**Protein kinase C promotes restoration of calcium homeostasis to platelet activating factor-stimulated human neutrophils by inhibition of phospholipase C**

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**Abstract**

**Background:** The role of protein kinase C (PKC) in regulating the activity of phospholipase C (PLC) in neutrophils activated with the chemoattractant, platelet-activating factor (PAF, 20 and 200 nM), was probed in the current study using the selective PKC inhibitors, GF10903X (0.5 - 1 μM) and staurosporine (400 nM).

**Methods:** Alterations in cytosolic Ca²⁺, Ca²⁺ influx, inositol triphosphate (IP₃), and leukotriene B₄ production were measured using spectrofluorimetric, radiometric and competitive binding radioreceptor and immunoassay procedures, respectively.

**Results:** Activation of the cells with PAF was accompanied by an abrupt increase in cytosolic Ca²⁺ followed by a gradual decline towards basal levels. Pretreatment of neutrophils with the PKC inhibitors significantly increased IP₃ production with associated enhanced Ca²⁺ release from storage vesicles, prolongation of the peak cytosolic Ca²⁺ transients, delayed clearance and exaggerated reuptake of the cation, and markedly increased synthesis of LTB₄. The alterations in Ca²⁺ fluxes observed with the PKC inhibitors were significantly attenuated by U73122, a PLC inhibitor, as well as by cyclic AMP-mediated upregulation of the Ca²⁺-resequestering endomembrane ATPase.

Taken together, these observations are compatible with a mechanism whereby PKC negatively modulates the activity of PLC, with consequent suppression of IP₃ production and down-regulation of Ca²⁺ mediated pro-inflammatory responses of PAF-activated neutrophils.

**Conclusion:** Although generally considered to initiate and/or amplify intracellular signalling cascades which activate and sustain the pro-inflammatory activities of neutrophils and other cell types, the findings of the current study have identified a potentially important physiological, anti-inflammatory function for PKC, at least in neutrophils.
Background
Chemoattractants, including the bioactive phospholipid, platelet-activating factor (PAF), interact with G-protein coupled receptors on the plasma membrane of human neutrophils to activate phospholipase C (PLC), which is followed by rapid and transient increases in cytosolic calcium concentrations [1,2]. Mobilization of the cation from intracellular stores is dependent on the PLC-mediated hydrolysis of membrane phospholipids, which generates inositol triphosphate (IP_3) and diacylglycerol (DAG). IP_3 interacts with its receptors on the membranes of calcium storage vesicles releasing Ca^{2+} into the cytosol [3]. The intracellular concentration of IP_3 peaks at about 10 - 15 sec following receptor ligation [2] and then declines towards basal levels consequent to both down-regulation of PLC activity [4] and intracellular metabolism of IP_3 by phosphomonoesterases [5-8].

Although PLC activity is modulated by depletion of enzyme substrate [4], and decay of receptor-mediated signaling [4], it has also been proposed that in some cell types, namely vascular endothelial cells [9] and platelets [10], protein kinase C (PKC) negatively regulates PLC. Diacylglycerol (DAG) and Ca^{2+}, both downstream products of PLC, activate PKC, which in turn, completes a negative feedback loop by inhibiting PLC. The existence and physiologic consequences of cross-talk between PKC and PLC in activated human neutrophils has, however, received little attention despite the potential of this mechanism to expedite restoration of Ca^{2+} homeostasis and attenuate the Ca^{2+}-dependent pro-inflammatory activities of these cells.

In the current study, we have utilized two selective PKC inhibitors to probe the interactions of PKC with PLC by determining the effects of these agents on intracellular IP_3 concentrations, cytosolic calcium fluxes and Ca^{2+}-dependent production of leukotriene B_4 by PAF-activated neutrophils. Our results are compatible with a mechanism to expedite restoration of Ca^{2+} homeostasis and attenuate the Ca^{2+}-dependent pro-inflammatory activities of these cells.

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Neutrophils
Purified human neutrophils were prepared from heparinised venous blood (five 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 neutrophil fraction was removed by sequential sedimentation with 3% gelatin in order to remove most of the erythrocytes. Following centrifugation (280 × g at 10°C for 10 min), residual erythrocytes were removed by selective lysis with 0.84% ammonium chloride at 4°C for 10 min. The neutrophils, which were routinely of high purity (>90%) and viability (>95%), were resuspended to 1 × 10^7.ml^{-1} in phosphate-buffered saline (PBS 0.15 M, pH 7.0) and held on ice until used.

Spectrofluorimetric measurement of cytosolic Ca^{2+}
Fura-2/AM was used as the fluorescent, Ca^{2+}-sensitive indicator for these experiments. 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 Hank’s 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 in the absence or presence of the PKC inhibitors (staurosporine at 400 nM, or GF10903X at 0.5 and 1 μM), 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 platelet-activating factor (PAF) at final concentrations of 20 and 200 nM.

A second chemoattractant, N-formyl-L-methionyl-L-leucyl-L-phenylalanine (FMLP, 1 μM, final) was used in a limited series of confirmatory experiments during which neutrophils were activated in the presence or absence of GF10903X (1 μM).

To determine the effects of the PKC inhibitors on cytosolic Ca^{2+} concentrations, uncomplicated by Ca^{2+} influx from extracellular reservoirs, the cells were treated with the Ca^{2+}-chelating agent, ethylene glycol-bis (β-aminoethyl ether)N,N,N',N'-tetraacetic acid (EGTA, 10 mM), added to the cells 1 min prior to PAF (200 nM).

Additional experiments were performed with U73122 (2 μM), a selective inhibitor of phospholipase C, added to the cells 10 - 15 sec after PAF (200 nM), when peak...
cytosolic Ca\(^{2+}\) concentrations had been reached, in the presence or absence of the PKC inhibitors staurosporine (400 nM) and GF10903X (1 \(\mu\)M). This experimental design was used to determine whether the putative target of PKC (following maximal mobilization of stored Ca\(^{2+}\)) is PLC or the intracellular phosphomonoesterases which metabolize IP\(_3\).

Further experiments were conducted to investigate the effects of the test agents on the rates of resequestration of cytosolic Ca\(^{2+}\) into storage vesicles mediated by the cAMP-sensitive endomembrane Ca\(^{2+}\)-ATPase. Fura-2-loaded cells were preincubated at 37°C with staurosporine (400 nM) or GF10903X (0.5 and 1 \(\mu\)M) for 5 min followed by addition of the phosphodiesterase 4 inhibitor, rolipram (2 \(\mu\)M), for 3 min prior to activation of the cells with PAF (20 nM), and the subsequent alterations in fura-2 fluorescence monitored over a 5 min time period.

**Mn\(^{2+}\) quenching of fura-2 fluorescence**

Cells loaded with fura-2 as described above were activated with PAF (20 and 200 nM) in HBSS containing 300 \(\mu\)M MnCl\(_2\) (added 5 min prior to PAF) and fluorescence quenching as a measure of Ca\(^{2+}\) influx was monitored at an excitation wavelength of 360 nm, which is an isosbestic wavelength, and at an emission wavelength of 500 nm [11]. This procedure was used to investigate the effects of GF10903X (0.5 and 1 \(\mu\)M) added to the cell suspensions 8 min before activation, on the rate and magnitude of Ca\(^{2+}\) influx.

**Radiometric assessment of Ca\(^{2+}\) fluxes**

\(^{45}\text{Ca}^{2+}\) (Calcium-45 chloride, specific activity 18.53 mCi.mg\(^{-1}\), Perkin Elmer Life Sciences, Inc.) was used as tracer to label 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 radiolabeled cation was used at a fixed, final concentration of 2 \(\mu\)Ci.ml\(^{-1}\), and the final assay volumes were 5 ml containing a total of 1 \(\times\) 10\(^7\) neutrophils. The standardization of the procedures used to load the cells with \(^{45}\text{Ca}^{2+}\), as well as a comparison with oil-based methods for the separation of labeled neutrophils from unbound isotope, have been described previously [12].

**Efflux of \(^{45}\text{Ca}^{2+}\) from neutrophils**

Neutrophils (1 \(\times\) 10\(^7\).ml\(^{-1}\)) were loaded with \(^{45}\text{Ca}^{2+}\) (2 \(\mu\)Ci.ml\(^{-1}\)) for 30 min at 37°C in HBSS which was free of unlabeled Ca\(^{2+}\). The cells were then pelleted by centrifugation, washed once with, and resuspended in ice-cold Ca\(^{2+}\)-replete HBSS and held on ice until used. Pre-loading with cold Ca\(^{2+}\) was undertaken to minimize spontaneous uptake of \(^{45}\text{Ca}^{2+}\) (unrelated to PAF activation) in the influx assay. The Ca\(^{2+}\)-loaded neutrophils (2 \(\times\) 10\(^6\).ml\(^{-1}\)), were then incubated for 10 min in the presence or absence of GF10903X (1 \(\mu\)M) at 37°C in HBSS containing 25 \(\mu\)M cold carrier Ca\(^{2+}\) (as CaCl\(_2\)), followed by simultaneous addition of PAF (20 or 200 nM) and \(^{45}\text{Ca}^{2+}\) (2 \(\mu\)Ci/ml) or \(^{45}\text{Ca}^{2+}\) only to control, unstimulated systems. Influx of \(^{45}\text{Ca}^{2+}\) into PAF-activated neutrophils was then monitored over a 5 min period, after which influx is complete and compared with the uptake of the radiolabeled cation by identically processed, unstimulated cells as described above.

**Inositol triphosphate (IP\(_3\))**

Neutrophils at a concentration of 5 \(\times\) 10\(^6\).ml\(^{-1}\) in Ca\(^{2+}\)-replete HBSS were preincubated for 10 min at 37°C in the presence or absence of GF10903X (1 \(\mu\)M), followed by the addition of PAF (20 or 200 nM) or FMLP (1 \(\mu\)M) 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 10 and 20 sec after addition of the chemoattractant, and the tubes transferred to an ice bath. These incubation times coincide with the early peak IP\(_3\) responses (10 sec) of PAF-activated neutrophils, as well as the subsequent decline (20 sec) towards basal levels which are reached at around 60 sec [1,2], determined in a series of preliminary experiments. In an additional series of experiments, the effects of the PKC activator, phorbol 12-myristate 13-acetate (PMA, 50 ng/ml final, added 2 min before PAF) on the IP\(_3\) responses of PAF (200 nM)-activated cells in the absence and presence of GF10903X (1 \(\mu\)M) were investigated.
Following 20 min incubation on ice, the tubes were centrifuged at 2000 × g for 15 min and the supernatants removed and brought to pH 7.5 with 5N KOH, followed by centrifugation at 2000 × g for 15 min to remove precipitated perchloric acid. The supernatants were assayed using the inositol-1,4,5-triphosphate [3H] radioreceptor assay procedure (Perkin Elmer Life Sciences, Inc., Boston, MA, USA), which is a competitive ligand binding assay, and the results expressed as pmol IP$_3$/10$^7$ cells.

**Measurement of LT$_B_4$**

A competitive binding enzyme immunoassay procedure (Correlate-EIA$^\text{TM}$; Assay Designs Inc., Ann Arbor, MI, USA) was used to measure LT$_B_4$ in the supernatants of neutrophils activated with PAF (20 and 200 nM) in the absence or presence of GF10903X (1 μM). Neutrophils (2 × 10$^6$.ml$^{-1}$, final) in HBSS were preincubated for 10 min at 37°C with the test agent after which PAF was added to the cells and the reactions stopped after 3 min incubation at 37°C (predetermined in preliminary time-course experiments) by the addition of an equal volume of ice-cold HBSS to the tubes which were then held in an ice-bath prior to pelleting the cells by centrifugation. The cell-free supernatants were then assayed for LT$_B_4$ using the enzyme immunoassay (EIA) procedure. Supernatants from cells activated with PAF were diluted 1:4 prior to assay. These results are expressed as picograms (pg)/10$^7$ cells.

**Statistical Analysis**

The results of each series of experiments ($n$ values represent the number of separate experiments in each series for which cells from a minimum of 3 different donors were used) are expressed as the mean value ± standard error of the mean (S.E.M.), with the exception of the fura-2/AM used) are expressed as the mean value ± standard error of the mean value of statistical significance were calculated using paired Student’s $t$ test when comparing two groups, or by analysis of variance (ANOVA) with subsequent Tukey-Kramer multiple comparisons test for multiple groups. A $P$-value < 0.05 was considered significant.

**Results**

**Effects of staurosporine and GF10903X on the fura-2 responses of PAF- or FMLP-activated neutrophils**

These results are shown in Figures 1 and 2. Exposure of neutrophils to PAF (20 nM) was accompanied by an abrupt increase in fura-2 fluorescence intensity, typical of G-protein-coupled receptor activation of phospholipase C and inositol triphosphate-mediated release of Ca$^{2+}$ from intracellular stores. Peak fluorescence intensity declined within a few seconds and continued to decrease steadily towards resting levels. Pretreatment of the cells with the PKC inhibitors, staurosporine and GF10903X, did not alter the magnitude of the peak fluorescence, but was associated with a sustained elevation in peak cytosolic Ca$^{2+}$ concentrations that declined towards resting levels at significantly slower rates than those observed for control systems (Figure 1).

Activation of neutrophils with FMLP resulted in an abrupt increase in fura-2 fluorescence intensity which coincided with the rise in cytosolic Ca$^{2+}$ concentrations, and quickly

![Figure 1](http://www.journal-inflammation.com/content/6/1/29)

**Figure 1**

Fura-2 fluorescence responses of PAF (20 nM)-activated neutrophils (A), pretreated with staurosporine 400 nM (B), GF10903X 0.5 μM (C) and 1 μM (D), in the presence ( _ _ _ ) or absence ( _____ ) of rolipram (2 μM), as well as those of FMLP (1 μM)-activated cells (E), with ( _ _ _ ) and without ( _____ ) GF10903X (1 μM).

These are traces from a single representative experiment with a total of 3-8 in each series. Addition of the chemoattractant is denoted by the arrow (↓).

![Figure 2](http://www.journal-inflammation.com/content/6/1/29)

**Figure 2**

Fura-2 fluorescence responses of PAF (200 nM)-activated neutrophils (A), pre-treated with staurosporine 400 nM (B), GF10903X 0.5 μM (C), and 1 μM (D) in the presence ( _ _ _ ), or absence ( _____ ) of EGTA or U73122 (2 μM) (.....) added 10 - 15 sec after PAF. These are traces from a single representative experiment with a total of 4 - 12 in each series. The arrows denote addition of PAF (↓) or U73122 (↑).
subsided, returning to base-line after several minutes. In the presence of GF10903X, the peak fluorescence intensity was not altered, but was followed by a sustained plateau phase of about 30 sec which subsequently declined towards basal levels at a significantly slower rate than that observed with control systems (Figure 1).

Addition of PAF at the higher concentration (200 nM) to neutrophils was accompanied by an abrupt increase in fura-2 fluorescence intensity due to elevation in the cytosolic Ca\(^{2+}\) concentration which also peaked rapidly, but which was followed by a sustained plateau phase lasting about 1 min with a subsequent gradual decline in fluorescence intensity towards basal levels (Figure 2). In the presence of staurosporine or GF10903X, the magnitudes of peak fluorescence intensity were not altered, but the duration of the plateau phase was significantly prolonged and the subsequent gradual decline in fluorescence intensity was slower than that observed for control systems.

**Effects of EGTA on fura-2 responses**

In the presence of the Ca\(^{2+}\)-chelating agent, EGTA, addition of PAF (200 nM), was also accompanied by the characteristic abrupt increase in fura-2 fluorescence, which subsequently declined rapidly towards basal levels without the sustained elevation in fluorescence intensity observed in the absence of EGTA (Figure 2). Treatment of neutrophils with the PKC inhibitors did not alter the magnitude of the initial peak cytosolic Ca\(^{2+}\) concentrations, but the rate of decline towards basal levels was slower. The effects of these agents on the rate of decline in fluorescence intensity were less pronounced than those observed in the absence of EGTA (preserved extracellular Ca\(^{2+}\) reservoirs). GF10903X (1 \(\mu\)M) had no effect on thapsigargin-mediated Ca\(^{2+}\) release from intracellular storage vesicles (results not shown).

**Effects of U73122 on fura-2 responses**

The effects of the phospholipase C (PLC) inhibitor, U73122 (2 \(\mu\)M) added to neutrophils 10 - 15 sec following addition of PAF (200 nM), are shown in Figure 2. At this concentration, U73122 abolishes receptor-mediated Ca\(^{2+}\) mobilization and IP\(_3\) generation by neutrophils [13], which were confirmed in a series of preliminary experiments (not shown). Addition of U73122 resulted in a rapid decline in fluorescence intensity with marked attenuation of the prolonged plateau phase. Similarly, in the presence of the PKC-inhibitors, addition of U73122 resulted in an almost immediate decline in fura-2 fluorescence intensity.

**Effects of rolipram on fura-2 responses**

These results are shown in Figure 2. Neutrophils were treated with the phosphodiesterase inhibitor, rolipram in order to investigate the effects of the PKC inhibitors on the rates of resequestration of Ca\(^{2+}\) into storage vesicles mediated by the protein kinase A (PKA)-sensitive Ca\(^{2+}\)-endomembrane ATPase. In the presence of rolipram, cAMP accumulates in neutrophils, activating PKA with consequent upregulation of the activity of the endomembrane Ca\(^{2+}\)-ATPase [14]. Neutrophils were pretreated with the PKC inhibitors for 5 min, followed by rolipram for 3 min. The magnitude of the peak fluorescence response was not altered by rolipram, but the rate of decline in cytosolic Ca\(^{2+}\) concentrations were markedly accelerated following attainment of peak fluorescence. Similar effects of rolipram were observed in neutrophils pretreated with the PKC inhibitors, suggesting that these agents do not interfere with endomembrane ATPase-mediated resequstration of Ca\(^{2+}\) into storage vesicles.

The consolidated data for all of the fura-2 fluorescence experiments described above are shown in Tables 1 and 2.

**Mn\(^{2+}\) quenching of fura-2 fluorescence**

These results are shown in Figure 3 and Table 3. In control cells, the decrease in fluorescence intensity, which indicates influx of Ca\(^{2+}\), occurred almost immediately after addition of PAF (20 and 200 nM). An initial abrupt linear decrease in fluorescence intensity over 2 - 3 min, of greater magnitude at the higher concentration of PAF, was followed by a slower decline for a further 2 - 3 min. In the presence of the PKC inhibitors, addition of PAF (20 nM) was followed by a rapid decline in fura-2 fluorescence intensity of significantly greater magnitude (measured 1, 3 and 5 min after addition of the chemoattractant) than that observed with untreated cells. In the presence of the PKC inhibitors, addition of PAF (200 nM), resulted in a slight, but insignificant increase in the magnitude of decline in fura-2 fluorescence.

The rate and magnitude of decline in fura-2 fluorescence for neutrophils activated with FMLP (1 \(\mu\)M), was significantly increased in the presence of GF10903X (1 \(\mu\)M), (results not shown).

**Effects of the PKC inhibitors on the net influx and net efflux of Ca\(^{2+}\)**

The magnitudes of net influx of Ca\(^{2+}\) following activation of neutrophils with 20 and 200 nM PAF are shown in Table 3. Treatment of neutrophils with GF10903X significantly increased the magnitude of store-operated influx of Ca\(^{2+}\) following activation of the cells with PAF at a concentration of 20 nM. No significant differences were observed for neutrophils activated with higher concentrations of PAF (200 nM). These results correspond closely with those obtained by means of the Mn\(^{2+}\) quenching of fura-2 fluorescence assays.

The net efflux of Ca\(^{2+}\) from PAF (20 nM)-activated neutrophils measured 5 min following addition of the chemoattractant was 4 ± 2% of the total amount of cell-
associated radiolabelled Ca²⁺ and this increased significantly to 12 ± 2% for cells pretreated with GF10903X, (P < 0.05 for comparison with the untreated control system).

Effects of the PKC inhibitors on inositol triphosphate production
These results are shown in Table 4. IP₃ concentrations increased significantly following exposure of neutrophils to PAF (20 and 200 nM) or FMLP (1 μM), peaking at 10 sec after addition of the chemoattractant. Pre-incubation of the cells with GF10903X (1 μM) resulted in significant increases in IP₃ concentrations.

Effects of GF10903X on LTB₄ production by activated neutrophils
LTB₄ production by PAF (20 nM)-activated neutrophils was markedly increased in the presence of GF10903X from 175 ± 31 to 794 ± 51 pg/10⁷ cells in the absence or presence of the PKC inhibitor respectively (P < 0.01), rising from a basal value of 24 ± 6 pg/10⁷ for resting cells.

Discussion
The results of the current study have identified a role for PKC in promoting restoration of Ca²⁺ homeostasis and down-regulation of Ca²⁺-dependent pro-inflammatory activity to chemoattractant-activated human neutrophils. Notwithstanding those which target IP₃ and its receptor, well-characterized mechanisms which promote efficient clearance of Ca²⁺ from the cytosol of activated neutrophils include: i) the electrical gradient created by the membrane depolarizing action of NADPH oxidase that restricts the influx of Ca²⁺ via store-operated Ca²⁺ channels [15-17] and ii) the combined action of two ATP-driven Ca²⁺ pumps, namely the Ca²⁺-resequestering endomembrane Ca²⁺-ATPase and the plasma membrane Ca²⁺-ATPase, that actively transports Ca²⁺ out of the cell [18,19]. However, based on the following observations, neither NADPH oxidase nor either of the Ca²⁺ pumps were considered to be putative targets for PKC in our experimental setting. Firstly, PAF, at the concentrations used in this study, does not activate NADPH oxidase [20], effectively excluding alterations in membrane potential as a mechanism for the prolonged cytosolic Ca²⁺ transients observed with the PKC inhibitors. Secondly, the apparent enhanced Ca²⁺ efflux in the presence of GF10903X is not compatible with inhibition of the plasma membrane-associated Ca²⁺-ATPase, which is upregulated by sustained elevations in cytosolic Ca²⁺ concentrations [21]. Thirdly, the sensitivity of the endomembrane Ca²⁺-ATPase to rolipram was preserved in PAF-activated neutrophils pretreated with the PKC inhibitors, suggesting that these agents do not significantly interfere with the refilling of Ca²⁺ stores.

From a mechanistic perspective however, treatment of neutrophils with GF10903X significantly elevated and prolonged the concentrations of the intracellular second messenger, IP₃, in chemoattractant-activated neutrophils. The apparent doubling of IP₃ concentrations in the pres-
ence of the PKC inhibitor observed in the current study likely maintains IP3 receptors in an open state for longer periods, facilitating sustained Ca2+ release by promoting shuttling of the cation between the stores and the cytosol [22].

Experiments performed in the presence of the extracellular Ca2+-chelating agent, EGTA, support this contention, as delayed Ca2+ clearance in the presence of the PKC inhibitors persisted in this setting, and could not be attributed to enhanced Ca2+ influx. Previous reports have suggested that PKC may modulate PAF-mediated activation of PLC by promoting desensitization of the PAF receptor [23]. This is an unlikely mechanism in human neutrophils, as similar effects of the PKC inhibitors were observed when the cells were activated with the formyl peptide, FMLP, a ligand which interacts with receptors considered resistant to PKC-mediated phosphorylation [24,25].

Sustained activation of IP3 receptors at higher concentrations of IP3 not only mobilizes stored Ca2+, but also activates store-operated influx mechanisms [26]. In addition, IP3 activates Ca2+ channels independently of the filling

Table 2: Effects of staurosporine and GF10903X, in the presence or absence of EGTA, on cytosolic Ca2+ concentrations of PAF-activated neutrophils, as well as the effects of U73122 added 10 - 15 sec after PAF on cytosolic Ca2+ concentrations.

| System | Peak (nM) | Plateau (min) | Magnitude of decrement (nM) from peak measured at: |
|--------|-----------|---------------|--------------------------------------------------|
|        |           |               | 1 min | 2 min | 3 min | 5 min |
| PAF (200 nM) | Control (n = 12) | 270 ± 8 | 0.8 ± 0.06 | 80 ± 5 | 116 ± 6 | 168 ± 8 |
|         | Staurosporine (400 nM) (n = 6) | 290 ± 16 | 1.03 ± 0.08 | 60 ± 11* | 92 ± 18 | 136 ± 20 |
|         | GF10903X (0.5 μM) (n = 9) | 274 ± 9 | 1.11 ± 0.05* | 52 ± 5* | 72 ± 6* | 112 ± 7* |
|         | GF10903X (1 μM) (n = 9) | 270 ± 8 | 1.2 ± 0.07* | 44 ± 6* | 64 ± 8* | 100 ± 8* |
| EGTA (n = 10) | 246 ± 4 | 100 ± 5 | 164 ± 6 |
| EGTA + Staurosporine (n = 7) | 246 ± 8 | 76 ± 5* | 136 ± 9* |
| EGTA + GF10903X (0.5 μM) (n = 7) | 238 ± 10 | 78 ± 5* | 132 ± 6* |
| EGTA + GF10903X (1 μM) (n = 7) | 246 ± 9 | 76 ± 3* | 132 ± 6* |
| U73122 (2 μM) (n = 4) | 266 ± 11 | 0.4 ± 0.05 | 120 ± 9 | 140 ± 7 |
| U73122 + Staurosporine (n = 3) | 290 ± 12 | 0.43 ± 0.06 | 112 ± 15 | 132 ± 9 |
| U73122 + GF10903X (0.5 μM) (n = 3) | 278 ± 11 | 0.43 ± 0.07 | 92 ± 16 | 116 ± 9 |
| U73122 + GF10903X (1 μM) (n = 3) | 262 ± 8 | 0.52 ± 0.05*** | 92 ± 11 | 108 ± 8*** |

The results are expressed as the mean percentage of control ± S.E.M. *P < 0.05 for comparison with the untreated control system and +P < 0.05 or **P < 0.05 for comparison with EGTA or U73122-treated neutrophils, respectively. Basal Ca2+ concentrations were 80 ± 8 nM.

Figure 3
Effects of GF10903X 0.5 μM (.....) or 1 μM (_ _ _) on the Mn2+ quenching of fura-2 fluorescence assay in PAF 20 nM (A)- or 200 nM (B)-activated neutrophils.

PAF was added as indicated (↓). These are traces from a single representative experiment with a total of 5 - 8 in each series.
state of Ca\textsuperscript{2+} stores [27]. These IP\textsubscript{3} dependent mechanisms are also likely to contribute to the prolonged cytosolic Ca\textsuperscript{2+} transients in GF10903X/staurosporine-treated cells. In support of this contention, the magnitudes of Ca\textsuperscript{2+} reuptake determined by means of both the Mn\textsuperscript{2+} quenching of fura-2 fluorescence assay and radiometric procedure were markedly increased in the presence of the PKC inhibitors when neutrophils were activated with PAF at 20 nM, but less at higher concentrations (200 nM). Ca\textsuperscript{2+} influx mechanisms are clearly submaximally activated at lower PAF concentrations and can be increased by potentiation of the IP\textsubscript{3} signal.

The magnitude and duration of the IP\textsubscript{3} response to chemotaxtrants reflect a balance between PLC activity and IP\textsubscript{3} metabolism by intracellular phosphomonoesterases [5-8]. Because PKC has been reported to activate 5'-phosphomonoesterases that metabolize IP\textsubscript{3} [8], we also investigated the effects of addition of U73122, a PLC inhibitor, to the cells 10 - 15 sec after PAF, when Ca\textsuperscript{2+} mobilization and IP\textsubscript{3} generation are complete. U73122 markedly attenuated the prolongation of cytosolic Ca\textsuperscript{2+} transients in the presence of the PKC inhibitors, suggesting that persistent PLC activity is primarily responsible for the exaggerated IP\textsubscript{3} production. Nevertheless, impaired activation of 5'-phosphomonoesterases cannot be conclusively excluded. Further evidence, albeit indirect, that PKC down-regulates PLC activity, is suggested by our previous observations that co-activation of neutrophils with PAF and a phorbol ester, a direct activator of PKC, attenuates PAF-mediated prolongation of peak cytosolic Ca\textsuperscript{2+} transients [20].

To determine the functional consequences of inactivation of PKC on the Ca\textsuperscript{2+}-dependent pro-inflammatory activities of neutrophils, we measured the effect of GF10903X on PAF-activated leukotriene B\textsubscript{4} (LTB\textsubscript{4}) production. Production of this highly pro-inflammatory eicosanoid was markedly enhanced by treatment of the cells with the PKC inhibitor, underscoring the role of PKC in down-regulating the Ca\textsuperscript{2+}-dependent pro-inflammatory activities of neutrophils. LTB\textsubscript{4} recruits and activates not only neutrophils...
trophils and other types of inflammatory cells, but also amplifies IP₃ production via a positive feedback autocrine loop, whereby LTB₄ released from the cell, interacts with its receptor on the plasma membrane to activate PLC [28,29]. Consequently, IP₃ generation is sustained and this in turn may exaggerate the pro-inflammatory activity of neutrophils.

**Conclusion**

In conclusion, the current study has demonstrated that PKC down-regulates Ca²⁺-dependent pro-inflammatory responses of chemoattractant-activated neutrophils, presumably by phosphorylative inactivation of PLC, resulting in termination of IP₃ production. This in turn, favours rapid restoration of Ca²⁺ homeostasis and attenuation of pro-inflammatory activity, a potentially important physiological mechanism of endogenous control of neutrophil inflammation.

**Competing interests**

The authors declare that they have no competing interests.

**Authors’ contributions**

GRT designed and conducted cytosolic calcium experiments and wrote the manuscript; AJT prepared neutrophil suspensions and assisted with experiments; HCS and RC conducted leukotriene B₄ and IP₃ experiments; LP assisted in formatting and editing the manuscript and RA assisted with experiments, interpretation of results and editing the manuscript.

All of the authors have read and approved the manuscript.

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