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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.

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

cells were stimulated with FMLP at 2 and 5 nM, followed by addition of DMSO or 2-APB immediately after the fura-2 fluorescence peak. Subsequent alterations in fura-2 fluorescence were monitored over a 3 min time course. The role of IP_3 in mediating these cytosolic Ca^{2+} responses in neutrophils stimulated with FMLP (2 and 5 nM) was investigated by pre-treating the cells for 2 min with U-73122 (1 μM) prior to addition of the chemoattractant.

In an additional series of experiments designed to determine the filling state of intracellular Ca^{2+} stores, measured 1.5 min after FMLP-mediated Ca^{2+} release, fura-2 loaded cells were incubated in Ca^{2+}-replete medium for 10 min at 37 °C to achieve complete filling of stores then transferred to nominally Ca^{2+}-free medium with EGTA (100 μM) and placed in disposable cuvettes. The cells were activated with 0.01 or 1 μM FMLP, followed 10 s later by addition of DMSO, 2-APB (100 μM) or U-73122 (1 μM). Subsequent alterations in fluorescence intensity were recorded and the calcium ionophore (4-bromo-A23187) was added to the cells at 1.5 min after FMLP in order to mobilize resequestered Ca^{2+}, and alterations in fluorescence intensity measured over a 3 min time course. Suspension of the cells in nominally Ca^{2+}-free HBSS containing 100 μM EGTA, as opposed to Ca^{2+}-replete HBSS + 10 mM EGTA, was undertaken to eliminate interference by EGTA/Ca^{2+} of penetration of 4-bromo-A23187 into the neutrophils [17].

2.4. Radiometric assessment of transmembrane Ca^{2+} fluxes

This procedure was used to compare the magnitude of efflux and store-operated influx of Ca^{2+} following the activation of neutrophils with 0.01 and 1 μM FMLP, as well as the effects of 2-APB (100 μM) or EGTA (10 mM), added as described above 10 s after, or 1 min before the chemoattractant, respectively.

For efflux studies, neutrophils (1×10^7 ml^{-1}) suspended in Ca^{2+}-free HBSS were loaded with $^{45}Ca^{2+}$ (Perkin Elmer Life Sciences, Inc; 490.8 mBq/mg; 10 μCi/ml final), for 15 min at 37 °C. The cells were then pelleted by centrifugation, washed with, and resuspended in Ca^{2+}-replete HBSS. The $^{45}Ca^{2+}$-loaded neutrophils (2×10^6 ml^{-1}) were then preincubated for 10 min at 37 °C in a final volume of 5 ml Ca^{2+}-replete HBSS, followed by addition of FMLP and measurement of efflux (decrease in cell-associated $^{45}Ca^{2+}$) at 60 s after the addition of the chemoattractant. This incubation period was based on our previous studies in which we established that FMLP-activated efflux of Ca^{2+} from neutrophils is a rapid response that terminates at about 30–60 s [4,5,12]. The reactions were stopped by the addition of 10 ml ice-cold Ca^{2+}replete HBSS to the tubes and the cells pelleted by centrifugation at $400 \times g$ for 5 min followed by washing with 15 ml ice-cold, Ca^{2+}-replete HBSS, and the cell pellets finally dissolved in 0.5 ml of 0.5% Triton X-100/0.05 M NaOH and the radioactivity assessed in a liquid scintillation spectrometer. The results are presented as the amount of $^{45}Ca^{2+}$ extruded from the cells (pmol $^{45}Ca^{2+}$/10^7 cells).

To measure net influx of $^{45}Ca^{2+}$ into FMLP-activated neutrophils, uncomplicated by concomitant efflux of the radiolabelled cation, the cells were preincubated for 15 min at 37 °C in Ca^{2+}-replete HBSS, then pelleted by centrifugation and resuspended to 1×10^7 ml^{-1} in HBSS containing 250 μM cold Ca^{2+}. Preloading of neutrophils with cold Ca^{2+} was undertaken to ensure that intracellular Ca^{2+} stores were replete, thereby minimizing spontaneous uptake of $^{45}Ca^{2+}$ (unrelated to FMLP activation) in the influx assay. The Ca^{2+}-loaded neutrophils (2×10^6 ml^{-1}) were then preincubated for 10 min at 37 °C in a final volume of 5 ml HBSS containing a final concentration of 50 μM cold, carrier Ca^{2+}. This was followed by the simultaneous addition of FMLP (0.01 or 1 μM) and $^{45}Ca^{2+}$ (2 μCi/ml), or $^{45}Ca^{2+}$ only to control, unstimulated systems. The influx of $^{45}Ca^{2+}$ was then measured as described above after 5 min incubation at 37 °C at which time store-operated uptake of Ca^{2+} by FMLP-activated neutrophils is complete [5].

This procedure was also used to investigate the effects of EGTA or 2-APB, added 1 min before and 10 s after FMLP (1 μM) respectively, on the store-operated influx of Ca^{2+}.

2.5. Inositol triphosphate (IP_3)

Neutrophils at a concentration of 5×10^6 ml^{-1} in Ca^{2+}-replete HBSS were preincubated for 10 min at 37 °C followed by the addition of FMLP (0.01 or 1 μM, final), or an equal volume of HBSS to control, unstimulated cells in a final volume of 2 ml, after which the reactions were terminated and the IP_3 extracted by the addition of 0.4 ml of 20% perchloric acid at 0, 5 and 10 s after addition of the chemoattractant, and the tubes transferred to an ice-bath. These incubation times coincide with the peak IP_3 responses of FMLP-activated neutrophils, which were determined in a series of preliminary experiments and are in agreement with previous reports on the time course of the IP_3 responses elicited by FMLP-activated neutrophils, which return to basal values at around 60 s [1,2]. Following a 20 min incubation on ice, the tubes were centrifuged at $2000 \times g$ for 15 min and the supernatants removed and brought to pH 7.5 with 5N KOH, followed by centrifugation at $2000 \times g$ for 15 min to remove precipitated perchloric acid. The supernatants were assayed using the inositol-1,4,5-triphosphate [^{3}H] radioreceptor assay procedure (Perkin Elmer Life Sciences, Inc), which is a competitive ligand binding assay, and the results expressed as pmol $IP_3/10^7$ cells.

2.6. Cellular ATP levels

To investigate the cytotoxic potential of 2-APB, neutrophils (1×10^6 ml^{-1}) were treated with this agent at a

fixed, final concentration of 100 μM for 10 min at 37 °C, followed by measurement of ATP in the lysates of control and 2-APB-treated cells using a luciferin/luciferase chemiluminescence procedure [18].

2.7. Statistical analysis

The results of each series of experiments are expressed as the mean value ± standard errors of the mean (S.E.M.). Levels of statistical significance were calculated by the Mann–Whitney *U*-test, and by ANOVA where appropriate. A computer-based software system was used for analysis. Significance levels were taken at a P value of <0.05.

3. Results

3.1. Effects of 2-APB, EGTA and U-73122 on the fura-2 fluorescence responses of FMLP-activated neutrophils

These results are shown in Fig. 1. Addition of FMLP to neutrophils was accompanied by the characteristic, abrupt increase in fura-2 fluorescence concomitant with the release of the cation from intracellular stores. Although the peak fura-2 fluorescence responses were similar in cells activated with 0.01 and 1 μM FMLP, the rate of decline in fluorescence intensity was more rapid in cells activated with the lower concentration of the chemoattractant. Treatment of the cells with either 2-APB or EGTA, added 10 s after or 1 min before FMLP, respectively, did not affect the magnitude of the peak fura-2 fluorescence responses of cells activated with FMLP. However, these agents dramatically accelerated the subsequent rate of decline in fluorescence intensity, their effects being more-or-less equivalent when the cells were activated with 0.01 μM of the chemoattractant, although in some cases 2-APB was slightly more potent than EGTA. When the cells were activated with 1 μM FMLP, however, there was a clear distinction between the effects of 2-APB and EGTA, with the IP_3 receptor antagonist being more effective than the Ca^{2+}-chelating agent in accelerating the decline in peak fura-2 fluorescence intensity (Fig. 1). Importantly, addition of 2-APB (100 μM) to unstimulated neutrophils did not affect baseline Ca^{2+} concentrations over the 5 min time course used in experiments with FMLP (not shown).

Data from a larger series of experiments summarizing post-peak cytosolic Ca^{2+} concentrations at 1 and 2 min after the addition of 1 μM FMLP to control neutrophils and those treated with 2-APB or EGTA are shown in Table 1. Both agents significantly ($P < 0.05$) decreased cytosolic Ca^{2+}, with 2-APB being more effective than EGTA ($P < 0.05$).

Addition of U-73122 10 s after FMLP, in the presence of EGTA (10 mM), did not alter the peak cytosolic Ca^{2+} concentrations, but significantly decreased the concentration of Ca^{2+} in the cytosol measured at 1 min following activation of the cells. The peak cytosolic Ca^{2+} concentrations, which are those measured 10–20 s after the addition of FMLP, were 299 ± 11 nM in the absence of U-73122 (1 μM) and 291 ± 3 nM in the presence of this agent, while the corresponding cytosolic Ca^{2+} concentrations measured 1 min after FMLP were 177 ± 7 and 106 ± 7 nM, respectively ($P < 0.05$). Importantly, the inactive analogue of U-73122, U-73343, was without effect (not shown), confirming that the observed effects of U-73122 in these experiments are due to inhibition of PLC.

The peak cytosolic Ca^{2+} concentrations of neutrophils, measured 10 s after the addition of either 0.01 or 1 μM FMLP, were not increased by inclusion of thapsigargin (1 μM) added simultaneously with the chemoattractant. The peak cytosolic calcium concentrations, rising from a basal value of 27 ± 5 nM, were 280 ± 19, 280 ± 11, 274 ± 11 and 287 ± 19 nM for cells activated with

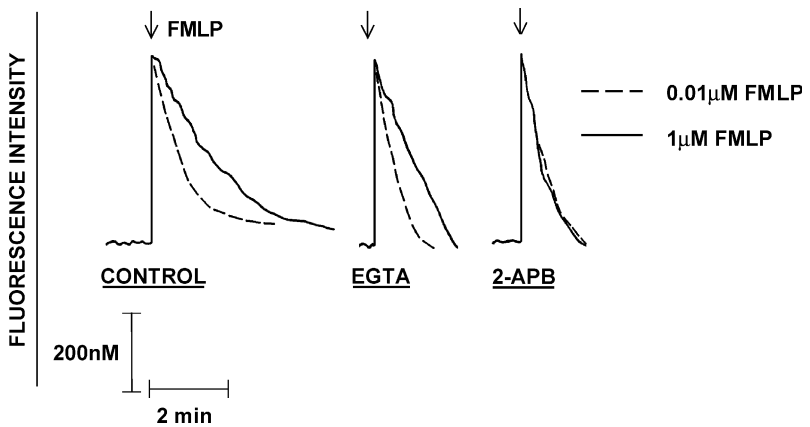

Fig. 1. Comparison of the fura-2 fluorescence responses of human neutrophils activated with 0.01 and 1 μM FMLP addition of which is denoted by the arrow, in the absence and presence of the Ca^{2+}-chelating agent EGTA (10 mM), or the IP_3 receptor antagonist, 2-APB (100 μM). EGTA was added to the cells 1 min prior to FMLP, while 2-APB was added 10 s after the chemoattractant to eliminate interference with IP_3-mediated mobilization of Ca^{2+} from neutrophil intracellular stores. These are traces from a single representative experiment with a total of 6 in the series using cells suspended in Ca^{2+}-replete HBSS.

Table 1
Peak and post-peak cytosolic Ca^{2+} concentrations in FMLP (1 μM)-activated neutrophils without and with 2-APB or EGTA

System	Peak	Cytosolic Ca^{2+} concentration at	
		1 min post-FMLP	2 min post-FMLP
FMLP-activated control neutrophils	321 ± 14[a]	193 ± 7	111 ± 4
FMLP-activated neutrophils + 2-APB (100 μM)	312 ± 18	$80 \pm 6^{+}$	$11 \pm 4^{+}$
FMLP-activated neutrophils + EGTA (10 mM)	288 ± 10	$146 \pm 6^{+,o}$	$51 \pm 4^{+,o}$

[a] The results of six different experiments are presented as the mean cytosolic Ca^{2+} concentrations (nM) ± S.E.M. measured at 10–20 s (peak) and at 1 and 2 min (post-peak) after the addition of FMLP, rising from a basal value of 29 ± 4 nM.
[+] $P < 0.05$ for comparison with the time-matched control system.
[o] $P < 0.05$ for comparison between the time-matched values for systems treated with 2-APB and EGTA.

Table 2
Peak and post-peak cytosolic Ca^{2+} concentrations in neutrophils activated with FMLP at 2 and 5 nM in Ca^{2+}-free medium without and with 2-APB (100 μM)

System	Peak	Cytosolic Ca^{2+} concentration at	
		30 s post-FMLP	60 s post-FMLP
FMLP (5 nM)-activated control neutrophils	238 ± 4[a]	154 ± 5	100 ± 3
FMLP (5 nM)-activated neutrophils + 2-APB	233 ± 3	$105 \pm 6^{*}$	$53 \pm 3^{*}$
FMLP (2 nM)-activated control neutrophils	220 ± 7	$127 \pm 7^{*}$	$86 \pm 5^{*}$
FMLP (2 nM)-activated neutrophils + 2-APB	222 ± 8	$103 \pm 5^{*}$	$66 \pm 6^{*}$

[a] The results of 8–10 experiments are presented as the mean cytosolic Ca^{2+} concentrations (nM) ± S.E.M. measured at 10 s (peak) and at 30 and 60 s (post-peak) after the addition of FMLP, rising from a basal value of 20 ± 3 nM.
[*] $P < 0.05$ for comparison with the time-matched control system.

0.01 μM FMLP only, 0.01 μM FMLP + thapsigargin, 1 μM FMLP only, and 1 μM FMLP + thapsigargin, respectively (data from four experiments).

The fura-2 fluorescence responses of neutrophils pre-treated with EGTA (10 mM) for 1 min prior to activation with lower concentrations of FMLP (2 and 5 nM) followed by addition of DMSO or 2-APB (100 μM) immediately after the peak fura-2 fluorescence responses are shown in Fig. 2. Although lower than those observed with 0.01 and 1 μM FMLP, 2-APB did not affect the peak cytosolic Ca^{2+} concentrations of neutrophils activated with either 2 or 5 nM FMLP. However, the subsequent decline in fura-2 fluorescence was significantly faster in 2-APB-treated cells. The cytosolic Ca^{2+} concentrations measured 30 and 60 s after addition of FMLP at 2 and 5 nM to neutrophils are shown in Table 2.

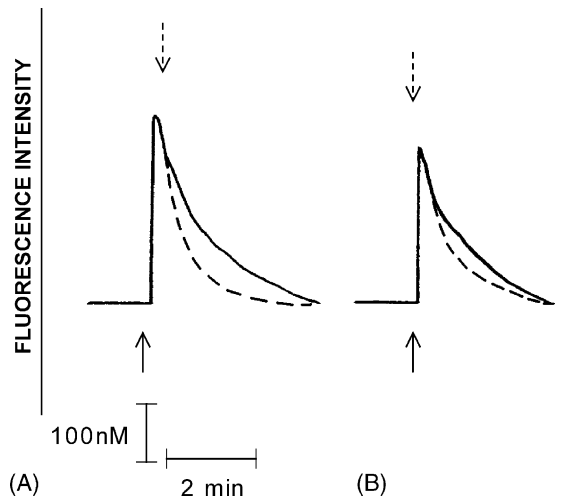

Fig. 2. Fura-2 fluorescence responses of neutrophils pretreated with EGTA (10 mM) for 1 min and then activated with FMLP (↑) 5 nM (A) and 2 nM (B) in the absence (—) and presence (- - - -) of 2-APB (100 μM) added immediately after the peak fura-2 fluorescence (⇣). These are traces from a single representative experiment with a total of 8–10 in the series.

Pre-treatment of neutrophils with U-73122 (1 μM) for 2 min before addition of FMLP (2 and 5 nM) abolished the fura-2 fluorescence responses (results not shown).

The peak fura-2 fluorescence responses of neutrophils suspended in Ca^{2+}-free HBSS + 100 μM EGTA and activated with either 0.01 or 1 μM FMLP (followed 1.5 min later by addition of 4-bromo-A23187), did not differ significantly being 266 ± 12 and 266 ± 19 nM, respectively (basal value 15 ± 3 nM). However, the increments in cytosolic Ca^{2+} concentrations (these being the difference between the measured values at the time of addition of the ionophore and the subsequent peak values) following addition of 4-bromo-A23187 1.5 min after the chemoattractant were significantly greater for cells activated with 0.01 compared to 1 μM FMLP, with values for the respective ionophore-mediated increases of 136 ± 5 and 82 ± 6 nM ($P < 0.05$).

The fura-2 fluorescence responses of neutrophils suspended in Ca^{2+}-free HBSS + 100 μM EGTA and treated in succession with FMLP (1 μM), 2-APB (100 μM) or DMSO (control system) 10 s later, and 4-bromo-A23187 (1 μM) 1.5 min after FMLP are shown in Fig. 3. Treatment of the cells with 2-APB subsequent to activation with FMLP accelerated the decline in cytosolic Ca^{2+} as observed with cells suspended in Ca^{2+} replete HBSS + 10 mM EGTA (Fig. 1). Importantly, the increment

Table 3
Net efflux and store-operated influx of $^{45}Ca^{2+}$ following activation of neutrophils with 0.01 and 1 μM FMLP

System	Net efflux of $^{45}Ca^{2+}$ (pmol/10^7 cells)	Net influx of $^{45}Ca^{2+}$ (pmol/10^7 cells)
Neutrophils activated with 0.01 μM FMLP	64 ± 6[a]	95 ± 5
Neutrophils activated with 1 μM FMLP	122 ± 3[*]	140 ± 4[*]

[a] Data from six experiments are presented as the mean value ± S.E.M. Net efflux of Ca^{2+} was measured 1 min after the addition of FMLP, the amount of cell-associated $^{45}Ca^{2+}$ being 212 pmol/10^7 cells. Net influx of $^{45}Ca^{2+}$ was measured 5 min after the addition of FMLP, the magnitude of influx for unstimulated, control systems being 24 pmol $^{45}Ca^{2+}$/10^7 cells.
[*] $P < 0.05$ for comparison between the systems activated with 0.01 and 1 μM FMLP.

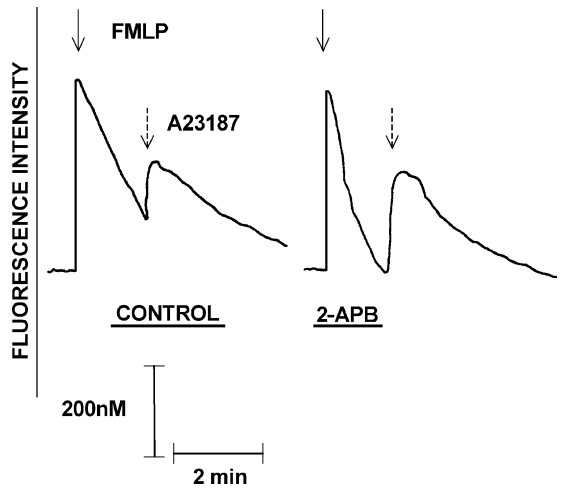

Fig. 3. Fura-2 fluorescence responses of neutrophils activated with 1 μM FMLP in the absence and presence of 2-APB (added 10 s after FMLP), followed 1.5 min later by 1 μM 4-bromo-A23187. These are traces from a single representative experiment with a total of 6 in the series using cells suspended in Ca^{2+}-free HBSS.

in fluorescence intensity observed following the addition of 4-bromo-A23187 1.5 min post-FMLP to 2-APB-treated cells was considerably higher than that observed in the corresponding control system. In a total of 10 experiments, in which U-73122 was also included, the mean peak cytosolic Ca^{2+} concentrations observed following activation of cells with FMLP were 274 ± 6, 269 ± 5 and 272 ± 9 nM for the control, 2-APB-treated and U-73122-treated systems, respectively. The corresponding increments in cytosolic Ca^{2+} following addition of 4-bromo-A23187 1.5 min post-FMLP were 74 ± 3, 148 ± 4 ($P < 0.05$) and 131 ± 13 nM ($P < 0.05$).

Table 4
Inositol 1,4,5-triphosphate in resting and FMLP (0.01 and 1 μM)-activated neutrophils

System	IP_3 (pmol) measured at	
	5 s after FMLP	10 s after FMLP
Resting neutrophils	34 ± 2[a]	ND
Neutrophils activated with 0.01 μM FMLP	39 ± 2	38 ± 1
Neutrophils activated with 1 μM FMLP	55 ± 6[*]	50 ± 3[*]

ND: not done.
[a] The results of four experiments are presented as the mean value ± S.E.M.
[*] $P < 0.05$ for comparison between resting neutrophils or those activated with 0.01 μM FMLP and those activated with 1 μM FMLP.

3.2. Measurement of net efflux and net influx of Ca^{2+}

The magnitudes of net efflux and net influx of Ca^{2+} following activation of neutrophils with 0.01 and 1 μM FMLP are shown in Table 3. Both of these were significantly ($P < 0.05$) lower when the cells were activated with 0.01 μM FMLP.

Treatment of neutrophils with 2-APB, but not EGTA, significantly ($P < 0.05$) decreased the magnitude of efflux from cells activated with 1 μM FMLP, the values for control cells, and for cells treated with 2-APB or EGTA being 94 ± 1, 66 ± 3 and 91 ± 3 pmol $^{45}Ca^{2+}$, respectively (data from six experiments).

Treatment of neutrophils with EGTA or 2-APB caused complete, and almost complete attenuation, respectively, of the FMLP (1 μM)-activated store-operated influx of Ca^{2+}, the respective values for unstimulated neutrophils, and for FMLP-activated control, 2-APB-treated, and EGTA-treated cells being 7 ± 1, 113 ± 4, 19 ± 1 and 10 ± 2 pmol $^{45}Ca^{2+}$ (data from six measurements).

3.3. IP_3 and ATP

IP_3 levels measured at 5 and 10 s following the activation of neutrophils with 0.01 and 1 μM FMLP are shown in Table 4. FMLP activation of neutrophils was accompanied by a dose-related increase in IP_3, with 1 μM of the chemoattractant being most effective. As reported previously, the relationship between the chemoattractant concentration and IP_3 formation was found to be non-linear [2].

ATP levels were unaffected by treatment of neutrophils with 100 μM 2-APB for 10 min at 37 °C, the values for control and 2-APB-treated neutrophils being 31 ± 2 and 29 ± 1 nmol ATP/10^7 cells, respectively.

4. Discussion

Although the exclusive involvement of IP_3 in mediating Ca^{2+} mobilization from the intracellular stores of neutrophils activated with FMLP is well-documented [1,2,4,14,19], less is known about the role of this second mes-

senger in prolonging post-peak cytosolic Ca^{2+} transients in these cells. In the current study we have attempted to address this issue by comparing the magnitude and duration of the post-peak elevations in cytosolic Ca^{2+} in neutrophils activated with FMLP at concentrations (0.01 and 1 μM) which cause maximum release of the cation from intracellular stores, but which differentially affect the production of IP_3 [1,2]. These experiments were performed in the absence and presence of EGTA or 2-APB. The former chelates extracellular Ca^{2+}, thereby preventing store-operated uptake of the cation by FMLP-activated neutrophils, while 2-APB, an IP_3-receptor antagonist, interferes with the release of Ca^{2+} from stores, as well as with the uptake of extracellular Ca^{2+}, possibly by uncoupling stores from the membrane [8], and/or other mechanisms [20]. To exclude inhibitory effects of 2-APB on the release of Ca^{2+} from intracellular stores, 2-APB was added to the cells 10 s after FMLP, at which time release of the cation from stores was maximal. Eliminating the effects of 2-APB on mobilization of Ca^{2+} from the intracellular stores of FMLP-activated neutrophils, together with the strategy of using EGTA to determine the contribution of store-operated influx of Ca^{2+}, enabled us to probe the involvement of IP_3 in sustaining post-peak cytosolic Ca^{2+} transients in these cells.

As expected [1,2], the abruptly-occurring peak increments in cytosolic Ca^{2+} were comparable in neutrophils activated with either 0.01 or 1 μM FMLP, with apparent total mobilization of the cation from intracellular stores, a contention which is supported by the observation that no further increments above the FMLP-activated peak values were observed when the cells were activated with FMLP combined with thapsigargin, a highly selective inhibitor of the endomembrane Ca^{2+}-ATPase [16]. However, the post-peak decline in cytosolic Ca^{2+} occurred more rapidly in cells activated with the lower concentration (0.01 μM) of the chemoattractant. IP_3 concentrations, although increased above basal levels, were also significantly lower in neutrophils activated with 0.01 μM FMLP in comparison with those activated with 1 μM of the chemoattractant. This is in keeping with previous reports that basal levels of IP_3 are maintained at fairly high levels in many cell types and that only modest increases in IP_3, of around 15% of maximal, are required to cause complete mobilization of intracellular Ca^{2+} [1,2,21]. Moreover, not only were IP_3 levels lower in cells activated with 0.01 μM FMLP, but the magnitudes of efflux and store-operated influx of Ca^{2+} were also considerably less, in the setting of maximal release of the cation from stores.

The effects of 2-APB on the post-peak cytosolic Ca^{2+} levels in neutrophils activated with lower concentrations of FMLP (2 and 5 nM), were similar to those observed at FMLP concentrations that caused maximal mobilization of stored Ca^{2+}. These observations suggest that similar mechanisms may be operative during the sustained phase of Ca^{2+} release at all concentrations of the chemoattractant tested and that the accelerated decline in post-peak cytosolic Ca^{2+} mediated by 2-APB also occurs following submaximal mobilization of the cation from intracellular stores. Although difficult to detect, probably because of a sub-maximal response coupled to rapid turnover, it is highly likely that IP_3 is generated at concentrations of the chemoattractant below 10 nM. This contention is based on the observation that Ca^{2+} release is abolished when neutrophils activated with 2 or 5 nM FMLP are pretreated with the phospholipase C inhibitor, U-73122.

We reasoned that IP_3-mediated shuttling of Ca^{2+} between the stores and the cytosol represented a possible unifying explanation for this set of observations. When IP_3 levels are low, Ca^{2+} released from stores is rapidly re-sequestered with an accompanying reduction in efflux and a lesser requirement for store-operated influx as a mechanism of store-refilling. At higher concentrations of IP_3, however, sustained activation of IP_3 receptors is accompanied by shuttling of Ca^{2+} between the stores and the cytosol, resulting in prolonged cytosolic Ca^{2+} transients. This in turn leads to increased efflux and compensatory store-operated influx of the cation.

Interestingly, the magnitude of the increase in cytosolic Ca^{2+} (albeit from a lower basal value) observed following the addition of the ionophore, 4-bromo-A23187, 1.5 min after activation of the cells with 0.01 μM FMLP was significantly greater than that observed in cells activated with 1 μM FMLP. These observations, in a system uncomplicated by Ca^{2+} influx, are compatible with accelerated-refilling of stores in cells activated with the lower concentration of the chemoattractant. Although not included in the current study, similar findings were observed when the chemoattractant, platelet-activating factor (PAF, 200 nM), was used as an alternative to A23187 to mobilize re-sequestered Ca^{2+} from neutrophil intracellular stores.

As an alternative strategy to probe the involvement of IP_3 in Ca^{2+} shuttling between the stores and the cytosol, we repeated the measurements of cytosolic Ca^{2+} in neutrophils activated with 0.01 and 1 μM FMLP in the absence and presence of EGTA or 2-APB. In neutrophils activated with 0.01 μM FMLP, neither of these agents affected peak cytosolic Ca^{2+} concentrations, but caused essentially comparable reductions in the magnitude and duration of the post-peak elevations in cytosolic Ca^{2+}, with 2-APB being slightly more effective in some experiments. In cells activated with the higher concentration (1 μM) of FMLP, there was, however, a clear difference in post-peak cytosolic Ca^{2+} concentrations between cells treated with 2-APB or EGTA, which were significantly less in cells treated with the IP_3-receptor antagonist. This observation could not be attributed to 2-APB-mediated inhibition of store-operated influx of Ca^{2+} since EGTA was at least equally effective in this respect. Taken together with the observation that 2-APB, but not EGTA, reduced the magnitude of efflux of Ca^{2+} from the cytosol of FMLP (1 μM)-activated neutrophils, these findings suggest that IP_3 con-

tributes to prolongation of Ca^{2+} transients in these cells by promoting shuttling of the cation between intracellular stores and the cytosol.

The most compelling evidence, however, in support of an IP_3-mediated shuttling mechanism was derived from experiments in which the Ca^{2+} ionophore, 4-bromo-A23187, was added to neutrophils 1.5 min post-FMLP in a system uncomplicated by Ca^{2+} influx. Treatment of the cells with 2-APB was accompanied by a significantly increased increment in cytosolic Ca^{2+} on addition of the ionophore. This increment, which relative to the control system originated from a lower basal value as expected, clearly demonstrates increased retention of resequestered Ca^{2+} in the stores of 2-APB-treated cells.

This contention is supported by experiments using the selective PLC inhibitor, U-73122. As was the case with 2-APB, addition of U-73122 10 s after FMLP to EGTA-treated neutrophils resulted in an accelerated decline of the peak fura-2 fluorescence response relative to that of cells treated with EGTA only. Again, this effect cannot be attributed to a reduction in capacitative Ca^{2+} influx (due to the presence of an excess of EGTA in the extracellular medium), nor to differences in the activities of the endomembrane and plasma membrane Ca^{2+}-ATPases. As observed with 2-APB, treatment of neutrophils with U-73122 was associated with increased retention of resequestered Ca^{2+}, demonstrated using 4-bromo-A23187 added to the cells 1.5 min post-FMLP.

The possibility that persistent IP_3 levels may counteract Ca^{2+} reuptake into stores mediated by the endomembrane Ca^{2+}-ATPase, has to our knowledge not been previously explored in neutrophils. Recent studies on pancreatic acinar cells found that the concentration of Ca^{2+} inside stores following the initial release phase remained constant and did not increase as expected in the presence of an active SERCA pump [22]. Other reports using endothelial cells have suggested that continuous IP_3 production throughout the decay phase of Ca^{2+} release from stores contributes to the Ca^{2+} transient [23]. The results of the current study support these contentions and suggest that similar mechanisms are operative in chemoattractant-activated human neutrophils. As 2-APB did not alter the cytosolic calcium concentration of resting neutrophils, it is probable that resting cells do not utilise IP_3-mediated shuttling mechanisms to maintain basal cytosolic Ca^{2+} levels.

Although we favour Ca^{2+} shuttling as an interpretation of our data, we do concede that alternative possibilities exist. These include the possible existence of different types of Ca^{2+} storage vesicles in human neutrophils which may vary with respect to sensitivity to IP_3, as well as possible activities of FMLP at high concentrations which may alter IP_3 receptors, negatively affecting sequestration/resequestration of cytosolic Ca^{2+} [24]. In the case of Ca^{2+} stores, there appear to be at least two distinct cellular locations in neutrophils that may have differential involvement in activation of proinflammatory functions, and may utilize different molecular/biochemical mechanisms of Ca^{2+} mobilization [25]. One site is located peripherally under the plasma membrane and appears to be involved in the activation of β_2-integrins, while the other is localized in the perinuclear space and is mobilized by chemoattractants, including FMLP [25]. Mitochondria may also serve as calcium-storage organelles [26], with neutrophils possessing a more extensive mitochondrial network than previously recognized [27].

Although concern has been expressed that 2-APB may lack specificity [20], this agent continues to be utilized as an intracellular probe and inhibitor of IP_3 receptors in various experimental designs [28–31]. Furthermore, we believe that the observed lack of cytotoxicity, the absence of effects on membrane potential (not shown in the current study) of 2-APB per se, the strategy of adding this agent to the cells when mobilization of Ca^{2+} from stores is complete, the rapid onset of the effects on post-peak cytosolic Ca^{2+}, as well as the comparable effects of U-73122, are compatible with a primary effect of this agent on IP_3-receptors in this experimental setting. This contention is underscored by the results obtained using FMLP at concentrations, which differentially affect PLC/IP_3.

In conclusion, the results of the current study have not only identified a role for PLC and IP_3 in maintaining cytosolic Ca^{2+} transients in FMLP-activated human neutrophils, but also indicate a mechanism, distinct from interference with conformational coupling [8] or antagonism of Ca^{2+} channels [20], by which inhibitors of PLC and IP_3-receptor antagonists may attenuate store-operated influx of Ca^{2+} into several cell types, including neutrophils. Decreased production of IP_3, as well as antagonism of IP_3-receptors, favours rapid re-uptake and retention in the stores of cytosolic Ca^{2+}, reduced efflux, and a consequent reduction in the magnitude of store-operated influx of the cation.

References

[1] Fruman DA, Gamache DA, Ernest MJ. Changes in inositol 1,4,5-triphosphate mass in agonist-stimulated human neutrophils. Agents Actions 1991;34:16–9.

[2] Thompson NT, Bonser RW, Tateson JE, Spacey GD, Randall RW, Hodson HF, et al. A quantitative investigation into the dependence of Ca^{2+} mobilisation on changes in inositol 1,4,5-triphosphate levels in the stimulated neutrophil. Br J Pharmacol 1991;103:1592–6.

[3] Ali H, Sozzani S, Fisher I, Barr AJ, Richardson RM, Haribabu B, et al. Differential regulation of formyl peptide and platelet-activating factor receptors: role of phospholipase Cβ3 phosphorylation by protein kinase A. J Biol Chem 1998;273:11012–6.

[4] Steel HC, Anderson R. Dissociation of the PAF-receptor from NADPH oxidase and adenylate cyclase in human neutrophils results in accelerated influx and delayed clearance of cytosolic calcium. Br J Pharmacol 2002;136:81–9.

[5] Anderson R, Goolam-Mahomed A. Calcium efflux and influx in f-met-leu-phe (fMLP)-activated human neutrophils are chronologically distinct events. Clin Exp Immunol 1997;110:132–8.

[6] Anderson R, Goolam-Mahomed A, Theron AJ, Ramafi G, Feldman C. Effect of rolipram and dibutyryl cyclic AMP on resequestration of cytosolic calcium in FMLP-activated human neutrophils. Br J Pharmacol 1998;124:547–55.
[7] Davies-Cox EV, Laffafian I, Hallett MB. Control of Ca^{2+} influx in human neutrophils by inositol 1,4,5-triphosphate (IP_3) binding: differential effects of micro-injected IP_3 receptor antagonists. Biochem J 2001;355(Pt 1):139–43.
[8] Ma H-T, Patterson RL, van Rossum DB, Birnbaumer L, Mikoshiba K, Gill DL. Requirement of the inositol triphosphate receptor for activation of store-operated Ca^{2+} channels. Science 2000;287:1647–51.
[9] Geiszt M, Kapus A, Német K, Farkas L, Ligeti E. Regulation of capacitative Ca^{2+} influx in human neutrophil granulocytes. Alterations in chronic granulomatous disease. J Biol Chem 1997;272:26471–8.
[10] Rada BK, Geiszt M, van Bruggen R, Német K, Roos D, Ligeti E. Calcium signaling is altered in myeloid cells with a deficiency in NADPH oxidase activity. Clin Exp Immunol 2003;132:53–60.
[11] Tintinger GR, Theron AJ, Steel HC, Anderson R. Accelerated calcium influx and hyperactivation of neutrophils in chronic granulomatous disease. Clin Exp Immunol 2001;123:254–63.
[12] Tintinger GR, Anderson R. Counteracting effects of NADPH oxidase and the Na^+/Ca^{2+} exchanger on membrane repolarisation and store-operated uptake of Ca^{2+} by chemoattractant-activated human neutrophils. Biochem Pharmacol 2004;67:2263–71.
[13] Grynkiewicz G, Poenie M, Tsien RY. A new generation of Ca^{2+} indicators with greatly improved fluorescence properties. J Biol Chem 1985;260:3440–50.
[14] Bishara NB, Murphy TV, Hill MA. Capacitative Ca^{2+} entry in vascular endothelial cells is mediated via pathways sensitive to 2-aminoethoxydiphenyl borate and xestospongin. Br J Pharmacol 2002;135:119–28.
[15] Cockeran R, Theron AJ, Steel HC, Matlola NM, Mitchell TJ, Feldman C, et al. Proinflammatory interactions of pneumolysin with human neutrophils. J Infect Dis 2001;183:604–11.
[16] Lytton J, Westlin M, Hanley MR. Thapsigargin inhibits the sarcoplasmic or endoplasmic reticulum Ca-ATPase family of calcium pumps. J Biol Chem 1991;266:17067–71.
[17] Erdahl WL, Chapman CJ, Taylor RW, Pfeiffer DR. Effects of pH conditions on Ca^{2+} transport catalyzed by ionophores A23187,4-br A23187 and ionomycin suggest problems with common applications of these compounds in biological systems. Biophys J 1995;69:2350–63.
[18] Holmsen HE, Storm E, Day HJ. Determination of ATP and ADP in blood platelets: a modification of the firefly luciferase assay for plasma. Anal Biochem 1972;46:481–502.
[19] Partida-Sanchez S, Iribarren P, Moreno-Garcia ME, Gao JL, Murphy PM, Oppenheimer N, et al. Chemotaxis and calcium responses of phagocytes to formyl peptide receptor ligands is differentially regulated by cyclic ADP ribose. J Immunol 2004;172:1896–906.
[20] Iwasaki H, Mori Y, Hara Y, Uchida K, Zhou H, Mikoshiba K. 2-Aminoethoxydiphenyl borate (2-APB) inhibits capacitative calcium entry independently of the function of inositol 1,4,5-triphosphate receptors. Receptors Channels 2001;7:429–39.
[21] Thomas AP, Alexander J, Williamson JR. Relationship between inositol polyphosphate production and the increase in cytosolic free Ca^{2+} induced by vasopressin in isolated hepatocytes. J Biol Chem 1984;259:5574–84.
[22] Mogami H, Tepikin AV, Petersen OH. Termination of cytosolic Ca^{2+} signals: Ca^{2+} reuptake into intracellular stores is regulated by the free Ca^{2+} concentration in the store lumen. EMBO J 1998;17:435–42.
[23] Moccia F, Berra-Romani R, Baruffi S, Spaggiari S, Signorelli S, Castelli L, et al. Ca^{2+} uptake by the endoplasmic reticulum Ca^{2+}-ATPase in rat microvascular endothelial cells. Biochem J 2002;364(Pt 1):235–44.
[24] Boehning D, Patterson RL, Sedaghat L, Glebova NO, Kurosaki T, Snyder SH. Cytochrome c binds to inositol (1,4,5) triphosphate receptors, amplifying calcium-dependent apoptosis. Nat Cell Biol 2003;5:1051–61.
[25] Pettit EJ, Hallett MB. Two distinct Ca^{2+} storage and release sites in human neutrophils. J Leukoc Biol 1998;63:225–32.
[26] Montero M, Lobatón CD, Gutierrez-Fernandez S, Morenao A, Alvarez J. Calcineurin-independent inhibition of mitochondrial Ca^{2+} uptake by cyclosporin A. Br J Pharmacol 2004;141:263–8.
[27] Fossati G, Moulding DA, Spiller DG, Moots RJ, White MRH, Edwards SW. The mitochondrial network of human neutrophils: role in chemotaxis, phagocytosis, respiratory burst activation and commitment to apoptosis. J Immunol 2003;170:1964–72.
[28] Pan BX, Zhao GL, Huang XL, Zhao KS. Calcium mobilization is required for peroxynitrite-mediated enhancement of spontaneous transient outward currents in arteriolar smooth muscle cells. Free Radic Biol Med 2004;37:823–38.
[29] Yanagida E, Shoji S, Hirayama Y, Yoshikawa F, Otsu K, Uematsu H, et al. Functional expression of Ca^{2+} signaling pathways in mouse embryonic cells. Cell Calcium 2004;36:135–46.
[30] Brown RA, Lever R, Jones NA, Page CP. Effects of heparin and related molecules upon neutrophil aggregation and elastase release in vitro. Br J Pharmacol 2003;139:845–53.
[31] Wang JP. GEA3162 stimulates Ca^{2+} entry in neutrophils. Eur J Pharmacol 2003;458:243–9.