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Relationship between calcium release and NADPH oxidase inhibition in human neutrophils

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ABSTRACT

The aim of this study was to investigate the possible relationship between NADPH oxidase activity and changes in cytosolic Ca^{2+} in response to different agonists. Treatment of neutrophils with leukotriene B4 (LTB_4) demonstrated characteristic changes to cytoslic Ca^{2+} yielding an EC_{50} of 4 nM. The pA_2 values for the specific LTB_4 receptor (BLT) antagonists, U-75302 and LY-255283 were 6.32 and 6.38, respectively. Similarly, neutrophils treated with *N*-formyl-L-methionyl-L-leucyl-L-phenylalanine (FMLP) and platelet activating factor (PAF) exhibited changes in cytoslic Ca^{2+} in a dose dependant manner with pD_2 values of 9.0 and 9.9, respectively. The phorbol ester PMA prevented elevations in cytosolic Ca^{2+} in response to LTB_4, FMLP and PAF with IC_{50} values of 5.88, 1.44 and 5.71 nM, respectively. In addition, potent NADPH oxidase inhibitors apocynin and diphenyleneiodonium (DPI) inhibited FMLP mediated cytosolic Ca^{2+} release. These results demonstrate that inhibition of the NADPH oxidase suppresses cytosolic Ca^{2+} release in FMLP activated human neutrophils.

Introduction

Polymorphonuclear neutrophils form a critically important component of the body's initial innate immune response and are implicated in several inflammatory processes. The NADPH oxidase is a key driving force in powering the microbicidal capacity of these cells. Mutations in any component of the NADPH oxidase prevent the effective functioning of the neutrophil, resulting in chronic granulomatous disease (CGD), which if not treated results in susceptibility to fatal infections [1]. Normal functioning of neutrophils therefore relies upon the concerted action between the NADPH oxidase and different ion channels, which enables neutrophils to prodigiously fulfil their important role when recruited to sites of inflammation or infection. At these sites, neutrophils can endogenously generate inflammatory mediators. One of these is the potent pro-inflammatory bioactive eicosanoid derived from arachidonate known as leukotriene B4 (LTB_4) [2].

Initially discovered in 1979 [3], LTB_4 has been implicated in the activation of several cellular functions. The most significant being the involvement of LTB_4 in the activation of several granulocyte responses. Neutrophils have been shown to exhibit a multitude of effects in response to LTB_4, some of which include activating neutrophil chemotaxis [4] and secretion of granule enzymes [5]. In addition to LTB_4, specific neutrophil responses can be stimulated by the potent chemo-attractant *N*-formyl-L-methionyl-L-leucyl-L-phenylalanine (FMLP) and platelet activating factor (PAF). Binding of LTB_4, PAF or FMLP to receptors on the neutrophil plasma membrane releases intracellular calcium ($[Ca^{2+}]_i$) via the phospholipase-inositol triphosphate pathway. This depletion of $[Ca^{2+}]_i$ in turn leads to Ca^{2+} influx via store operated channels [6] resulting in elevated Ca^{2+} levels that are thought to be implicated in several Ca^{2+} dependant pro-inflammatory responses.

In neutrophils, refilling of these stores via store operated channels, a phenomenon known as capacitative Ca^{2+} entry, is mediated by the NADPH oxidase [6]. An important feature of the neutrophil NADPH oxidase is that it generates an electron current [7,8] which depolarise the membrane. This electron current depolarises the plasma membrane at a rate of 2.3 V s^{-1} if there was no counter-ion to oppose the membrane depolarisation [7]. Under current clamp conditions proton fluxes thorough voltage-gated proton channels [7] and/or chloride flux via tamoxifen insensitive swell activated chloride channels can oppose the extreme membrane depolarisation generated by the NADPH oxidase [9,10]. The electrogenic characteristic of the NADPH oxidase means that its inhibition will also abrogate membrane depolarisation [7] and augments Ca^{2+} entry into the cell [11]. Depolarisation thereby alters the electrochemical gradient across the neutrophil plasma membrane and limits Ca^{2+} entry into the cell. Taken together, these findings are thought to suggest that the NADPH oxidase puts a protective restraint on Ca^{2+} entering into the neutrophil.

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The role of LTB_4, PAF and FMLP in initiating and modulating various neutrophil responses has been under examination by several groups in recent years, however, very little data exists in human neutrophils that investigates to what extent the mobilisation of calcium release in response to these agonists is mediated by the NADPH oxidase. This work was pursued because it would be important to understand the effect NADPH oxidase inhibitors on changes in $[Ca^{2+}]_i$ release in response to different agonists. The objectives in the current paper were 2-fold. First, to provide a detailed spectrofluorometric analysis which examines the changes in $[Ca^{2+}]_i$ release in response to LTB_4, PAF and FMLP. Second, to investigate the role of the NADPH oxidase in modulating changes in $[Ca^{2+}]_i$ release in response to FMLP in activated human neutrophils.

Materials and methods

Materials. Leukotriene B4 (LTB_4) was purchased from Tocris (UK) at a ready made stock concentration of 50 μg/ml. Platelet activating factor (C16 PAF) and LY-255283 were purchased from Tocris (UK) and made to stock concentrations of 5 mM (water) and 25 mM (DMSO), respectively. U-75302 was purchased from Biomol (UK) and made up to a stock concentration of 1 mM in DMSO. FURA-2AM was purchased from Invitrogen (UK) as a ready made 1 mM solution. Unless otherwise indicated, all other reagents were purchased from Sigma, Chemicals, UK. A stock concentration of phorbol 12-myristate 13-acetate (PMA, 1 mg/ml), *N*-formyl-L-methionyl-L-leucyl-L-phenylalanine (FMLP, 10 mM), apocynin (100 mM) and diphenyleneiodonium chloride (DPI, 10 mM) were made up in DMSO.

Neutrophil isolation. Human neutrophils were isolated from heparin anti-coagulated venous blood as described [7] from healthy consenting adults according to the appropriate ethical guidelines at University of East London. Purified neutrophils were re-suspended in Dulbecco's PBS (D-PBS, Sigma, UK) pH 7.2 prior to use.

Calcium dye loading. FURA-2AM was used as the fluorescent Ca^{2+} sensitive indicator for these experiments [12]. Neutrophils were loaded with FURA-2AM (2 μM, final) by sedimenting for 2 min at 2000 rpm in a microcentrifuge followed by re-suspension in D-PBS containing 1 mM $CaCl_2$. After incubation at 37 °C for 30 min in the dark, neutrophils were washed twice in Ca^{2+} free D-PBS and stored at room temperature and protected from light.

Spectrofluorimetric measurement of Ca^{2+} fluxes. Intracellular $[Ca^{2+}]$ was monitored by spectrofluorimetry by the ratiometric method as described [12] and was adapted for use in a micro-titre spectrofluorimetric 96 well plate reader (Thermo Labsystems Fluoroskan Ascent FL Fluoresence microplate reader). Ninety microliters of neutrophil suspension (2×10^7/ml) were added to each well of a flat-bottomed black 96 well micro-titre plate. This was followed by 10 μl of each addition of agonist and/or inhibitor and orbital shaking for 5 s at 1200 rpm. The micro-titre plate reader was programmed to pre-incubate neutrophils at 37 °C for 3 min to obtain a stable base-line before the first addition of agonist and/or inhibitor was made using an automatic multi-channel pipette. Changes in FURA-2AM fluorescence were monitored at 340 and 380 nm excitation wavelengths and 510 nm emission, with a time between reading at each wavelength of 3.4 s. Each reading was integrated for 20 ms and a plate acceleration of 10 ms was used, and the time between reading adjacent wells was 50 ms. The time between two readings of a block of wells (typically 12 wells) at both wavelengths was 8.1 s. Alterations in fluorescence intensity were then monitored over a 3–12 min period. The final volume in each well was 100 μl containing a total of 1.8×10^6 neutrophils. Calibration at the end of an experiment was performed by lysing cells by the addition of 0.1% Triton X-100 and 1 mM $CaCl_2$ to determine $F_{340\ nm}/F_{380\ nm} = R_{max}$. R_{min} was determined by the subsequent addition of 25 mM EGTA. Intracellular $[Ca^{2+}]$ was calculated using the formula [12]: $[Ca^{2+}]_i = K_d \{(R\text{-}R_{min})/(R_{max}\text{-}R)\}.\{S_{f2}/S_{b2}\}$, S_{f2} is the fluorescence of free FURA-2AM at 380 nm, determined in the presence of 25 mM EGTA and S_{b2} is the fluorescence at 380 nm of FURA-2AM saturated with Ca^{2+} measured in the presence of 1 mM Ca^{2+} in cells lysed with Triton X-100. The K_d of FURA-2AM was taken to be 224 nM at 37 °C [12].

Data analysis. Statistical data was analysed using the one-way ANOVA Bonferroni test of multiple comparisons on Graph Pad Prism 4.0 software.

Results

Effect of BLT receptor antagonists on $[Ca^{2+}]_i$ mobilisation in response to LTB_4 in human neutrophils

Pre-treatment of neutrophils loaded with FURA-2AM with LTB_4 (20 nM) evoked an sharp increase in $[Ca^{2+}]_i$ from a basal value of approximately 100 nM to a peak of 753 nM ± 12 ($n = 3$). After the initial transient peak, $[Ca^{2+}]_i$ declined rapidly to basal values (Fig. 1A). The EC_{50} of the LTB_4 induced responses was 4.02 nM (Fig. 1B). The effect of BLT receptor antagonists on $[Ca^{2+}]_i$ responses induced by LTB_4 were studied using antagonists U-75302 and LY-255283 (Fig. 1B). The respective pA_2 values were 6.32 and 6.38, respectively, estimated using the Gaddum equation; *pA_2 = log(concentration ratio1)-log[antagonist]* assuming competitive antagonism.

Effect of FMLP and PAF on $[Ca^{2+}]_i$ mobilisation in human neutrophils

Neutrophils treated with FMLP evoked a rapid increase in $[Ca^{2+}]_i$ from resting values of about 100 nM and rapidly rising to peak values in excess of 700 nM (Fig. 2A). After the initial elevation there was a decrease of $[Ca^{2+}]_i$ to basal levels that took longer in comparison to LTB_4. The EC_{50} of the FMLP induced response was 0.11 nM ($pD_2 = 9.96 \pm 0.09$, $n = 3$) (Fig. 2B). Similarly, PAF resulted in a sharp increase in $[Ca^{2+}]_i$ from resting values of 80 nM to a peak in excess of 500 nM (Fig. 2C). After the initial peak in response to PAF, $[Ca^{2+}]_i$ mobilisation took longer to return to steady-state values when compared to FMLP. The EC_{50} value of PAF mediated $[Ca^{2+}]_i$ was 0.87 nM ($pD_2 = 9.05 \pm 0.24$, $n = 4$) (Fig. 2D).

Inhibition by PMA of $[Ca^{2+}]_i$ release in response to LTB_4, PAF and FMLP in human neutrophils

The phorbol ester PMA potently inhibited in a dose dependent manner $[Ca^{2+}]_i$ elevations in response to LTB_4 (Fig. 3A), FMLP (Fig. 3B) and PAF (Fig. 3C), with IC_{50} values of 5.88, 1.44 and 5.71 nM respectively. Using the Cheng–Prussoff relationship [13], K_i values for inhibition of FMLP, LTB_4 and PAF were 1.33×10^{-10} M, 1.34×10^{-10} M and 2.53×10^{-9} M, respectively.

NADPH oxidase inhibition suppresses $[Ca^{2+}]_i$ release in FMLP stimulated human neutrophils

Experiments were carried out on neutrophils incubated with the potent NADPH oxidase inhibitors DPI [14] and apocynin [15] prior to stimulation of $[Ca^{2+}]_i$ responses with FMLP. Apocynin significantly inhibited FMLP stimulated $[Ca^{2+}]_i$ release (Fig. 4A) with an IC_{50} of 21 μM (Fig. 4B). Similarly, in the presence of DPI, neutrophils that had been stimulated using FMLP, demonstrated significantly reduced levels of $[Ca^{2+}]_i$ release in comparison to untreated cells (Fig. 4C). DPI inhibited $[Ca^{2+}]_i$ release from peak values from 568 nM ± 83 ($n = 3$) to 141 nM ± 18 ($n = 3$) (Fig. 4D).

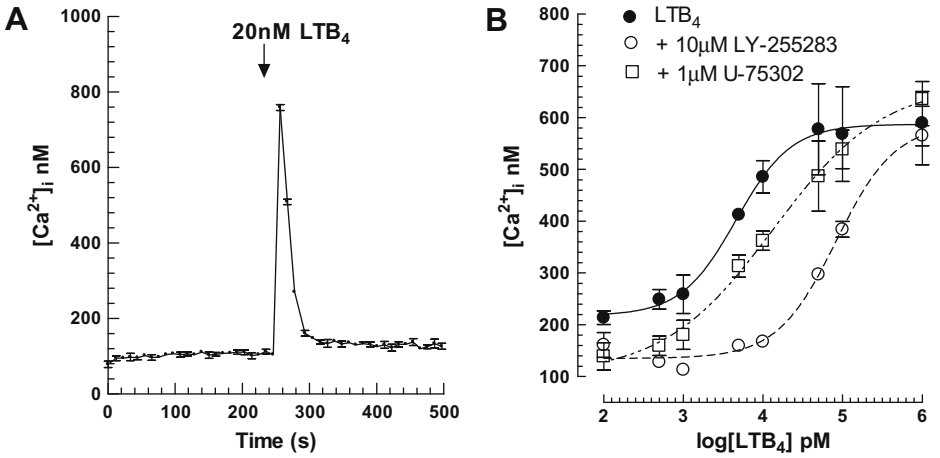

Fig. 1. Dependence of $[Ca^{2+}]_i$ responses with LTB_4 in human neutrophils in the absence and presence of BLT receptor antagonists. Transient peak in response to 20 nM LTB_4 ($n = 3$) (A). BLT receptor antagonist dose response curves in response to LTB_4 ($n = 3$) (B). Error bars show the ±SEM.

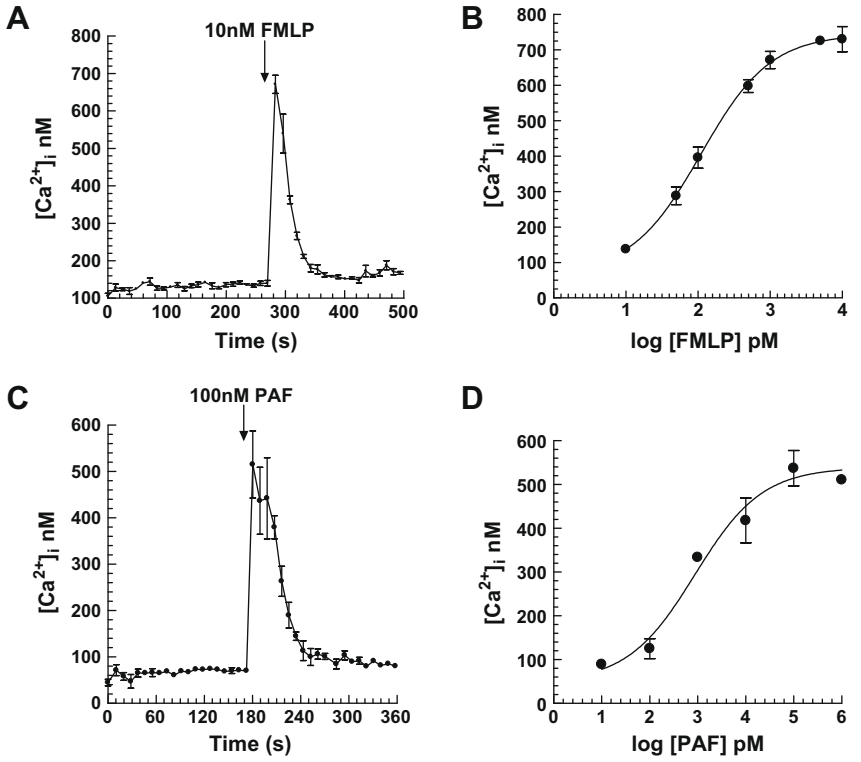

Fig. 2. Dependence of $[Ca^{2+}]_i$ on FMLP and PAF responses in human neutrophils. Time course of changes in $[Ca^{2+}]_i$ in response to 10 nM FMLP (A) and the concentration dependence curve of $[Ca^{2+}]_i$ to FMLP ($n = 3$) (B). Time course of changes in $[Ca^{2+}]_i$ in response to 100 nM PAF (C) and the concentration dependence curve of $[Ca^{2+}]_i$ to PAF ($n = 4$) (D). Error bars show the ±SEM.

Discussion

Very little has been documented on the effect of NADPH oxidase activity on agonist induced calcium release. This is in part due to the practical time constraints of conventional fluorimetric techniques. In this report, the development of a method of intracellular $[Ca^{2+}]$ measurement using a microtitre plate fluorimetric reader capable of ratiometric kinetic measurements has allowed more extensive and detailed analysis. Using this method, the results of the current study has revealed novel features of the inter-relationships between mechanisms of calcium release in human neutrophils stimulated by pro-inflammatory lipid mediators (LTB_4 and PAF) and the chemotactic peptide FMLP. In this study we have shown for the first time that potent inhibitors of the NADPH oxidase can suppress FMLP mediated $[Ca^{2+}]_i$ release in human neutrophils.

In addition, our study provides extensive analysis of LTB_4 induced $[Ca^{2+}]_i$ release in human neutrophils. Previously, there has been a lack of Schild analysis in identifying receptor mechanisms that mediate the effect of LTB_4 induced $[Ca^{2+}]_i$ changes in human neutrophils. Similar apparent affinity values (pA_2) have been infrequently calculated for BLT receptors in neutrophils. The action of LTB_4 is mediated by two G-protein receptors. Of these receptors BLT1 is only activated by LTB_4 whereas BLT2 can be activated by

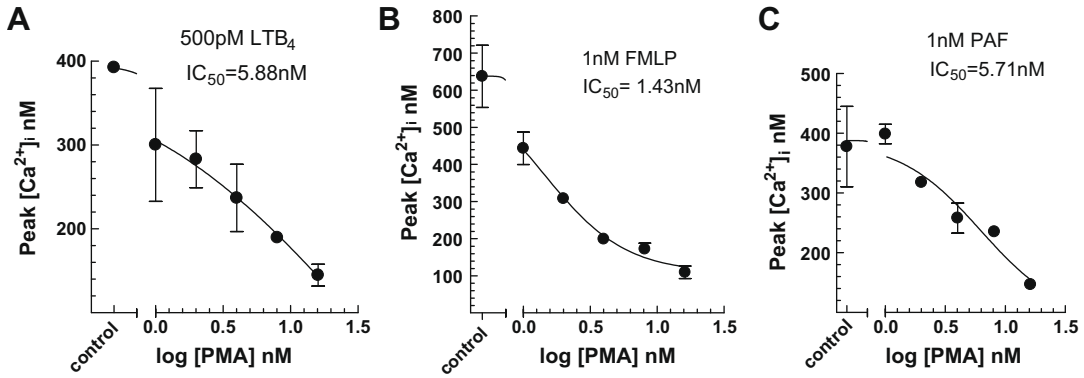

Fig. 3. Effect of PMA on changes in cytosolic calcium in response to LTB_4, FMLP and PAF in activated human neutrophils. PMA inhibited $[Ca^{2+}]_i$ release in LTB_4 (A), FMLP (B) and PAF (C) stimulated neutrophils. Cells were pre-incubated for 3 min and PMA was added at time zero. Three minutes later, 0.5 nM LTB_4 ((A) $n = 3$), 1 nM FMLP ((B) $n = 3$) or 1 nM PAF ((C) $n = 3$) were added. The maximum $[Ca^{2+}]_i$ recorded after addition of agonist are plotted. The error bars show ±SEM.

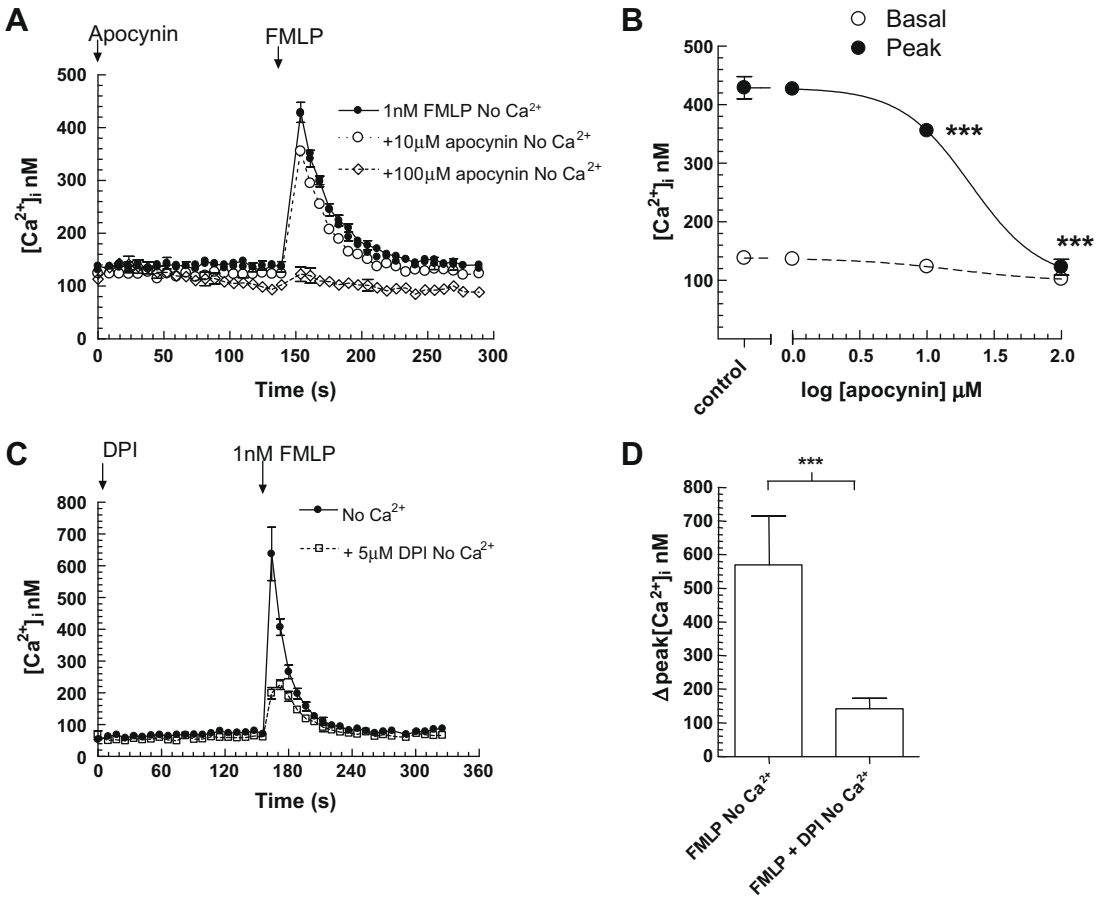

Fig. 4. Inhibition by DPI and apocynin of calcium release in response to 1 nM FMLP. Neutrophils were pre-incubated at 37 °C and the indicated concentration of apocynin added at zero time and FMLP was added as indicated by the arrow in the absence of calcium (A). (B) The concentration dependence of inhibition of basal and peak $[Ca^{2+}]_i$ by apocynin. The IC_{50} of apocynin was 21 μM. (C) The time courses of $[Ca^{2+}]_i$ in response to 1 nM FMLP ($n = 3$) with DPI (5 μM) added as indicated by the arrow in the absence of calcium. The corresponding peak changes in $[Ca^{2+}]_i$ recorded (peak-basal) in the absence and presence of DPI (5 μM) with 1 nM FMLP (D). Error bars indicate ±SEM ($n = 3$).

not only LTB_4 but also by other hydroxyeicosotetraenoic acids [16]. In this study we used two antagonists of the BLT receptors; U-75302 a BLT antagonist that is specific for the high affinity BLT1 receptor and LY-255283, which is specific for the low affinity BLT2 receptor [17]. Both of these antagonists inhibited the binding of LTB_4 to its receptor in a dose dependant manner (Fig. 1B). We found that Schild–Gaddum analysis of these antagonists yielded similar pA_2 values of approximately 6.3 supporting the idea that both antagonists bind to similar sites on the BLT receptor to antagonise the calcium mediated release in human neutrophils. From the present data we cannot conclude if the LTB_4 mediated responses in neutrophils are mediated by a particular type of BLT receptor. There is growing evidence that BLT1 and BLT2 may function as heterodimers, which would suggest that both of these selective antagonists could inhibit calcium mediated responses in neutrophils, even at concentrations where they inhibit LTB_4 binding specifically to BLT1 or BLT2.

In the current study, activation of neutrophils with LTB_4 (20 nM), FMLP (10 nM) and PAF (100 nM) resulted in the characteristic immediate increase in $[Ca^{2+}]_i$. The peak concentration of

$[Ca^{2+}]_i$ in LTB_4 stimulated neutrophils was very short lived (60 s, Fig. 1A) in comparison to FMLP treated cells, which took longer to reach basal levels (Fig. 2A). The prolongation of the peak $[Ca^{2+}]_i$ to reach basal levels in FMLP stimulated cells probably reflects a delayed extrusion of $[Ca^{2+}]_i$ from intracellular stores in comparison to $[Ca^{2+}]_i$ responses with LTB_4. This prolongation of $[Ca^{2+}]_i$ to return to basal values was the most notable with neutrophils stimulated with PAF in which a much broader response was observed that took much longer to reach basal levels (Fig. 2C). This biphasic component in response to PAF is in agreement with a previous study [18] and has been attributed to the secondary production of LTB_4 after PAF stimulation.

In rabbit neutrophils it has been established that PMA activates the NADPH oxidase and causes membrane depolarization with no apparent rise in $[Ca^{2+}]_i$ [19]. In addition, in a more recent study it was shown that phorbol esters potently inhibit divalent cation influx (PMA IC_{50} ~50 pM) at concentrations lower than required for stimulation of delayed superoxide formation [20]. The results in the present study are in agreement with these previous studies. In addition, the binding affinity values of PMA were very similar for all the agonists. Our findings show that PMA inhibits $[Ca^{2+}]_i$ release in neutrophils stimulated with FMLP. Previously, it was shown that PMA only partially inhibited $[Ca^{2+}]_i$ release in response to 10 nM FMLP [21]. In the present study we demonstrate that PMA at concentrations greater than 1 nM completely inhibited $[Ca^{2+}]_i$ release in response to FMLP at concentrations of 10-fold less than that was observed in the previous study [21].

Our data show that the NADPH oxidase inhibitors DPI and apocynin can suppress $[Ca^{2+}]_i$ release in human neutrophils stimulated with FMLP. An interesting variation of our current results is the ability of NADPH oxidase inhibitors to suppress $[Ca^{2+}]_i$ release. Neutrophils treated with these inhibitors mimic the CGD phenotype and CGD cells are proposed to have higher levels of $[Ca^{2+}]_i$ influx [11,22]. In one study it was shown that CGD cells stimulated with FMLP had higher levels of cytosolic calcium in comparison to healthy cells and therefore led to the proposal that CGD neutrophils are prone to calcium overload [11]. In contrast, our findings demonstrate that DPI and apocynin treated neutrophils had significantly lower $[Ca^{2+}]_i$ release levels than untreated cells. The proposed theory of the NADPH oxidase in preventing Ca^{2+} overload [11,22] needs to be treated with caution. A previous report using Quin-2 to measure changes in $[Ca^{2+}]_i$ release from patients with CGD [23] demonstrated that $[Ca^{2+}]_i$ changes induced by FMLP in CGD cells, were quantitatively and kinetically similar to those observed in normal cells suggesting that a functional oxidase was not responsible for preventing $[Ca^{2+}]_i$ overload. This finding along with the data presented in the current study therefore casts some uncertainty on the theory proposed by other studies [11,22]. Taken together these findings suggest that the role for a functional NADPH oxidase in preventing Ca^{2+} overload in FMLP stimulated human neutrophils is not as clear as was previously thought. For the purposes of the present study we have predominantly focussed on the direct affect of specific NADPH oxidase inhibitors on FMLP induced changes in $[Ca^{2+}]_i$ release in human neutrophils. FMLP is considered to be the gold standard for investigating different responses in human neutrophils and in addition our study shows that LTB_4 and PAF can increase $[Ca^{2+}]_i$. Investigating the direct interaction of NADPH oxidase inhibitors on LTB_4 and PAF induced changes in $[Ca^{2+}]_i$ release is not the purpose of the present study but no doubt would be an important avenue to explore in future studies.

What do our currents finding suggest for the neutrophil NADPH oxidase and changes in $[Ca^{2+}]_i$ release? A possible interpretation of the current findings could be linked to the role of membrane potential in regulating G-protein coupled receptors. It has been documented that FMLP receptors are dependent on G_i, whereas both LTB_4 receptor and PAF receptors utilise both G_i and pertussis toxin insensitive G-proteins [24]. In addition, membrane potential is thought to have an important role in regulating G-protein coupled receptors in several cell types [25]. Although this has not yet been documented in neutrophils, findings from another non-excitable cell; the megakaryocyte have demonstrated that membrane depolarisation can modulate Ca^{2+} mobilisation evoked by G-protein coupled receptors [26]. It is possible in neutrophils that after activation of the NADPH oxidase, concomitant membrane depolarisation may differentially regulate the activities of G-proteins coupled to FMLP receptors and therefore modulate the $[Ca^{2+}]_i$ release. In the presence of NADPH oxidase inhibitors, membrane depolarisation would be abrogated [7] and therefore, would effect how different G-protein coupled receptors modulate the way $[Ca^{2+}]_i$ is released from stores in response to different agonists.

In conclusion, our study demonstrates that inhibition of the NADPH oxidase can suppress $[Ca^{2+}]_i$ release in response to FMLP in human neutrophils and suggests a complex interaction occurs between FMLP receptors and the NADPH oxidase in activated human neutrophils.

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