Actin polymerization in neutrophils is triggered without a requirement for a rise in cytoplasmic Ca^{2+}

Futwan A. AL-MOHANNA and Maurice B. HALLETT*

Department of Surgery, University of Wales College of Medicine, Heath Park, Cardiff CF4 4XN, U.K.

Stimulation of rat neutrophils with the peptide fMetLeuPhe caused (i) the appearance of a 40 kDa protein in the Triton-X-100-insoluble cytoskeleton, (ii) the disappearance of DNAase inhibition from the cytosol and (iii) the appearance of *N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)phalloidin (NBD-phalloidin) binding sites. All three observations were consistent with a rapid and transient assembly of polymerized actin, peaking at approximately 5 s and returning to near resting levels within 40 s. By experimentally depleting the cells of Ca^{2+} and increasing the cytoplasmic Ca^{2+} buffering capacity, the peptide-induced Ca^{2+} transient was reduced from a peak of 900 nM to 250 nM, without inhibiting actin polymerization, and this peak was sustained for at least 2 min. A further dissociation between the triggering of actin polymerization and peptide-induced Ca^{2+} elevation and oxidase activation was demonstrated at low concentrations of peptide (1–100 μM), actin polymerization being triggered without an elevation in Ca^{2+} or activation of the oxidase. Two other agents which induced actin polymerization, phorbol 12-myristate 13-acetate and latex beads, failed to elevate cytoplasmic Ca^{2+} . It was therefore concluded that neither Ca^{2+} nor those intracellular messengers which act with Ca^{2+} to trigger the neutrophil oxidase are responsible for triggering actin polymerization in neutrophils.

INTRODUCTION

Neutrophils play a key role in combatting infection by chemotaxis towards an inflammatory site, phagocytosis of micro-organisms and activation of a bactericidal oxidase system. Inhibitors of actin polymerization such as cytochalasins and botulinum C_2 toxin have profound effects on these responses. Whereas phagocytosis and chemotaxis are inhibited, activation of the oxidase system is not. Furthermore, with stimuli which operate through a rise in cytoplasmic Ca^{2+} , inhibition of actin polymerization results in exocytosis of secondary granules and a marked amplification of the oxidase response (Becker *et al.*, 1979; Al-Mohanna & Hallett, 1987; Al-Mohanna *et al.*, 1987), with subsequent extracellular production of reactive oxygen metabolites (Hallett & Campbell, 1983a). The control of actin polymerization therefore represents an important step in determining the nature and magnitude of the response by neutrophils to a particular stimulus. Elucidation of the relationship between the intracellular triggers for actin polymerization and for other cellular events may thus be crucial for an understanding of the role of these cells in inflammatory disease.

Although the polymerization of G-actin to F-actin is provoked by many neutrophil stimuli, the trigger for the event has not been established. It has however been suggested that cytoplasmic Ca^{2+} , perhaps acting through gelsolin (Stossel *et al.*, 1985), may be the initial trigger. The aim of the work presented in this paper was therefore to test whether cytoplasmic Ca^{2+} triggers or plays other roles in controlling the extent or duration of actin polymerization induced by the chemotactic and oxidase-stimulating peptide, fMetLeuPhe.

MATERIALS AND METHODS

Materials

Fura2-acetoxymethyl ester, bis-(*o*-aminophenoxy)ethane-*NNN'*-tetra-acetic acid (BAPTA)-acetoxymethyl ester and *N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)-phalloidin (NBD-phalloidin) were purchased from Molecular Probes, Junction City, OR, U.S.A. Ionomycin was purchased from Calbiochem, Cambridge, U.K. DNA type DNAase I, phorbol 12-myristate 13-acetate (PMA), cytochalasin B and the peptide fMetLeuPhe were purchased from Sigma Chemical Co., Poole, Dorset, U.K. Fluorescein-conjugated latex beads were purchased from Uniscience, Northampton, U.K. All other reagents were of analytical grade. Botulinum C_2 toxin was kindly provided by Professor I. Ohishi, University of Osaka, Sakai, Japan.

Preparation of rat peritoneal neutrophils

Neutrophils were isolated from the peritoneal exudate of rats injected intraperitoneally with sterile sodium caseinate (12%, w/v) as described previously (Hallett *et al.*, 1982). After centrifugation through Ficoll-Hypaque and lysis of erythrocytes with NH_4Cl , the purified neutrophils (> 99%) were resuspended in Hepes-buffered Krebs medium containing NaCl (120 mM), KCl (4.8 mM), K_2HPO_4 (1.2 mM), Mg_2SO_4 (1.3 mM), CaCl_2 (1.3 mM), bovine serum albumin [0.1% (w/v)] and Hepes (25 mM), with the pH adjusted to 7.4 by the addition of NaOH.

Measurement of cytoplasmic Ca^{2+}

Rat neutrophils were loaded with fura2 to an intracellular concentration of approx. 100 μM using the

Abbreviations used: BAPTA, bis-(*o*-aminophenoxy)ethane-*NNN'*-tetra-acetic acid; NBD-phalloidin, *N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)-phalloidin; PMA, phorbol 12-myristate 13-acetate.

* To whom correspondence should be addressed.

acetoxymethoxyl ester as described previously (Al-Mohanna & Hallett, 1988). The cellular location of the indicator was cytoplasmic, and the cytoplasmic Ca^{2+} buffering capacity was raised by approx. 10%. The cytoplasmic Ca^{2+} concentration was calculated from the ratio of fluorescence intensity at 505 nm measured at excitation wavelengths of 340 nm and 380 nm using a Spex Fluorolog III with dual-excitation optics (Glen Spectra, Stanmore, Middx., U.K.). The signal was calibrated using ionomycin ($2\text{ }\mu\text{M}$) followed by extracellular EGTA to produce maximum and minimum ratio values respectively as described previously (Gryniewicz *et al.* 1985).

Measurement of cellular actin state

Neutrophils [$(2 \times 10^7) - (2 \times 10^8)/\text{ml}$] were placed in a stirred temperature-controlled chamber maintained at 37°C . After equilibrium for 3–5 min, the stimulus was added. Samples ($100\text{ }\mu\text{l}$) were withdrawn at defined time points and treated as described below.

(i) **40 kDa cytoskeletal protein.** The sample was placed in a cytoskeletal extraction medium ($100\text{ }\mu\text{l}$) as described by White *et al.* (1983) containing Triton X-100 (2%), KCl (20 mM), imidazole/HCl (20 mM), EGTA (20 mM) and sodium azide (4 mM), pH 7.0, for 10 min on ice. The sample was then centrifuged for 10 min at 13000 g , and the pellet was dissolved in $200\text{ }\mu\text{l}$ of medium containing SDS (10%, w/v), mercaptoethanol (13 mM) and glycerol (20%, v/v) by vortex-mixing for 60 min, followed by boiling for 30 min. Portions of the sample were diluted 1:1 with tris(hydroxymethyl)aminomethane (63 mM), glycerol (10%, v/v), SDS (2%, w/v), mercaptoethanol (5%, w/v) and Bromophenol Blue (0.02%). After boiling for a further 2 min and centrifugation, a $20\text{ }\mu\text{l}$ sample was taken for electrophoresis through a 10% polyacrylamide slab gel. The gels were then stained with Coomassie Blue and the amount of actin was quantified by densitometry and comparison with actin standards.

(ii) **DNAase inhibition.** The sample was placed into lysis buffer ($100\text{ }\mu\text{l}$) containing NaCl (154 mM), tris(hydroxymethyl)aminoethane (2.5 mM), Triton X-100 (2%, v/v) and DNAase I ($40\text{ }\mu\text{g}/\text{ml}$), pH 7.0, as described by Varani *et al.* (1983). After solubilization, the sample was centrifuged at 2800 g for 5 s and the supernatant was transferred to an assay solution containing ethidium bromide ($1.5\text{ }\mu\text{g}/\text{ml}$), DNA ($60\text{ }\mu\text{g}/\text{ml}$), CaCl_2 (0.1 mM) and tris(hydroxymethyl)aminomethane (2.5 mM), pH 8.0. The rate of decrease in fluorescence intensity at 602 nm (excitation 520 nm) was monitored and the percentage DNAase inhibition was calculated. The amount of G-actin was calculated from actin standards and the percentage of actin polymerized was calculated from the value obtained after 3 h in depolymerizing conditions.

(iii) **NBD-phalloidin staining.** The sample was added to 40% formaldehyde ($20\text{ }\mu\text{l}$), and after 15 min at room temperature, it was added to staining solution ($10\text{ }\mu\text{l}$) containing NBD-phalloidin ($3.3\text{ }\mu\text{M}$) and lysophosphatidylcholine (5 mg/ml) as described by Howard & Oresajo (1985). After 30 min in the dark, the samples were analysed by flow cytometry on a Becton–Dickinson FACS III, and the mode fluorescence of the distribution was determined.

Assay of phagocytosis

Fluorescein-conjugated latex beads ($1\text{ }\mu\text{m}$ diam.) were incubated with neutrophils in a stirred temperature-controlled chamber at 37°C . At defined time points, samples of the cell suspension were diluted by a factor of 100 with ice-cold medium and the number of fluorescent beads phagocytosed was counted under fluorescent microscopy.

Fig. 1. Effect of fMetLeuPhe on actin polymerization

The Figure shows (a) 40 kDa protein associated with the Triton-X-100-insoluble cytoskeleton (control = 1), (b) DNAase-inhibitory activity of the cytosol and (c) NBD-phalloidin binding to fixed cells. At time zero on each graph, fMetLeuPhe ($1\text{ }\mu\text{M}$) was added to the cells. The data shown are representative from a series of at least three different cell preparations.

Fig. 2. Effect of fMetLeuPhe on the population distribution of NBD-phalloidin binding

The distribution of fluorescence (x -axis, arbitrary units) in the population is shown for the times after addition of the peptide fMetLeuPhe ($1 \mu\text{M}$) indicated on the z -axis. The x and y -axes are constant for each spectra, and in each case 20000 cells were analysed at a flow rate of approx. 2000/s. The mean fluorescence of the population shows maximal displacement to higher fluorescence at 5 s, before returning to resting levels, and must therefore be compared with the time course shown in Fig. 1(c).

RESULTS

Temporal relationship between polymerization and cytoplasmic Ca^{2+}

Stimulation of rat neutrophils with the peptide fMetLeuPhe ($1 \mu\text{M}$) resulted in (i) an increase in the amount of 40 kDa protein associated with the Triton-X-100-insoluble cytoskeleton, (ii) a loss of DNAase-inhibitory activity from the cytosol, and (iii) an increase in binding of an F-actin-specific toxin, NBD-phalloidin. All three events were inhibited by cytochalasin B or botulinum C_2 toxin. These observations were therefore consistent with the assembly of Triton-insoluble F-actin, which binds phalloidin, from DNAase-inhibiting cytosolic G-actin. From calibration curves of DNAase inhibition and Coomassie Blue staining density, it was estimated that the rat neutrophil contained $6.3 \pm 0.7 \text{ pg}$ of actin/cell, of which approx. $44 \pm 11 \%$ ($n = 7$) was polymerized in the resting cell. The assembly of actin was transient, peaking at 5–10 s after stimulation and decaying to a prolonged plateau level (Fig. 1). Flow cytometry of NBD-phalloidin-stained cells produced no evidence for a subpopulation of cells responding with different kinetics, and it was thus concluded that population measurements provided a reflection of events occurring within individual cells (Fig. 2). At the peak of polymerization, 80–95 % of the total cellular actin was in the polymerized F-actin form. Microscopic examination of NBD-phalloidin-stained cells showed that the majority of this polymerized actin was at the cortical margins.

The cytoplasmic free Ca^{2+} in cells stimulated with the peptide also rose transiently; the peak concentration was approx. $0.9 \mu\text{M}$, being reached after 6 s and decaying to a low plateau with a half-time of approx. 30 s (Fig. 3). Although the time resolution for the detection of actin polymerization was limited to approx. 5 s, its temporal relationship with cytoplasmic Ca^{2+} elevation did not exclude the possibility that Ca^{2+} elevation triggered the formation of F-actin.

Fig. 3. Relationship between cytoplasmic free Ca^{2+} and actin polymerization triggered by fMetLeuPhe

Neutrophils were incubated with either fura2-AM in Ca^{2+} -free medium to give a final intracellular fura concentration of 5 mM (■ and lower Ca^{2+} trace) or fura2-AM in Ca^{2+} -containing medium to give final concentration of approx. $100 \mu\text{M}$. (▲ and upper Ca^{2+} trace). The effect of stimulation with fMetLeuPhe ($1 \mu\text{M}$) is shown on (a) actin polymerization (control = 1) and (b) cytoplasmic Ca^{2+} .

Effect of buffering cytoplasmic Ca^{2+}

In order to test whether the peptide-induced rise in cytoplasmic Ca^{2+} triggered actin polymerization, the cytoplasmic Ca^{2+} buffering capacity of the cells was increased experimentally with the intracellular Ca^{2+} chelators fura2 and BAPTA. The chelators were loaded as their acetoxymethyl esters in Ca^{2+} -free medium to produce Ca^{2+} -depleted cells. Under these conditions, the resting Ca^{2+} concentration was less than 50 nM and stimulation with the peptide failed to elevate the cytoplasmic Ca^{2+} concentration to more than 250 nM . However, despite this Ca^{2+} -depleted state, the peptide was able to trigger actin polymerization. Moreover, removal of the Ca^{2+} signal resulted in a slight elevation and marked prolongation of the association of actin with the

Fig. 4. Relationship between fMetLeuPhe concentration and actin polymerization, cytoplasmic Ca^{2+} and oxidase activation

The effect is shown of the concentration of the peptide fMetLeuPhe and maximum incorporation of 40 kDa protein into the Triton-X-100-insoluble cytoskeleton ($\blacktriangle$), maximum cytoplasmic free Ca^{2+} concentration ($\blacksquare$), and the maximum luminol-dependent chemiluminescent response ($\bullet$). The peptide was diluted in dimethyl sulphoxide and added to the cell suspension to give a constant dimethyl sulphoxide concentration of 0.1% (v/v). Each point is expressed as $(R - R_d)/(R_{\text{max}} - R_d) \times 100\%$, where R is the parameter measured at a particular peptide concentration, R_d is the parameter measured after addition of dimethyl sulphoxide alone, and R_{max} is the maximum achieved value of the parameter. The error bars show S.E.M. of at least three determinations where greater than the size of the symbol.

Triton-X-100-insoluble cytoskeleton (Fig. 2). Inhibition of DNAase from the cytosol was also reduced, consistent with an elevation in actin polymerization from 78% to 84%. Under these conditions, therefore, the triggering of the polymerization of actin was dissociated from the Ca^{2+} signal.

Relationships between Ca^{2+} , actin polymerization and oxidase activation and peptide receptor occupancy

A dissociation between actin polymerization and cytoplasmic Ca^{2+} was also demonstrated in neutrophils at low concentrations of the peptide fMetLeuPhe. Whereas the concentration of peptide required to produce 50% of the maximum Ca^{2+} transient ($K_{0.5}$) was approx. 30 nM, maximum actin polymerization was achieved with peptide concentrations of less than 0.1 nM, the $K_{0.5}$ value being approx. 15 pM (Fig. 3). The concentration of the peptide required for oxidase activation was greater than for either actin polymerization or the Ca^{2+} rise, the $K_{0.5}$ value for oxidase activity being approx. 0.5 μM (Fig. 4).

Effect of other actin-polymerizing stimuli on cytoplasmic Ca^{2+}

The experimental agent PMA produced a slow polymerization of actin in the absence of a detectable increase in Ca^{2+} (Figs. 5a and 5b). Also, no rise in Ca^{2+} could be demonstrated after the addition of latex particles, despite the internalization of these particles by phagocytosis within 100 s (Figs. 5c and 5d). It was therefore concluded that these actin-polymerizing stimuli

Fig. 5. Effect of PMA and unopsonized latex beads on cytoplasmic Ca^{2+} and actin polymerization

The effects are shown of PMA (0.1 $\mu\text{g}/\text{ml}$) on (a) the incorporation of 40 kDa protein into the Triton-X-100-insoluble cytoskeleton (control = 1) and (b) cytoplasmic free Ca^{2+} , and of latex beads (1.01 μm diam., 10/ml) on (c) phagocytosis and (d) cytoplasmic Ca^{2+} . The vast excess of latex particles ensured that every neutrophil had phagocytosed at least one bead by 100 s. The error bars show the standard error of at least three determinations where greater than the size of the symbol.

also trigger actin polymerization with intracellular messengers other than Ca^{2+} .

DISCUSSION

The results presented in this paper have demonstrated that the polymerization of actin does not require an elevation in cytoplasmic Ca^{2+} . Actin polymerization in response to the peptide fMetLeuPhe occurred in cells containing the intracellular Ca^{2+} buffers BAPTA and fura2, and also at nanomolar concentrations of the peptide. Furthermore, phagocytosis of latex beads and stimulation by the experimental agent PMA, which produced a slow polymerization of actin, did not require a rise in cytoplasmic Ca^{2+} .

Sha'afi *et al.* (1986) have reported previously that quinacrine abolished peptide-induced Ca^{2+} influx without inhibiting the accompanying actin polymerization. They raised the question of the requirement of actin polymerization for cytoplasmic Ca^{2+} . Light scattering, associated with cytoskeletal changes, was also reported to occur in neutrophils depleted of Ca^{2+} by quin2 (Sklar *et al.*, 1985). Bengtsson *et al.* (1986), however, demonstrated a reduction in the extent of actin polymerization in neutrophils in which the Ca^{2+} -buffering capacity was increased by quin2, although even in this report significant actin polymerization occurred in the absence of a Ca^{2+} transient. The reason for this discrepancy with the work reported here is not clear, but may be related to an inhibitory effect of quin2 unrelated to its Ca^{2+} -chelating properties (see e.g. DiVirgilio *et al.*, 1988). Using fura2, which can be used to measure cytoplasmic Ca^{2+} in these cells with or without significantly affecting the Ca^{2+} buffering capacity of the cytoplasm (Al-Mohanna & Hallett, 1988), and BAPTA as cytoplasmic Ca^{2+} chelators, the peptide-induced Ca^{2+} transient was significantly inhibited without reducing the peptide-induced actin polymerization. Furthermore, at picomolar concentrations of the peptide, actin polymerization was triggered without any detectable elevation in cytoplasmic Ca^{2+} . Thus it was concluded that occupancy of the peptide receptor is coupled to actin polymerization through a pathway that does not involve an elevation in cytoplasmic Ca^{2+} . It was further concluded that both inositol trisphosphate, in concentrations which release Ca^{2+} from intracellular stores, and non- Ca^{2+} messengers in concentrations which trigger the oxidase system (this probably includes diacylglycerols and fatty acids), are also not involved in triggering actin polymerization. The possibility, however, cannot be excluded that at low receptor occupancy these or other mediators are generated in sufficient local intracellular concentrations to trigger actin polymerization without triggering Ca^{2+} release or oxidase activation. Another possibility suggested by the displacement of the concentration-response curve for actin polymerization to lower concentrations than for Ca^{2+} elevation or oxidase activation is that there exist three types of peptide receptor: one type of receptor, with a K_d of approx. 15 pM, is coupled to actin polymerization, another type with a K_d of approx. 30 nM which is coupled to Ca^{2+} elevation, and a third type with a K_d of approx. 0.5 μM which is coupled to oxidase activation. Binding studies have suggested the existence of peptide receptors with two affinities on human neutrophil membranes, K_d values being approx. 0.5 nM and 24 nM (Koo *et al.*, 1982).

However, no demonstration of a third type of receptor or of specific coupling to a specific intracellular event has yet been made.

The absence of a requirement of actin polymerization for elevated cytoplasmic Ca^{2+} was predicted from observations of phagocytosis, which is mediated by actin assembly (Sheterline *et al.*, 1984, 1986). Although phagocytosis often accompanies chemotaxis, there are several reports of a dissociation of phagocytosis from an elevation of cytoplasmic Ca^{2+} . For example, unopsonized latex beads (Hallett & Campbell, 1982; Campbell & Hallett, 1983) and fungal hyphae (Levitz *et al.*, 1987) are phagocytosed by neutrophils without an elevation in cytoplasmic Ca^{2+} , and intracellular Ca^{2+} buffers do not prevent neutrophil phagocytosis of unopsonized latex beads (Hallett & Campbell, 1984; Cooke *et al.*, 1989), iCb-opsonized latex (Lew *et al.*, 1985) or Fc-coated latex beads (Sheterline & Rickard, 1989). Furthermore, macrophage phagocytosis of latex beads (Hallett & Campbell, 1983b) or IgG-coated erythrocytes (DiVirgilio *et al.*, 1988) is not prevented by intracellular Ca^{2+} buffers. However, in chemotaxis, where actin assembly also occurs, an elevation of cytoplasmic Ca^{2+} may be necessary to generate cell movement. It may be suggested that Ca^{2+} could act at at least three sites: (i) restriction of actin chain length at particular cellular sites, by acting through gelsolin, (ii) activation of myosin light-chain kinase to produce myosin chains with the potential of overlapping with shorter actin chains and (iii) generation of contraction by interaction with actinomyosin. Evidence that Ca^{2+} may restrict actin polymerization induced by the peptide was provided by the prolongation of actin polymerization in Ca^{2+} -buffered neutrophils (Fig. 3), and is also suggested by the increase in the total amount of actin polymerized under these conditions.

We therefore propose that actin polymerization in neutrophils in response to phagocytic or chemotactic stimuli is not triggered by cytoplasmic Ca^{2+} . The possibility exists that the polymerization of actin is triggered by messengers distinct from those required for elevation of cytoplasmic Ca^{2+} or for activation of the oxidase system. Cytoplasmic Ca^{2+} may, however, regulate the extent and duration of the transient assembly of actin observed in cells in suspension, and may thus play an important role in the assembly of actinomyosin and its contraction during chemotaxis.

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