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Dissociation of the PAF-receptor from NADPH oxidase and adenylate cyclase in human neutrophils results in accelerated influx and delayed clearance of cytosolic calcium

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1 The magnitude and duration of the abruptly occurring increases in cytosolic Ca^{2+} in human neutrophils following activation with PAF (20 and 200 nM) and FMLP (1 μM), have been compared and related to alterations in NADPH oxidase activity, membrane potential and intracellular cyclic AMP.

2 Cytosolic Ca^{2+} and membrane potential were measured by spectrofluorimetry, transmembrane fluxes of Ca^{2+} by radiometric procedures, and NADPH oxidase activity and cyclic AMP by chemiluminescence and radioimmunoassay respectively.

3 Activation of neutrophils with both PAF (200 nM) and FMLP (1 μM) was accompanied by an abrupt increase in cytosolic Ca^{2+}, which was of similar magnitude for each activator (393 ± 9 and 378 ± 17 nM respectively). Unlike FMLP-activated cells in which Ca^{2+} was rapidly removed from the cytosol, peak levels of cytosolic Ca^{2+} were sustained for longer (0.14 ± 0.02 vs 1.16 ± 0.04 min, $P \leqslant 0.0001$) and declined at a slower rate in PAF-treated neutrophils.

4 The prolonged elevation of cytosolic Ca^{2+} in PAF-treated cells was due to accelerated store-operated influx of extracellular cation and was attenuated by dibutyryl cyclic AMP (4 mM), the Ca^{2+}-chelator, EGTA (5 mM), and SKF 96365 (10 μM). In contrast to FMLP, basal levels of superoxide production and cyclic AMP were unaltered in PAF-activated neutrophils, while only moderate membrane depolarization was detected.

5 These observations demonstrate that mechanisms which restore Ca^{2+} homeostasis to FMLP-activated neutrophils, *viz*. activation of NADPH oxidase and adenylate cyclase, are not operative in PAF-treated cells, presenting the potential hazard of Ca^{2+} overload and hyperactivity.
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Keywords: Calcium; CGS 21680; cyclic AMP; membrane depolarization; dibutyryl cyclic AMP; elastase; FMLP; neutrophils; PAF; superoxide

Abbreviations: cyclic AMP, adenosine 3′,5′ cyclic monophosphate; CB, cytochalasin B; CGS 21680, 2(4-[(2-carboxyethyl)phenyl]ethylamino)-5′-N-ethylcarboxamido adenosine; Di-O-C_5(3), dipentyloxacarbocyanine; DMSO, dimethyl sulphoxide; EGTA, ethylene glycol-bis(β-aminoethyl ether) N,N,N′,N′-tetraacetic acid; FMLP, N-formyl-L-methionyl-L-leucyl-L-phenylalanine; HBSS, Hanks balanced salt solution; LECL, lucigenin-enhanced chemiluminescence; MAPk, mitogen-activated protein kinase; PBS, phosphate-buffered saline; PLC, phospholipase C; PMA, phorbol 12-myristate 13-acetate; r.l.u., relative light units; SKF 96365, (1-[β-)3-[4-methoxyphenyl]-1H-imidazole hydrochloride; U 73122, (1-[6-([17β)-3-methoxyestra-1,3,5(10)-trien-17-yl]amino) hexyl]-1H-pyrrole-2,5-dione

Introduction

Platelet-activating factor (PAF; 1-O-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine) is arguably the most potent of the pro-inflammatory, bioactive phospholipids (Zimmerman *et al*., 1992; Kravchenko *et al*., 1995) with the potential to reproduce all of the clinical manifestations of bronchial asthma (Barnes & Chung, 1987). Receptor-mediated pro-inflammatory activities of PAF include pro-adhesive and chemotactic activities for eosinophils and neutrophils, as well as sensitization ('priming') of these cells for increased production of reactive oxidants and release of granule constituents on subsequent exposure to chemoattractants and other activators (Peplow, 1999). PAF has also been reported to sensitize monocytes/macrophages for increased production of leukotriene B_4 (Shindo *et al*., 1998) and interleukin-8 (Hilger *et al*., 1996; Arbabi *et al*., 1999) and to activate the proinflammatory, cytosolic nuclear transcription factor NF-κB in a variety of cell types (Kravchenko *et al*., 1995).

Although many of these pro-inflammatory activities of PAF are believed to be achieved through receptor-mediated increases in cytosolic Ca^{2+} in target cells, relatively little is known about the sites (extracellular or intracellular) from which the cation is mobilized, or the mechanisms used by PAF-activated cells to restore Ca^{2+} homeostasis. In the case of neutrophils activated with chemoattractants such as N-formyl-L-methionyl-L-leucyl-L-phenylalanine (FMLP), the plasma membrane and endo-membrane Ca^{2+}-ATPases, which

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are regulated by calmodulin and adenosine 3′,5′ cyclic monophosphate (cyclic AMP)-dependent protein kinase respectively (Lagast *et al*., 1984; Tao *et al*., 1992), promote clearance of cytosolic Ca^{2+} by efflux and resequestration of the cation (Anderson *et al*., 1998; Pettit & Hallett, 2000). The efficiency of these Ca^{2+}-clearance pumps is enhanced by the membrane depolarizing actions of NADPH oxidase which restrict the uptake of extracellular Ca^{2+} (Di Virgilio *et al*., 1987; Geiszt *et al*., 1997; Tintinger *et al*., 2001). In contrast to FMLP-activated neutrophils, neither NADPH oxidase (Nick *et al*., 1997) nor adenylate cyclase (Ali *et al*., 1998) undergoes significant activation on exposure of neutrophils to PAF, suggesting that Ca^{2+} handling by cells activated with this bioactive phospholipid is likely to differ from that of cells exposed to the chemotactic tripeptide.

In the current study we have compared the magnitude and duration of the changes in cytosolic Ca^{2+} concentrations which accompany activation of human neutrophils with PAF and FMLP, as well as the origins of the cytosolic cation mobilized by both activators. In addition, we have investigated the potential of the cell-permeable cyclic AMP-analogue, dibutyryl cylic AMP, the type 4 phosphodiesterase inhibitor, rolipram and the subtype A_{2A} receptor agonist, 2(4-[(2-carboxyethyl)phenyl]ethylamino)-5′-N-ethylcarboxamido adenosine (CGS 21680), to restore Ca^{2+} homeostasis to PAF-activated neutrophils.

Methods

Neutrophils

Neutrophils were prepared from heparinized (5 U of preservative-free heparin ml^{-1}) venous blood taken from healthy, adult volunteers. The heparinized blood was fractionated by centrifugation (400 *g* for 25 min) on Histopaque®-1077 (Sigma Diagnostics) cushions and the resultant neutrophil fraction removed by sequential sedimentation with 3% gelatin and selective lysis with 0.84% ammonium chloride (Anderson *et al*., 1998). The neutrophils, which were routinely of high purity (>90%) and viability (>95%) were resuspended to a concentration of 10^7 ml^{-1} in phosphate-buffered saline (PBS) and held on ice until use.

Spectrofluorimetric measurement of Ca^{2+}-fluxes

Fura-2/AM was used as the fluorescent, Ca^{2+}-sensitive indicator for these experiments (Grynkiewicz *et al*., 1985). Neutrophils (10^7 ml^{-1}) were pre-loaded with fura-2 (2 μM) for 30 min at 37°C in PBS (0.15 M, pH 7.4), washed twice and resuspended in indicator-free Hanks balanced salt solution (HBSS, pH 7.4) containing 1.25 mM $CaCl_2$ (Ca^{2+}-replete HBSS). The fura-2 loaded cells (2×10^6 ml^{-1}) were then pre-incubated for 10 min at 37°C, after which they were transferred to cuvettes, which were maintained at 37°C in a Hitachi 650 10S fluorescence spectrophotometer with excitation and emission wavelengths set at 340 nm and 500 nm, respectively. After a stable baseline was obtained ($\pm$1 min), PAF (2 pmol–200 nM) or FMLP (1 μM) was added to the neutrophils and the subsequent increase in fura-2 fluorescence intensity was monitored over a 5 min period. The final volume in each cuvette was 3 ml containing a total of 6×10^6 neutrophils. Based on the results of these experiments, PAF was used at 20 and/or 200 nM in subsequent investigations since at these concentrations the phospholipid caused elevations in neutrophil cytosolic Ca^{2+} equivalent to those induced by FMLP at 1 μM, a concentration of this agent which was found in preliminary experiments to be maximally effective with respect to Ca^{2+} mobilization, membrane depolarization and superoxide generation (concentrations of 0.001, 0.01, 0.1 and 1 μM were evaluated). The values shown in the text and tables are those for the peak increments observed within 30 s of addition of FMLP or PAF to the cells and were calculated as described previously (Grynkiewicz *et al*., 1985).

The effects of the extracellular Ca^{2+}-chelating agent ethylene glycol-bis(β-aminoethyl ether) N,N,N′,N′-tetraacetic acid (EGTA, 5 mM), (1-[β-)3-[4-methoxyphenyl]-1H-imidazole hydrochloride (SKF 96365, 10 μM) a selective inhibitor of store-operated Ca^{2+} influx (Merritt *et al*., 1990), (1-[6-([17()-3-methoxyestra-1,3,5(10)-trien-17-yl]amino)hexyl]-1H-pyrrole-2,5-dione (U 73122, 10 μM) a phospholipase C (PLC) inhibitor (Tatrai *et al*., 1994), the cell permeable analogue of cyclic AMP, dibutyryl cyclic AMP (4 mM), the type 4 phosphodiesterase inhibitor, rolipram (0.5 μM), the subtype A_{2A} receptor agonist, CGS 21680 (1 μM) and the protein kinase C activator, phorbol 12-myristate 13-acetate (PMA, 40 nM) on the PAF (200 μM)-activated fura-2 fluorescence responses of neutrophils were also investigated.

Mn^{2+}-quenching of fura-2 fluorescence

Cells loaded with fura-2/AM as described above were activated with FMLP (1 μM) or PAF (20 and 200 nM) in HBSS supplemented with 300 μM $MnCl_2$ (added 5 min prior to FMLP or PAF) and fluorescence quenching, as a measure of Ca^{2+} influx, was determined at an excitation wavelength of 360 nm, which is an isosbestic wavelength, and at an emission wavelength of 500 nm (Geiszt *et al*., 1997). This procedure was also used to investigate the effects of CGS 21680 (1 μM), dibutyryl cyclic AMP (4 mM), rolipram (0.5 μM) and SKF 96365 (10 μM) on the influx of Ca^{2+} into PAF (200 nM)-activated neutrophils.

Radiometric assessment of Ca^{2+} fluxes

$^{45}Ca^{2+}$ (Calcium-45 chloride, specific activity 18.53 mCi mg^{-1}, Du Pont NEN Research Products, Boston, MA, U.S.A.) was used as tracer to lable the intracellular Ca^{2+} pool and to monitor Ca^{2+} fluxes in resting and PAF-stimulated neutrophils. In the assays of Ca^{2+} influx and efflux described below, the radiolabelled cation was used at a fixed, final concentration of 2 μCi ml^{-1}, containing 50 nM cold carrier Ca^{2+} (as $CaCl_2$) and the final assay volumes were 5 ml containing a total of 1×10^7 neutrophils. The standardization of the procedures used to load the cells with $^{45}Ca^{2+}$, as well as a comparison with oil-based methods for the separation of labelled neutrophils from unbound isotope, have been described (Anderson & Goolam Mohamed, 1997).

Efflux of $^{45}Ca^{2+}$ from neutrophils exposed to PAF

To measure net efflux of $^{45}Ca^{2+}$ from neutrophils uncomplicated by concomitant influx of the radiolabelled cation, the

cells (10^7 ml^{-1}) were loaded with $^{45}Ca^{2+}$ (2 μCi ml^{-1}) for 20 min at 37°C in HBSS. The neutrophils were then pelleted by centrifugation, washed once 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 followed by activation with FMLP (1 μM) or PAF (20 and 200 nM). In additional experiments, neutrophils were preincubated for 10 min at 37°C in the presence and absence of dibutyryl cyclic AMP (4 mM), CGS 21680 (1 μM), rolipram (0.5 μM) or SKF 96365 (10 μM) followed by addition of PAF (20 and 200 nM) and measurement of the net efflux of $^{45}Ca^{2+}$ over a 60 s time-course.

Reactions were stopped by adding 10 ml ice-cold Ca^{2+}-replete HBSS to the tubes, which were then transferred immediately to an ice-bath. The cells were then pelleted by centrifugation at 400 *g* for 5 min followed by washing with ice-cold Ca^{2+}-replete HBSS and the cell pellets finally dissolved in 0.5 ml of Triton X-100/0.05 M NaOH and the radioactivity assayed in a scintillation spectrometer.

Influx of $^{45}Ca^{2+}$ into PAF-exposed neutrophils

To measure net influx of $^{45}Ca^{2+}$ from neutrophils, uncomplicated by concomitant efflux of the radiolabelled cation, the cells (10^7 ml^{-1}) were suspended in Ca^{2+}-replete HBSS and incubated for 15 min at 37°C, after which they were pelleted by centrifugation, then washed once and resuspended in ice-cold Ca^{2+}-free HBSS and held on ice until use. Preloading with cold Ca^{2+} was undertaken to minimize spontaneous uptake of $^{45}Ca^{2+}$ in the efflux assay. The efficiency of this loading procedure was demonstrated by measurement of the FMLP-activated fura-2 responses of the Ca^{2+}-loaded neutrophils, which did not differ from those of neutrophils maintained in Ca^{2+}-replete HBSS (Anderson & Goolam Mahomed, 1997). The Ca^{2+}-loaded neutrophils (2×10^6 ml^{-1}) were then incubated for 10 min at 37°C followed by simultaneous addition of PAF (200 nM) and $^{45}Ca^{2+}$ (2 μCi ml^{-1}) or FMLP (1 μM) and $^{45}Ca^{2+}$, or by $^{45}Ca^{2+}$ only to control systems. Influx of $^{45}Ca^{2+}$ into FMLP- or PAF-activated neutrophils was then monitored over a 5 min period, after which influx is complete (Anderson & Goolam Mohamed, 1997), and compared with the uptake of the radiolabelled cation by identically processed, unstimulated cells. The effects of CGS 21680 (1 μM), dibutyryl cyclic AMP (4 mM), rolipram (0.5 μM) and SKF 96365 (10 μM) on PAF-activated influx of $^{45}Ca^{2+}$ into neutrophils were also investigated.

Reactions were stopped by adding 10 ml ice-cold Ca^{2+}-replete HBSS to the tubes. The cells were then pelleted by centrifugation at 400 *g* for 5 min followed by washing with ice-cold Ca^{2+}-replete HBSS and the cell pellets finally dissolved in 0.5 ml of Triton X-100/0.05 M NaOH and the radioactivity assayed in a scintillation spectrometer.

Superoxide production

This was measured by use of a lucigenin (bis-N-methylacridinium nitrate)-enhanced chemiluminescence (LECL) method (Minkenberg & Ferber, 1984). Neutrophils (10^6 ml^{-1}) were preincubated for 15 min at 37°C in 900 μl HBSS (pH 7.4, 1.25 mM $CaCl_2$) containing 0.2 mM lucigenin after which PAF (20 and 200 nM) or FMLP (1 μM) was added. LECL responses were measured using a model 2010R Biocounter (Lumac Systems, Titusville, Flo, U.S.A.) and the results expressed as relative light units (r.l.u.).

Elastase release

Neutrophil degranulation was measured according to the extent of release of the primary granule-derived enzyme, elastase. PAF (200 nM) was added to neutrophils which were preincubated at a concentration of 2×10^6 ml^{-1} in HBSS for 10 min at 37°C. FMLP, (0.1 μM final) in combination with a submaximal concentration of cytochalasin B (CB, 1 μM), was then added to the cells, which were incubated for a further 15 min at 37°C. The tubes were then transferred to an ice bath, followed by centrifugation at 400 *g* for 5 min to pellet the cells. The neutrophil-free supernatants were then decanted and assayed for elastase activity using a micromodification of a standard colourimetric procedure (Beatty *et al*., 1982). Briefly, 125 μl of supernatant were added to an equal volume of the elastase substrate N-succinyl-L-alanyl-L-alanyl-L-alanine-p-nitroanilide (3 mM) in 0.05 M Tris-HCl (pH 8.0) and elastase activity monitored at a wavelength of 405 nm.

The effects of dibutyryl cyclic AMP (4 mM), CGS 21680 (1 μM) and rolipram (0.5 μM) on the release of elastase from neutrophils exposed to PAF (200 nM) were also investigated. These agents were added to the neutrophils 5 min prior to addition of PAF.

Membrane potential

The potential sensitive fluorescent dye, dipentyloxacarbocyanine (di-O-C_5(3)) was used to measure changes in membrane potential in activated neutrophils (Seligmann & Gallin, 1980). The cells (10^6 ml^{-1}) were preincubated for 10 min at 37°C in HBSS containing 80 nM di-O-C_5(3), 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 460 nm and 510 nm respectively. The neutrophils were then activated with FMLP (1 μM) or PAF (20 and 200 nM) and subsequent alterations in fluorescence intensity monitored over a 5–10 min period.

Measurement of Intracellular cyclic AMP

Neutrophils (10^7 ml^{-1}) in HBSS were preincubated for 10 min at 37°C with and without the phosphodiesterase inhibitor, rolipram (0.5 μM). Following preincubation, the cells were activated with PAF (20 and 200 nM), in a final volume of 1 ml. The reactions were terminated and the cyclic AMP extracted by the addition of ice-cold ethanol (65% v v^{-1}) at 0, 10 and 30 s and 1, 3 and 5 min after addition of the PAF. The resultant precipitates were washed twice (2000 *g* for 15 min at 4°C) with ice-cold ethanol and the supernatants pooled and evaporated at 60°C under a stream of air. The dried extracts were reconstituted in assay buffer (0.05 M acetate buffer, pH 5.8) and assayed for cyclic AMP using the Biotrak cyclic AMP [^{125}I] scintillation proximity assay system (Amersham International plc.), which is a competitive binding radioimmunoassay procedure. These results are expressed as pmoles cyclic AMP 10^{-7} neutrophils.

Drugs and reagents

CGS 21680 and rolipram were kindly provided by Dr Malcolm Johnson (Glaxo Smith Kline, Stockley Park West, London, U.K.), while all other chemicals and reagents were purchased from the Sigma Chemical Co. Dimethyl sulphoxide (DMSO) was used as the solvent for PAF, rolipram, SKF 96365 and U 73122 and appropriate solvent control systems were included for these agents.

Statistical analysis

The results of each series of experiments are expressed as the mean value ± the standard error of the mean (s.e.mean). Where appropriate, levels of statistical significance were calculated using the Mann-Whitney test. P values of $\leqslant 0.05$ were considered significant.

Results

Fura-2 responses of neutrophils

In preliminary experiments, PAF at concentrations of 20 pmol and higher caused dose-related, transient, increases in neutrophil cytosolic Ca^{2+} which were maximal at 20 nM. Concentrations of 20 and 200 nM were used in all subsequent experiments. The traces shown in Figure 1 are representative of the FMLP (1 μM)- and PAF (20 and 200 nM)-activated fura-2 responses of neutrophils. Addition of FMLP or PAF to neutrophils was accompanied by an abrupt increase in fura-2 fluorescence intensity due to elevations in the cytosolic concentrations of Ca^{2+}, which were of similar magnitude for each activator. In the case of FMLP, peak fluorescence intensity occurred at around 10 s and was followed by an initially linear decrease in fluorescence intensity, reflecting clearance of Ca^{2+} from the cytosol, which reached baseline values approximately 5 min after addition of FMLP. However, in the case of PAF-treated neutrophils, peak cytosolic Ca^{2+} concentrations were either sustained for longer (200 nM PAF) or subsided briefly to be followed by a second peak (20 nM PAF). In both cases, the prolonged peaks (relative to FMLP) in fluorescence intensity were followed by an initially linear decrease in fluorescence. The effects of EGTA (5 mM), SKF 96365, PMA and U 73122 on the fura-2 responses of PAF (200 nM)-activated neutrophils are shown in Figure 2. EGTA, SKF 96365 and PMA did not affect the peak increases in cytosolic Ca^{2+} in PAF-activated neutrophils. However, all three agents abolished the prolongation in peak fluorescence intensity, suggesting that this is due to influx of extracellular Ca^{2+}. Treatment of neutrophils with U 73122 completely abrogated the PAF-activated increase in cytosolic Ca^{2+}.

EGTA and SKF 96365 also dramatically hastened the return to basal fura-2 fluorescence intensity in FMLP-activated neutrophils without affecting the magnitude of peak fluorescence intensity, while in the case of cells treated with U 73122 there was complete attenuation of the fura-2 fluorescence response (not shown).

The effects of dibutyryl cyclic AMP (4 mM), rolipram (0.5 μM) and CGS 21680 (1 μM) on the fura-2 fluorescence responses of PAF (200 nM)-activated neutrophils are shown in Figure 3. Although none of the test agents affected the peak changes in fluorescence, all three agents, in particular dibutyryl cyclic AMP and CGS 21680, hastened the rate of decline in peak fluorescence intensity, indicative of accelerated clearance of Ca^{2+} from the cytosol.

Peak increments in cytosolic Ca^{2+} concentrations, time taken for fluorescence intensity to decline to half peak values, as well as the rate of Ca^{2+} clearance for neutrophils from eight different individuals activated with FMLP or PAF (200 nM), in the absence or presence of EGTA, SKF 96365, PMA, U 73122, CGS 21680, dibutyryl cyclic AMP and rolipram, are shown in Table 1.

Influx of Ca^{2+} using Mn^{2+} quenching of fura-2 fluorescence

The results of the indirect measurement of Ca^{2+} influx into FMLP- and PAF-activated neutrophils using Mn^{2+} quench-

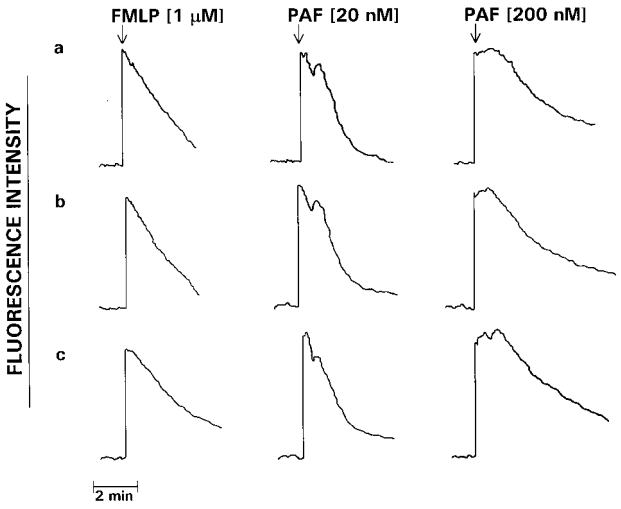

Figure 1 Fura-2 fluorescence responses of FMLP (1 μM)- and PAF (20 and 200 nM)-activated neutrophils. Following loading with fura-2, the cells were preincubated for 10 min at 37°C prior to the addition of FMLP or PAF and subsequent alterations in fluorescence intensity monitored over a 5 min period. The results shown are three typical sets of traces of 15 obtained. The arrow (↓) denotes addition of FMLP or PAF.

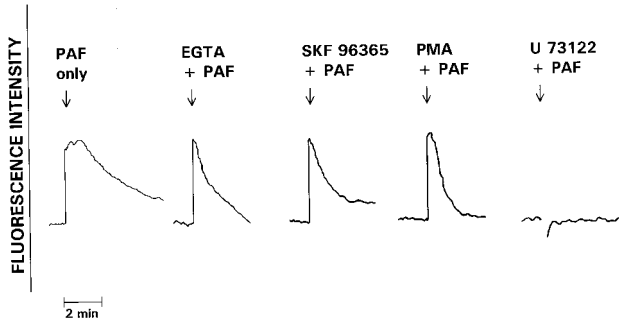

Figure 2 The effects of pretreatment with EGTA (5 mM), SKF 96365 (10 μM), PMA (40 nM) and U 73122 (10 μM) on the fura-2 responses of PAF (200 nM)-activated neutrophils. The responses of cells treated with these various agents were compared with those of the corresponding control system activated with PAF in the absence of these agents. The results shown are representative traces of between three and six sets of results obtained. The arrow (↓) denotes addition of PAF.

ing of fluorescence are shown in Figure 4. In the case of FMLP-activated cells, addition of the chemoattractant was accompanied by a lag phase of about 20–30 s duration followed by an almost linear decrease, indicative of influx of Ca^{2+}, over 3–4 min. In the case of PAF-activated neutrophils, the decrease in fluorescence intensity occurred almost immediately and proceeded at a much faster rate than that observed in FMLP-activated cells.

Efflux of $^{45}Ca^{2+}$

The kinetics of efflux of Ca^{2+} from PAF (20 and 200 nM)- and FMLP (1 μM)-activated neutrophils over a 60 s time-course are shown in Figure 5. No significant loss of $^{45}Ca^{2+}$ was observed in the unstimulated, control neutrophils over the 60 s incubation period during which efflux was measured. Exposure of the neutrophils to PAF and FMLP resulted in rapid efflux of the radiolabelled cation from the neutrophils in the first 10 s incubation period, followed by a slow efflux of Ca^{2+} to a maximum loss of 43, 54 and 42% of cell-associated cation for 20 nM, 200 nM PAF and 1 μM FMLP respectively at the end of the 60 s time-course of the experiment.

The effects of dibutyryl cyclic AMP, CGS 21680, rolipram and SKF 96365 on the efflux of $^{45}Ca^{2+}$ from PAF-activated neutrophils are shown in Table 2. Pretreatment of the neutrophils with dibutyryl cyclic AMP, CGS 21680 or rolipram significantly reduced the extent of PAF-mediated efflux of $^{45}Ca^{2+}$ from the cells, while the discharge of the cation was not significantly affected by SKF 96365.

Influx of $^{45}Ca^{2+}$

The kinetics of influx of $^{45}Ca^{2+}$ into PAF- and FMLP-activated neutrophils are shown in Figure 6. Modest influx of $^{45}Ca^{2+}$ (64 pmol 10^{-7} cells) was observed in control neutrophils over the 5 min time-course while, in the case of FMLP- and PAF-activated neutrophils, significant influx of Ca^{2+} above this level was noted ($P \leqslant 0.0001$). The net influx of Ca^{2+} into FMLP- and PAF (20 and 200 nM)-activated neutrophils was 275 ± 7 pmol 10^{-7} cells, 175 ± 27 pmol 10^{-7} cells and 293 ± 62 pmol 10^{-7} cells, respectively.

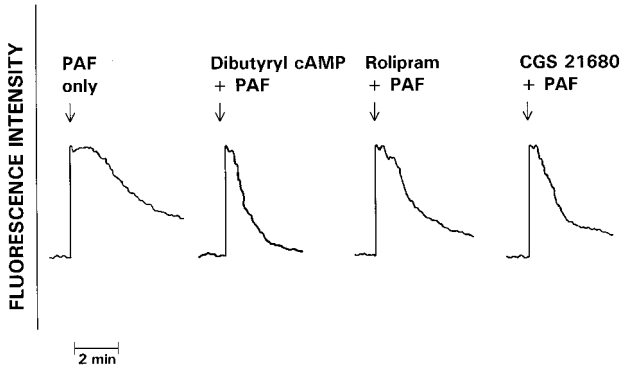

Figure 3 The effects of pretreatment with dibutyryl cyclic AMP (4 mM), rolipram (0.5 μM) and CGS 21680 (1 μM) on the fura-2 responses of PAF (200 nM)-activated neutrophils. The responses of cells treated with these various agents were compared with those of the corresponding control system activated with PAF in the absence of these agents. The results shown are representative traces of 12 sets of results obtained. The arrow (↓) denotes addition of PAF.

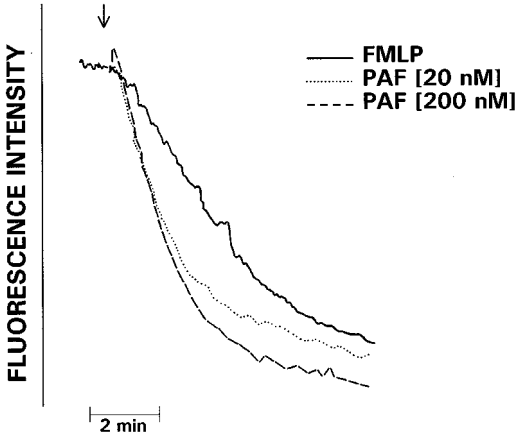

Figure 4 FMLP (1 μM)- and PAF (20 and 200 nM)-activated Mn^{2+}-quenching of the fura-2 responses of neutrophils. A direct relationship exists between the magnitude of Mn^{2+}-quenching of fura-2 fluorescence and the extent of store-operated influx of Ca^{2+} into the cells. The results shown are representative traces of five sets of results obtained. The arrow (↓) denotes addition of FMLP or PAF.

Table 1 Peak increments in cytosolic Ca^{2+} concentrations, time taken for fura-2 fluorescence intensity to decline to half peak values and the rate of Ca^{2+} clearance in FMLP- and PAF-activated neutrophils

System	*Peak increments in cytosolic Ca^{2+}* (nM)	*Time taken to decline to half peak values* (min)	*Clearance rate of cytosolic Ca^{2+}* (nM min^{-1})
FMLP (1 μM)	378±17	1.6±0.1	118±1
PAF (20 nM)	386±13	1.4±0.1	138±1
PAF (200 nM)	393±9	3.4±0.1*	58±0*
EGTA (1 nM) + PAF (200 nM)	303±26**	0.9±0.1**	169±2**
SKF 96365 (10 μM) + PAF (200 nM)	304±3**	1.1±0.1**	138±0**
PMA (40 nM) + PAF (200 nM)	310±1**	1.1±0.1**	141±9**
U 73122 (10 μM) + PAF (200 nM)	0.0**	0.0**	0.0**
Dibutyryl cAMP (4 mM) + PAF (200 nM)	362±6	0.7±0.0**	259±1**
CGS 21680 (1 μM) + PAF (200 nM)	375±12	1.7±0.2**	110±1**
Rolipram (0.5 μM) + PAF (200 nM)	388±13	1.1±0.1**	176±2**

The results are expressed as the mean value±s.e.mean for activated neutrophils with and without EGTA, SKF 96365, PMA, U 73122, dibutyryl cyclic AMP, CGS 21680 and rolipram. *$P \leqslant 0.0001$, for comparison between the FMLP-activated control neutrophils. **$P \leqslant 0.0001$, for comparison between the PAF-activated neutrophils.

The effects of CGS 21680, dibutyryl cyclic AMP, rolipram and SKF 96365 on influx of $^{45}Ca^{2+}$ into PAF-activated neutrophils are shown in Table 3. Influx of $^{45}Ca^{2+}$ into PAF-activated neutrophils was almost completely attenuated by SKF 96365, and partly attenuated by dibutyryl cyclic AMP, rolipram and CGS 21680. These findings were confirmed using the Mn^{2+} quenching of fura-2 fluorescence procedure (not shown).

Superoxide generation

PAF, at the concentrations used in this study, did not activate oxidant production by neutrophils. The absolute peak values ± s.e.mean for superoxide production observed over a 5 min time-course for the control system (in the absence of FMLP or PAF) and FMLP (1 μM)-activated neutrophils were 3929 ± 245 and 16066 ± 857 r.l.u., while those for PAF at concentrations of 20 and 200 nM were 3766 ± 287 and 3853 ± 297 r.l.u., respectively.

Elastase release

Although PAF *per se* did not activate the release of elastase from neutrophils, this phospholipid, at a concentration of 200 nM, caused significant enhancement of elastase release from FMLP/CB-activated neutrophils ($P \leqslant 0.001$). The effects of pre-incubation of neutrophils with dibutyryl cyclic AMP, CGS 21680 and rolipram on the release of elastase from PAF (200 nM)-sensitised, FMLP/CB-activated neutrophils are shown in Table 4. All three of these agents caused significant ($P \leqslant 0.001$), but incomplete, attenuation of the sensitising effects of PAF on elastase release from neutrophils.

Membrane potential

FMLP- and PAF-activated alterations in neutrophil membrane potential are shown in Figure 7. Exposure of neutrophils to FMLP resulted in rapid membrane depolarization which terminated at around 30 s and was followed approximately 30–60 s later by gradual membrane repolarization which was complete by 5 min, but which did not recover to preactivation values. In contrast, the PAF-activated decrease in membrane potential was considerably less (averages of 16 ± 1% and 46 ± 5% of the FMLP response for 20 nM and 200 nM PAF respectively).

Intracellular cyclic AMP levels

Intracellular cyclic AMP levels in control and rolipram (0.5 μM)-treated unstimulated neutrophils were 122 ± 8 and 321 ± 28 pmol 10^{-7} cells immediately prior to the addition of PAF (200 nM) to the cells and were not significantly altered throughout the subsequent 5 min time-course of the experiment, the maximum values for PAF-treated cells in the absence and presence of rolipram being 135 ± 8 and 351 ± 38 pmol 10^{-7} cells respectively (data from five experiments).

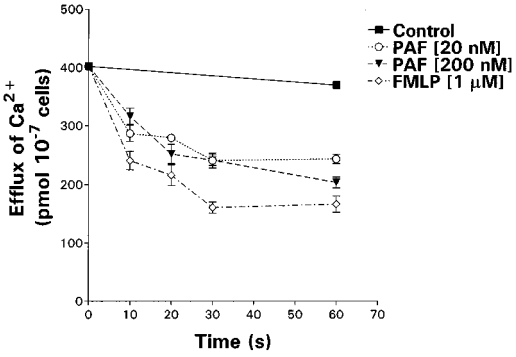

Figure 5 Kinetics of efflux of $^{45}Ca^{2+}$ from unstimulated neutrophils and from neutrophils activated with PAF (20 and 200 nM) or FMLP (1 μM) over a 60 s time-course. The results of three different experiments are expressed as the mean amount of cell-associated $^{45}Ca^{2+}$ (pmol 10^{-7} cells) with vertical lines showing s.e.mean.

Table 2 Effects of dibutyryl cyclic AMP, CGS 21680, rolipram and SKF 96365 on the efflux of $^{45}Ca^{2+}$ from PAF-activated neutrophils

System	*Amount of $^{45}Ca^{2+}$ released from neutrophils (pmol 10^{-7} cells)*
PAF (200 nM)	119 ± 3*
Dibutyryl cAMP (4 mM) + PAF (200 nM)	44 ± 2**
CGS 21680 (1 μM) + PAF (200 nM)	64 ± 8**
Rolipram (0.5 μM) + PAF (200 nM)	91 ± 1**
SKF 96365 (10 μM) + PAF (200 nM)	92 ± 4

The results are expressed as the mean value ± s.e.mean. The concentration of $^{45}Ca^{2+}$ in the control, unstimulated cells was 266 ± 5 pmol 10^{-7} cells after the 60 s time-course of the experiment. *$P \leqslant 0.0001$, for comparison between the control, unstimulated neutrophils. ** $P \leqslant 0.0001$, for comparison between the PAF-activated neutrophils.

Discussion

Although both FMLP and PAF activate neutrophils *via* binding to members of the seven trans-membrane spanning G-protein coupled receptor family, the repertoire of functional responses elicited by these chemoattractants is distinctly different (Nick *et al*., 1997). Activation of

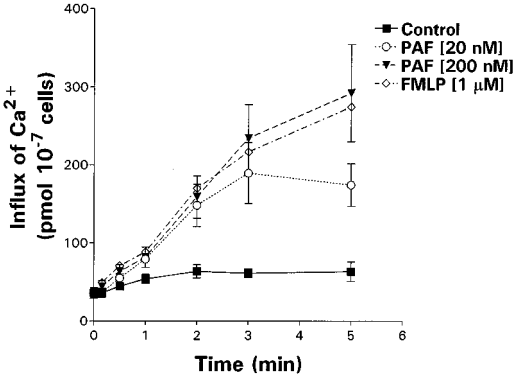

Figure 6 Kinetics of influx of $^{45}Ca^{2+}$ into unstimulated neutrophils and into cells activated with PAF (20 and 200 nM) or FMLP (1 μM) over a 5 min time-course. The results of four different experiments are expressed as the mean amount of cell-associated $^{45}Ca^{2+}$ (pmol 10^{-7} cells) with vertical lines showing s.e.mean.

Table 3 Effects of dibutyryl cyclicAMP, CGS 21680, rolipram and SKF 96365 on the influx of $^{45}Ca^{2+}$ into PAF-activated neutrophils

System	*Amount of $^{45}Ca^{2+}$ taken up by neutrophils (pmol 10^{-7} cells)*
PAF (200 nM)	141±2*
Dibutyryl cAMP (4 mM) + PAF (200 nM)	48±4**
CGS 21680 (1 μM)+PAF (200 nM)	103±4**
Rolipram (0.5 μM)+PAF (200 nM)	85±4**
SKF 96365 (10 μM)+PAF (200 nM)	27±7

The results are expressed as the mean value±s.e.mean. The uptake of $^{45}Ca^{2+}$ by control, unstimulated cells was 60±3 pmol 10^{-7} cells over the 5 min time-course of the experiment. *$P \leqslant 0.0001$, for comparison between the control, unstimulated neutrophils. ** $P \leqslant 0.0001$, for comparison between the PAF-activated neutrophils.

Table 4 Effects of dibutyryl cyclic AMP, CGS 21680 and rolipram on the release of elastase by PAF-treated FMLP/CB-activated neutrophils

System	*Elastase release (% control)*
PAF (200 nM) only	137±3*
Dibutyryl cAMP (4 mM) only	32±2
Dibutyryl cAMP (4 mM) + PAF (200 nM)	47±3*
CGS 21680 (1 μM) only	56±1
CGS 21680 (1 μM)+PAF (200 nM)	82±3*
Rolipram (0.5 μM) only	49±1
Rolipram (0.5 μM)+PAF (200 nM)	73±4*

The results of four experiments, with six replicates in each, are expressed as the mean percentage±s.e.mean of the PAF and drug-free, FMLP/CB-activated control system. The absolute values for release of elastase from control, unstimulated and FMLP/CB-activated neutrophils were 15±1 and 123±13 milliunits enzyme 2×10^{-6} cells respectively. *$P \leqslant 0.0001$, for comparison with the respective control systems.

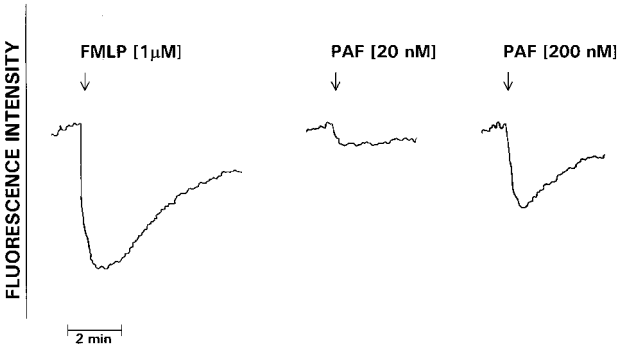

Figure 7 Alterations in neutrophil membrane potential following activation of the cells with PAF (20 and 200 nM) or FMLP (1 μM). The neutrophils were preincubated for 10 min at 37°C with the potential-sensitive, fluorescent dye di-O-C_5(3) followed by addition of PAF or FMLP (denoted by ↓) and measurement of alterations in fluorescence intensity. Membrane depolarization is documented as a decrease in fluorescence intensity, while recovery towards pre-activation values demonstrates repolarization. The results shown are representative traces from one of five separate experiments.

neutrophils with FMLP elicits a broad range of responses including Ca^{2+} mobilization, membrane depolarization, actin assembly, adhesion to vascular endothelium, chemotaxis, superoxide generation, granule enzyme release and synthesis of cyclic AMP (Snyderman & Uhing, 1992). Exposure of neutrophils to PAF, on the other hand, is accompanied by receptor-mediated activation of a more limited range of responses, with no meaningful alterations in superoxide generation, granule enzyme release or intracellular cyclic AMP (Nick *et al*., 1997; Ali *et al*., 1998).

These differences in the profiles of the proinflammatory responses of human neutrophils activated by FMLP and PAF have been attributed to differential utilization of G proteins by the two chemoattractants. FMLP utilizes a receptor-associated, pertussis-sensitive G-protein which initiates a series of events leading to activation of p38 and p42/44 mitogen-activated protein kinases (MAPk), while the PAF receptor is coupled to a G protein, which is at least partially pertussis insensitive and results in predominant activation of p38 MAPk (Nick *et al*., 1997; Ali *et al*., 1998).

The inability of PAF to cause meaningful receptor-mediated activation of NADPH oxidase and adenylate cyclase suggests that the mechanisms used by neutrophils to restore Ca^{2+} homeostasis following exposure to this proinflammatory phospholipid may differ from those utilized by FMLP-activated cells. FMLP-activation of neutrophils is accompanied by activation of phospholipase C and inositol triphosphate-mediated mobilization of Ca^{2+} from intracellular stores (Prentki *et al*., 1984). Ca^{2+} overload is prevented by the combined action of the calmodulin-dependent plasma membrane Ca^{2+}-ATPase (Lagast *et al*., 1984) and the cyclic AMP-dependent protein kinase-upregulated Ca^{2+}-ATPase (Tao *et al*., 1992) which promote efflux and resequestration of cytosolic Ca^{2+} respectively. The efficiency of these cytosolic Ca^{2+} clearance pumps is potentiated by the electrogenic, membrane depolarizing action of NADPH oxidase, which restricts the influx of extracellular cation by abolishing the electrical component of the electrochemical gradient for Ca^{2+} (Di Virgilio *et al*., 1987; Geiszt *et al*., 1997; Tintinger *et al*., 2001).

In the current study, activation of neutrophils with FMLP (1 μM) resulted in the characteristic, immediate increase in cytosolic Ca^{2+} which was completely abolished by pre-treatment of the cells with U 73122, underscoring the dependence of this event on activation of phospholipase C. As reported previously, the peak concentration of cytosolic Ca^{2+} in FMLP-activated neutrophils was short-lived (of about 10 s duration) and subsided rapidly, reaching basal levels within several minutes due to efflux and resequestration of the cation (Anderson & Goolam Mahomed, 1997). Net influx of the cation was only detectable at around 30–60 s, proceeded over a 5 min time-course coincident with membrane repolarization as reported previously (Anderson & Goolam Mahomed, 1997; Tintinger *et al*., 2001), and was abolished by treatment of the cells with SKF 96365, all of which support a store-operated uptake mechanism. Although the effects of EGTA and PMA were not investigated in the current study, both agents have been reported to attenuate the store-operated influx of Ca^{2+} into FMLP-activated neutrophils, underscoring the respective requirements for extracellular Ca^{2+} and membrane repolarization in this process (Di Virgilio *et al*., 1987; Geiszt *et al*., 1997).

Although not shown in the current study, we have observed that activation of neutrophils with a lower concentration of FMLP (0.01 μM), which did not activate superoxide generation and caused only modest membrane depolarization, resulted in accelerated influx of the Ca^{2+} relative to the corresponding responses of cells activated with 1 μM of the chemoattractant. These observations appear to support the proposed relationship between activation of NADPH oxidase resulting in membrane depolarization and restricted influx of extracellular Ca^{2+}. Activation of neutrophils with PAF (20 and 200 nM) also resulted in U 73122-inhibitable mobilization of Ca^{2+} from intracellular stores, the peak cytosolic concentrations of the cation being similar in magnitude to those observed with FMLP. However, in contrast to FMLP, peak levels of cytosolic Ca^{2+} were sustained for about 1 min in PAF-activated neutrophils. This relative prolongation of the peak cytosolic Ca^{2+} response in PAF-activated neutrophils was not a consequence of delayed/decreased extrusion of the cation since the magnitude and kinetics of efflux of Ca^{2+} were similar to those observed with FMLP-activated neutrophils. Accelerated influx of the cation as an alternative mechanism of prolongation of the peak cytosolic Ca^{2+} concentration was supported by kinetic data from Ca^{2+} influx experiments using both the radiometric and Mn^{2+} quenching of fura-2 fluorescence procedures. This contention was also supported by observations that the sustained elevations in cytosolic Ca^{2+} in PAF-activated neutrophils were attenuated by pre-treatment of the cells with SKF 96365, or by inclusion of EGTA in the cell-suspending medium.

PAF, at the highest concentration used (200 nM), depolarized the neutrophil membrane. However, the magnitude of the depolarization response was considerably less ($<50\%$) than that observed with FMLP-activated neutrophils, being insufficient to prevent early influx of extracellular Ca^{2+}. Moreover, exposure of neutrophils to PAF was not accompanied by detectable superoxide production by the cells, suggesting the existence of a mechanistic relationship between failure of the phospholipid to activate NADPH oxidase (with consequent inadequate membrane depolarization) and accelerated influx of Ca^{2+}. This was supported by data from experiments in which neutrophils were coactivated with PAF and PMA, the latter being a potent activator of NADPH oxidase which does not cause fluctuations in cytosolic Ca^{2+} (Naccache *et al*., 1985; Goolam Mahomed & Anderson, 2000). PMA, which caused membrane depolarization equivalent to or greater than that mediated by FMLP, did not affect the PAF-mediated abrupt increase in cytosolic Ca^{2+}, but did attenuate the prolongation of peak levels of the cation.

The mechanism of the membrane depolarization response observed in PAF-activated neutrophils remains to be established, but does not appear to be due to trivial activation of NADPH oxidase since it was unaffected by treatment of the cells with staurosporine, a potent inhibitor of superoxide production by phagocytes, nor was it attenuated in PAF-treated neutrophils from two individuals with chronic granulomatous disease (results not included).

The failure of PAF to activate adenylate cyclase in neutrophils, as previously described by others (Ali *et al*., 1998), is in contrast to the well-documented transient increase in cyclic AMP observed in FMLP-activated cells (Iannone *et al*., 1989; Goolam Mahomed *et al*., 1998). This transient increase in cyclic AMP has been linked to deactivation of the proinflammatory activities of neutrophils as a consequence of up-regulation of the activity of the endo-membrane Ca^{2+}-ATPase (Anderson *et al*., 1998) and/or inactivation of phospholipase C (Ali *et al*., 1998). We reasoned that the inability of PAF to activate adenylate cyclase may also contribute to prolonged Ca^{2+} transients in neutrophils through failure to up-regulate the endo-membrane Ca^{2+}-ATPase. This contention was supported by observations, from fura-2 fluorescence experiments, that dibutyryl cyclic AMP, rolipram and CGS 21680 hastened the clearance of Ca^{2+} from the cytosol of PAF-activated neutrophils, without affecting release from stores, as has previously been reported for FMLP-activated neutrophils (Anderson *et al*., 1998, 2000). Using the radiometric procedure, all three agents were found to reduce the magnitude of both the PAF-activated efflux and influx of Ca^{2+}. These observations are compatible with up-regulation of the endo-membrane Ca^{2+}-ATPase, which in turn favours re-sequestration of cytosolic Ca^{2+} with a corresponding reduction in magnitude of both efflux and store-operated influx of the cation (Anderson *et al*., 1998, 2000). The lesser activity of rolipram, relative to that of CGS 21680, which activates adenylate cyclase *via* subtype A_{2A} receptors, and dibutyryl cyclic AMP, in potentiating the clearance of Ca^{2+} from the cytosol of PAF-activated neutrophils, may be explained by the inability of the phospholipid to activate synthesis of cyclic AMP. It is noteworthy, however, that cyclic AMP-dependent protein kinase has been reported to inhibit phospholipase C (Yue *et al*., 1998), a mechanism, which if operative in activated neutrophils, may also contribute to the reduction in cytosolic Ca^{2+} concentrations in neutrophils treated with dibutyryl cyclic AMP, CGS 21680 or rolipram.

The functional consequences of the altered handling of Ca^{2+} by PAF-activated neutrophils (relative to FMLP and possibly other chemoattractants) remain to be conclusively established, but may result in hyper-adhesion of the cells to vascular endothelium (Ruchaud-Sparagano *et al*., 2000) or 'priming' of other pro-inflammatory activities of the cells such as oxidant production and release of granule enzymes (Zimmerman *et al*., 1992). A possible relationship between PAF-mediated mobilization of Ca^{2+} and 'priming' of neutrophils for activation with a second stimulus is supported by the observations that dibutyryl cyclic AMP, CGS 21680 and rolipram all attenuated the sensitizing effects of PAF on FMLP/CB-activated release of elastase by the cells. These findings also suggest that cyclic AMP-elevating agents, presumably by enhancement of clearance of Ca^{2+} from the cytosol of activated neutrophils, may represent a possible therapeutic alternative to PAF-receptor antagonists and -hydrolyzing enzymes (Kuijpers *et al*., 2001).

In conclusion, the results of the current study demonstrate that the efficiency of clearance of Ca^{2+} from the cytosol of PAF-activated human neutrophils is not optimal due to the failure of the proinflammatory phospholipid to activate adenylate cyclase and NADPH oxidase. The resultant prolongation of the peak cytosolic Ca^{2+} concentration, which is amenable to attenuation by cyclic AMP-elevating agents, may contribute to hyper-activity of the cells.

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