₄ for 2 min at 37°C before stimulation with 10⁻⁷ M-FMLP. The change in the rate of O₂ production (measured as nmol of O₂/10⁷ cells per min) has been expressed as the percentage increase above the corresponding control rates for neutrophils from the same donor exposed to the stimulus without pretreatment with InsP₄. The effect of the priming was to increase the rate of O₂ production 50-40% at the higher concentrations of InsP₄. Included also in Fig. 1 are data for the neutrophils from another three normal subjects in which the isolated cells were first exposed to InsP₄ (250 μM) for 2 min and 5 min periods before stimulation with 10⁻⁷ M-FMLP. Here the percentage increase in the rate of O₂ production above that for control cells exposed only to the stimulus lay in the range 27-200%.

Since there is a general agreement that the stimulation of neutrophil actin polymerization by chemotactic agents, such as FMLP (when applied at concentrations > 10⁻⁸ M), is a rapid event, with maximum formation of F-actin occurring between 30 and 60 s after the stimulus, followed by slow depolymerization during the next 3-10 min, we investigated this time course for both FMLP and InsP₄ using the NBD-phallacidin binding assay for F-actin. Freshly isolated neutrophils were divided into three aliquots and exposed to FMLP at either a priming concentration (10⁻¹⁰ M) or a stimulating concentration (10⁻⁸ M), and to a priming concentration of InsP₄ (100 μM). The amount of F-actin present in the FMLP-treated cells was measured at 10, 45 and 300 s after addition of the stimulatory peptide and at 1, 2, 5 and 10 min after addition of the InsP₄. The results are shown in Fig. 2(a) where it can be seen that for both the priming and stimu-
lating concentrations of FMLP, the polymerization of actin was very rapid with maximum assembly occurring in 45 s. The increase in F-actin was \( \sim 30\% \) higher with the priming dose of FMLP (a concentration that did not induce \( \mathbf{O}_2^- \) generation) and over \( 60\% \) higher for the stimulatory concentration when compared with the values for resting control cells. There was evidence of depolymerization occurring at 5 min, and this was modest with \( 10^{-10} \) M-FMLP and substantial with \( 10^{-8} \) M-FMLP. With \( \text{InsP}_3 \) priming, the results are similar to those presented in Fig. 1, but the rate of polymer formation was slower. A substantial increase in F-actin content occurred 2 min after the stimulus and the polymerization was maximum at 5 min. Again there was evidence for depolymerization after a longer period of exposure to \( \text{InsP}_3 \).

Using this time course information, the neutrophils from a number of normal subjects were studied for the priming effect of pretreatment with \( \text{InsP}_3 \) on subsequent FMLP-stimulated F-actin polymerization. The cells were exposed to 100 \( \mu\)M-InsP, for 2 min, FMLP was added and the content of F-actin measured after 45 s. Three different concentrations of FMLP were used, \( 10^{-9} \) M, \( 10^{-8} \) M and \( 10^{-7} \) M, in order to span the variability in response to this stimulant previously observed with different donor neutrophils and to also cover the functional heterogeneity in the circulating pool seen in single individuals. Typical results for three different normal donor neutrophils are presented in Fig. 2(b). The unprimed cells from all three subjects showed significant FMLP-stimulated increases in F-actin content at all three stimulant concentrations measured 45 s after addition of FMLP. In all experiments \( \text{InsP}_3 \) pretreatment for 2 min resulted in a significant enhancement of polymerization over unprimed controls when these were also measured 45 s after addition of the stimulant. In the morphological studies using rhodamine-phalloidin staining of F-actin in fixed and permeabilized neutrophils, the cells were attached to glass coverslips before treatment with priming agents, agonist or control buffer. They were fixed, permeabilized and stained \textit{in situ} with the fluorochrome-labelled phalloidin. Photographs taken were of random fields selected by different observers to reduce subjective bias.

Glass attachment is of course itself a stimulus for certain neutrophil motile functions such as spreading and the formation of filopodia arising from the surface membrane. Although this introduces interpretational difficulties it also allows one to follow motile phenomena morphologically in parallel with changes in the status of F-actin. Fig. 3 shows micrographs of attached neutrophils observed 45 s, 2 min and 10 min after exposure of the attached cells to FMLP (\( 10^{-7} \) M) and \( \text{InsP}_3 \) (100 \( \mu\)M).

It can be seen that 45 s after exposure to \( \text{InsP}_3 \), the attached cells remain essentially spheroid with a prominent circumferential network of F-actin adjacent to the surface membrane and visualized with the fluorochrome-labelled phalloidin. In contrast, the FMLP-treated neutrophils have begun to spread after 45 s and the peripheral distribution of F-actin is more patchy with membrane filopodia forming. After 2 min exposure to \( \text{InsP}_3 \), these neutrophils too have begun to spread and after 10 min most of the actin is now present as punctate spots suggesting that cleavage of the longer F-actin filaments into shorter oligomeric fragments has occurred. In contrast, 2 min and 10 min after exposure to FMLP substantial depolymerization has occurred with very few cells showing phalloidin-stained structures.

**Discussion**

A major barrier to our understanding of the complex intracellular mechanisms controlling the assembly and disassembly of the neutrophil micro-filament protein actin during motile functions is the heterogeneity in the circulating pool of cells. This heterogeneity, which may be in part senescence dependent, and part due to random factors, is expressed with respect to surface receptor status and their linked signal transduction processes and also to the co-existence of primed and unprimed (or resting state) neutrophil subpopulations. The action of priming agents has generally been demonstrated by pre-incubating neutrophils with certain agonists at low concentrations insufficient to trigger chemo-taxis or superoxide (\( \mathbf{O}_2^- \)) production. When the cells are subsequently exposed to agonists at stimulatory concentrations, the responses are substantially enhanced above those seen with the stimulant only. This priming phenomena has been demonstrated with stimuli as molecularly diverse as FMLP, PAF, LTB\(_4\), diacylglycerol, granulocyte-macrophage colony-stimulating factor, lactoferrin, tumour necrosis factor-\( \alpha \) and endotoxins, but there is as yet no satisfactory explanation of why such pretreatment should enhance subsequent responses. Up-regulation of surface receptors for agonists has been suggested as well as modulation of intracellular protein kinase C, but such explanations do not fit all aspects of priming or the many different priming agents known to exist physiologically. Changes in neutrophil Ca\(^{2+}\) haemostasis through extracellular Ca\(^{2+}\) mobilization has been
Fig. 3

Changes in molecular organization and intracellular disposition of F-actin visualized by rhodamine-phalloidin staining of glass-attached neutrophils treated with 100 μM-InsP₄ (a, b and c) or 10⁻⁶ M-FMLP (d, e and f)

The photomicrographs were taken at 45 s (a and d), 2 min (b and e) and 10 min (c and f) after adding the InsP₄ or FMLP to the adherent neutrophils. Note the uniform spheroid appearance of the InsP₄-treated cells at 45 s with pronounced cortical staining of F-actin in contrast to the shape changes and filopodia formation seen in the FMLP-treated neutrophils. After 2 and 10 min, the FMLP-treated cells show substantial depolymerization, whereas the InsP₄-primed neutrophils still show extensive staining after 10 min, but the intracellular distribution is now punctate. Pretreatment with InsP₄ followed by FMLP stimulation (10⁻⁶ M) gave essentially the same sustained distribution of F-actin as seen in a, b and c over the same time course (micrographs not shown here).

questioned by the finding that, as with certain stimulants, priming can be achieved with neutrophils in the presence of EGTA [7, 15]. Moreover no significant changes in inositol phosphate metabolism during FMLP priming have been reported. Effects upon various membrane G-proteins is being actively considered. An added complexity arises from the fact that what may be a priming concentration for a particular agonist (such as FMLP) with one normal donor's neutrophils, showing no change in O₂⁻ production over basal rats, may be a stimulant concentration for another, with substantial production of O₂⁻ as a result. In fact, the neutrophil literature is replete with findings where this between-donor heterogeneity in responsiveness has not been fully appreciated. This has added some measure of confusion and controversy to this area of research. In a few published reports it is clear that most of the data have been derived from neutrophils of a single donor pool. The importance of these concentration-dependent difficulties in interpreting priming and stimulus-induced changes in the level of organization and disposition of the cytoskeletal protein actin is paramount. This is particularly so now that it is fairly well established that, for example, with FMLP stimulation, the rate of the
rapid polymerization of actin is not dose dependent, whereas the rate of depolymerization appears to be more closely related to the FMLP concentration [3].

In earlier studies we reported the priming function for human neutrophils of InsP₆ [8]. Of a range of other inositol phosphates studied, with the possible exception of inositol 1,2,6-trisphosphate (which has a weak priming action), no other compound tested showed any priming action towards FMLP or phorbol 12-myristate 13-acetate-stimulated respiratory burst, or towards bacteria or zymosan-induced phagocytosis [8]. Priming with InsP₆ appears to be unique in this respect, giving after 2 min preincubation, enhanced responses to all these stimuli when they were subsequently presented to the neutrophils at activation dose levels. Since InsP₆ alone appears to be without effect on any of these functions at concentrations up to 500 µM, we felt it may have some advantages over other more concentration-dependent priming agents in exploring any intracellular changes that may be observable during priming.

In the present investigations we have used a quantitative procedure for the measurement of total neutrophil F-actin content based upon the binding of a fluorescent derivative of phalloidin, NBD-phallacidin. At a priming dose of FMLP (10⁻¹⁰ M), a concentration which has no effect upon the rate of O₂⁻ production, there is a rapid increase in the F-actin content of the neutrophils reaching a level after 45 s 25–30% higher than the content of resting neutrophils. Priming doses of InsP₆ (100 µM) gave similar increases in polymerization, but the time course was slower, reaching peak polymer formation at or around 5 min. In both priming circumstances there is evidence of depolymerization of the F-actin occurring with increased times of exposure to the priming agents. At stimulatory doses of FMLP (10⁻⁶ M) the content of F-actin is increased over 60% above resting control cell levels, but at these stimulant concentrations depolymerization is substantial after 5 min of exposure to FMLP.

The morphological studies performed on attached neutrophils have confirmed the reports of others [13, 14] that exposure of neutrophils to both priming and stimulating levels of FMLP results in the rapid and sequential polymerization/depolymerization of F-actin as quantitatively demonstrated in the F-actin assays of cells in suspension. Concomitant with these assembly/disassembly events, protrusions form from the spreading cells and although the force-generating mechanisms for these shape changes are not known, some initial dislocation of the membrane-associated cortical network appears to occur which may liberate membrane domains from cytoskeletal constraints. We have previously demonstrated that domain-specific decreases in F-actin occur during pig neutrophil phagocytosis [5] in the polymer content of phagocytic vesicle membranes, while the plasma membrane regions not involved in the phagocytic event and surface membranes from resting neutrophils show less depolymerization.

The use of InsP₆, which has been shown to have priming action for agonist-induced functions, may have particular value in studying cytoskeletal dynamics. During priming with InsP₆, the polymerization of F-actin occurs on a much slower time scale than with FMLP. Moreover, unlike other priming agents, high concentrations of InsP₆ do not generate O₂⁻ production, yet prime the cells for respiratory burst induced by other agonists. It may therefore also be a useful tool for further investigating the relationship between the dynamic features of the neutrophil's cytoskeletal elements and the membrane-associated enzyme systems which produce reactive oxygen intermediates on stimulation. This link has not yet been firmly established.

In comparison with other physiologically occurring primers which operate at low concentrations (e.g. PAF 10⁻¹⁰ M, FMLP 10⁻¹⁰ M), InsP₆ priming concentrations are relatively high (50–100 µM). However, such high intracellular concentrations are known to occur in mammalian cells and its local release from lysed or effete neutrophils may well have a physiologically relevant modulatory role for neutrophil behaviour.

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Generation of plasma membrane domains in polarized epithelial cells: role of cell–cell contacts and assembly of the membrane cytoskeleton

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Introduction

Polarized epithelial cells form barriers between two biological compartments in the body and regulate the ionic composition of these compartments by vectorial transport of ions and solutes. In tissues such as the kidney, the epithelium separates the lumen of the tubule from the blood supply. Ions and solutes are reabsorbed from the tubule lumen (ultrafiltrate) and vectorially delivered to the blood supply on the other side of the epithelium. This unique function of the epithelium requires that the space between cells is sealed by the tight junction, and that there is a non-random distribution of proteins between the membrane domains, apical and basal-lateral, that face different biological compartments. Proteins involved in the absorption of ions and solutes, including ion channels and cotransporters, are restricted to the apical membrane domain, whereas proteins involved in signal transduction, cell-cell and -cell-substratum contact, and some ion transporters (e.g. Na+/K+-ATPase), are restricted to the basal-lateral domain.

Much of the current interest in polarized epithelial cells has focused on the mechanisms involved in the establishment and maintenance of these restricted distributions of membrane proteins (for reviews, see [1, 2]). Studies of polarized epithelial cells in culture have yielded important insights into the mechanisms involved. Madin-Darby canine kidney (MDCK) cells, a cell line derived from canine renal epithelium, form continuous monolayers of cells that are structurally and functionally polarized, and exhibit a polarized distribution of membrane proteins between the apical and basal-lateral membrane domains similar to that found in the cells of origin [1, 2]. The majority of recent studies have focused on the delivery of newly synthesized proteins from the Golgi complex to the plasma membranes in these cells. Results have shown clearly that in this cell type proteins arrive efficiently and directly at the appropriate membrane domains; this indicates that apical and basal-lateral membrane proteins are sorted in the trans-Golgi network before delivery to the cell surface [3].

Studies of other cell types, including hepatocytes [4] and intestinal epithelia (e.g. Caco 2 cells [5]), have demonstrated more complex pathways of protein delivery to the cell surface involving endocytosis and transcytosis of proteins inserted into the ‘incorrect’ membrane domain.

While these studies address the question of the mechanism(s) involved in the maintenance of polarized distributions of membrane proteins, they do not provide direct insight into those mechanisms involved in the establishment of cell polarity. This too is a complex problem since, in vivo, polarized epithelial cells arise from non-polarized precursor cells [2, 6, 7]. The formation of a polarized epithelial cell requires several fundamental changes in the structural and functional organization of the precursor cell: the formation of an apical cell surface; development of specific cell–cell, and, probably, cell–substratum contacts; and the remodelling of the distribution of proteins constitutively expressed in the non-polarized precursor cell to a more restricted distribution in the polarized cell [2].

Ideally, we would like to address the mechanisms involved in the development of a polarized epithelial cell by studying the process in vivo. Recent studies of the formation of the trophecto-