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Inositol 1,4,5-triphosphate-mediated shuttling between intracellular stores and the cytosol contributes to the sustained elevation in cytosolic calcium in FMLP-activated human neutrophils

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Abstract

The current study was designed to probe Ca^{2+} shuttling between intracellular stores and the cytosol as a potential mechanism contributing to the prolongation of elevated Ca^{2+} transients in *N*-formyl-L-methionyl-L-leucyl-L-phenylalanine (FMLP)-activated human neutrophils. Cytosolic Ca^{2+} concentrations and transmembrane fluxes of the cation were measured using spectrofluorimetric and radiometric procedures, respectively, while inositol 1,4,5-triphosphate (IP_3) was measured using a radioreceptor assay. The Ca^{2+}-chelating agent, ethylene glycol-bis (β-aminoethyl ether) *N,N,N′N′*-tetraacetic acid (EGTA; 10 mM), was used to exclude store-operated influx of Ca^{2+} into neutrophils, while the IP_3 receptor antagonist, 2-aminoethoxydiphenyl borate (2-APB, 100 μM), added to the cells 10 s after FMLP (0.01 and 1 μM), at which time the increases in IP_3 and cytosolic Ca^{2+} were maximal, was used to eliminate both sustained release from stores and influx of Ca^{2+}. Addition of FMLP at 0.01 or 1 μM resulted in equivalent peak increases in cytosolic Ca^{2+}, while the increase in IP_3 was greater and the rate of clearance of Ca^{2+} from the cytosol slower, in cells activated with 1 μM FMLP. Treatment of the cells with either EGTA or 2-APB following addition of 1 μM FMLP, completely (EGTA) or almost completely (2-APB) abolished the influx of Ca^{2+} and accelerated the rate of clearance of the cation from the cytosol. Post-peak cytosolic Ca^{2+} concentrations were lower, and the Ca^{2+} content of the stores higher, in cells treated with 2-APB. The involvement of IP_3 was confirmed by similar findings in cells treated with U-73122 (1 μM), a selective inhibitor of phospholipase C. Taken together, these observations are compatible with IP_3-mediated Ca^{2+} shuttling in neutrophils activated with FMLP.
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Keywords: 2-Aminoethoxydiphenyl borate; Calcium; Calcium shuttling; Inositol 1,4,5-triphosphate; Neutrophils

1. Introduction

Exposure of human neutrophils to chemoattractants results in an abrupt increase in cytosolic Ca^{2+}, primarily by activation of phospholipase C (PLC) and consequent inositol 1,4,5-triphosphate (IP_3)-mediated mobilization of the cation from intracellular stores [1,2]. Notwithstanding the influence of the type and concentration of the chemoattractant [2–4], the major determinants of the duration of the elevation in cytosolic Ca^{2+} are the efficiency of the Ca^{2+} clearance systems operative in activated neutrophils, particularly the plasma membrane and endomembrane Ca^{2+}-ATPases, as well as the time of onset, rate and magnitude of store-operated influx of extracellular cation [2–6]. The magnitude of store-operated influx of Ca^{2+} into chemoattractant-activated human neutrophils appears to be directly related to the intracellular IP_3 concentration [7], compatible with a conformational coupling mechanism of influx [8], while the extent and duration of activation of the electrogenic NADPH oxidase regulates the rate of influx of the cation [9–12].

Repetitive release of Ca^{2+} from intracellular stores by IP_3 represents an additional, albeit unexplored mechanism,

Abbreviations: 2-APB, 2-aminoethoxydiphenyl borate; DMSO, dimethylsulphoxide; EGTA, ethylene glycol-bis (β-aminoethyl ether) *N,N,N′N′*-tetraacetic acid; FMLP, *N*-formyl-L-methionyl-L-leucyl-L-phenylalanine; IP_3, inositol 1,4,5-triphosphate; U-73122, 1-[6[((17β)-3-methoxyestra-1,3,5(10)-trien-17-yl)amino]hexyl]-1-H-pyrrole-2,5-dione; U-73343, 1-[6[((17β)-3-methoxyestra-1,3,5[10]-trien-17-yl)amino]hexyl]-2,5-pyrrolidinedione

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which may contribute to maintaining elevated cytosolic Ca^{2+} concentrations and oscillations of the cation in chemoattractant-activated neutrophils. Accordingly, the current study was undertaken to investigate Ca^{2+} shuttling between intracellular stores and the cytosol as a possible mechanism of prolongation of Ca^{2+} transients in *N*-formyl-L-methionyl-L-leucyl-L-phenylalanine (FMLP)-activated human neutrophils, as well as the involvement of IP_3 in mediating these events.

2. Materials and methods

2.1. Chemicals and reagents

Unless otherwise indicated these were purchased from Sigma.

2.2. Neutrophils

These cells were isolated from heparinized venous blood (5 units of preservative-free heparin per ml of blood) from healthy adult volunteers. Neutrophils were separated from mononuclear leukocytes by centrifugation on Histopaque-1077 (Sigma Diagnostics) cushions at 400 × *g* for 25 min at room temperature. The resultant pellet was suspended in phosphate-buffered saline (PBS, 0.15 M, pH 7.4) and sedimented with 3% gelatin to remove most of the erythrocytes. Following centrifugation (280 × *g* at 10 °C for 10 min), residual erythrocytes were removed by selective lysis with 0.83% ammonium chloride at 4 °C for 10 min. The neutrophils, which were routinely of high purity (>90%) and viability (>95%), determined by light microscopy and fluorescence microscopy (exclusion of ethidium bromide) respectively, were resuspended to 1×10^7 ml^{-1} in PBS and held on ice until used.

2.3. Spectrofluorimetric measurement of cytosolic Ca^{2+}

Fura-2/AM was used as the fluorescent, Ca^{2+}-sensitive indicator for these experiments [13]. Neutrophils (1×10^7 ml^{-1}) were incubated with fura-2/AM (2 μM) for 30 min at 37 °C in PBS, washed and resuspended in indicator-free Hanks balanced salt solution (HBSS, pH 7.4), containing 1.25 mM $CaCl_2$. The fura-2-loaded cells (2×10^6 ml^{-1}) were then preincubated for 10 min at 37 °C after which they were transferred to disposable reaction cuvettes, which were maintained at 37 °C in a Hitachi 650 10S fluorescence spectrophotometer with excitation and emission wavelengths set at 340 and 500 nm, respectively. After a stable baseline was obtained (±1 min), the neutrophils were activated by addition of the chemotactic tripeptide, FMLP at final concentrations of 0.01 or 1 μM (the former being the lowest concentration of the chemoattractant which caused maximal release of Ca^{2+} from intracellular stores), followed 10 s later by 2-aminoethoxydiphenyl borate (2-ABP, 100 μM final), an IP_3 receptor antagonist [14], or an equal volume (3 μl) of the solvent, dimethylsulphoxide (DMSO), and measurement of alterations of cytosolic Ca^{2+} over a 5 min time course. Delayed addition of 2-aminoethoxydiphenyl borate was used to prevent interference by this agent with the peak IP_3-mediated increase in cytosolic Ca^{2+} following exposure of the cells to the chemoattractant. These responses were compared with those of matched ethylene glycol-bis (β-aminoethyl ether) *N*,*N*,*N*′*N*′-tetraacetic acid (EGTA; 10 mM)-treated cells, with the Ca^{2+}-chelating agent being added to the cells 1 min prior to FMLP. EGTA-treated cells also received DMSO, but not 2-APB, 10 s after the addition of FMLP. Cytosolic Ca^{2+} concentrations were calculated as described previously [13].

Additional experiments were performed with U-73122 (1 μM), a selective inhibitor of phospholipase C, and its inactive analogue, U-73343, or an equal volume of DMSO, added 10 s after FMLP (1 μM) (when peak cytosolic Ca^{2+} concentrations have been reached) in the presence of 10 mM EGTA.

The rationale underlying this experimental design is that EGTA should eliminate the influx of extracellular Ca^{2+} without influencing the mobilization of the cation from intracellular stores, while 2-APB, as well as U-73122, added at the time of the peak Ca^{2+} response should eliminate not only the IP_3-activated store-operated influx of Ca^{2+}, but also residual IP_3-mediated mobilisation of the cation from stores. The difference, if any, in the post-peak cytosolic Ca^{2+} concentrations between FMLP-activated neutrophils treated with 2-APB or EGTA should, therefore, reflect sustained release from stores mediated by IP_3.

To confirm that EGTA, at the concentration used (10 mM), removed all available Ca^{2+} from the extracellular fluid, the pore-forming pneumococcal toxin (8.35 ng/ml, final), pneumolysin, which rapidly permeabilizes neutrophils to Ca^{2+} [15], was added to neutrophils 1 min after EGTA. As expected, treatment of the cells with pneumolysin caused substantial influx of Ca^{2+}, which was completely attenuated by EGTA, excluding any residual Ca^{2+} influx (not shown).

To confirm that activation of neutrophils with 0.01 or 1 μM FMLP results in mobilisation of the total pool of stored Ca^{2+}, neutrophils were activated simultaneously with FMLP (0.01 or 1 μM) combined with 1 μM thapsigargin, a highly selective inhibitor of the endomembrane Ca^{2+}-ATPase [16], in the presence and absence of EGTA (10 mM), and peak cytosolic Ca^{2+} concentrations compared with those of EGTA-treated cells activated with FMLP alone in the absence of thapsigargin.

To determine the effects of 2-APB (100 μM) on the post-peak cytosolic Ca^{2+} concentrations of neutrophils activated with lower concentrations of FMLP (<10 nM) that are chemotactic for neutrophils, but which may not maximally mobilise stored calcium, a further series of experiments was performed during which EGTA-treated

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