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Synchronous free Ca^{2+} changes in individual neutrophils stimulated by leukotriene B_4

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Calcium (Ca^{2+}) signals were monitored in individual neutrophils using ratio imaging of fura-2. In contrast to *N*-formyl-L-leucyl-L-phenylalanine (f-met-leu-phe), which produced grossly asynchronous Ca^{2+} signals with delays in response (up to 60 s), leukotriene B_4 (LTB_4) provoked synchronous and immediate elevations in cytosolic free Ca^{2+}. Some individual neutrophils which responded immediately to LTB_4, subsequently displayed delayed Ca^{2+} signals in response to f-met-leu-phe. A sub-population of neutrophils failed to respond to both LTB_4 and f-met-leu-phe. The asynchrony of the Ca^{2+} signalling to f-met-leu-phe is not, therefore, an obligatory property of signal transduction in neutrophils.

Neutrophil; Ca^{2+} signalling; Ratio imaging; Leukotriene

1. INTRODUCTION

The oxidase response to f-met-leu-phe of individual neutrophils within a population is heterogeneous and asynchronous [1,2] and results from heterogeneity in the timing of the cytosolic Ca^{2+} signal [2,3]. In response to f-met-leu-phe, neutrophils also generate a range of biologically active molecules, such as LTB_4 and platelet activating factor [4,5], which may act as intercellular messengers, activating bystander cells. The question now arises as to whether the origin of the asynchrony of the Ca^{2+} signal in response to f-met-leu-phe results from (a) properties within individual neutrophils which produce a delayed and time variable step in the receptor-Ca^{2+} signal transduction, or (b) the opening of Ca^{2+} channels on some cells indirectly, by intercellular messengers, such as leukotriene B_4 (LTB_4), generated by other neutrophils in response to f-met-leu-phe. Here we show, using single cell imaging, that the Ca^{2+} signal generated in response to LTB_4 is synchronous and immediate in all neutrophils within the population. Furthermore, some neutrophils, which fail to respond to f-met-leu-phe, are responsive to LTB_4. The possibility therefore exists that part of the asynchrony to f-met-leu-phe results from indirect stimulation by LTB_4 generated by nearby cells.

2. MATERIALS AND METHODS

2.1. *Materials*

Fura-2/AM and pluronic F-127 were purchased from Molecular Probes, Oregon, USA, and LTB_4 and f-met-leu-phe from Sigma Chemicals, Poole, Dorset, UK.

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2.2. *Neutrophil isolation*

Neutrophils were isolated from heparinized blood of healthy volunteers as described previously [2]. Following dextran sedimentation, centrifugation through Ficoll-Paque (Pharmacia) and hypotonic lysis of red cells, neutrophils were washed, resuspended in Krebs buffer (120 mM NaCl, 4.8 mM KCl, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 1.3 mM $CaCl_2$, 25 mM HEPES and 0.1% bovine serum albumin, adjusted to pH 7.4 with NaOH).

2.3. *Measurement of cytosolic Ca^{2+}*

Neutrophils (2×10^7 cells/ml) were loaded with fura-2 as previously described [6]. Ratio fluorescence measurements and ratio imaging of fura-2 loaded neutrophils adherent to glass coverslips was performed at 37°C as previously described [2,3,6]. Excitation wavelengths at 350 nm and 380 nm were achieved using a Spex Fluorolog dual wavelength fluorometer (Glen Spectra, Stanmore, UK). Ratio images were acquired by an ISIS intensified CCD camera (Photonic Science, Tunbridge, UK) coupled to a Spex IM201 analysis system [2,3,6].

3. RESULTS

3.1. *Characteristics of LTB_4 triggered cytosolic free Ca^{2+} rises*

LTB_4 triggered transient or sustained but oscillatory rises in cytosolic free Ca^{2+} in individual neutrophils within 4 s. No neutrophils showed delays before producing a Ca^{2+} signal (Fig. 1). This was in contrast to f-met-leu-phe, where responses in individual neutrophils were either (a) immediate (within 6 s), with a transient or sustained but oscillating rise in cytosolic free Ca^{2+}, or (b) delayed, onset of the rise in cytosolic free Ca^{2+} occurring after 21–56 s. In response to LTB_4, cytosolic free Ca^{2+} rose uniformly throughout the cell cytosol (Fig. 2). As with f-met-leu-phe [2,7], in the absence

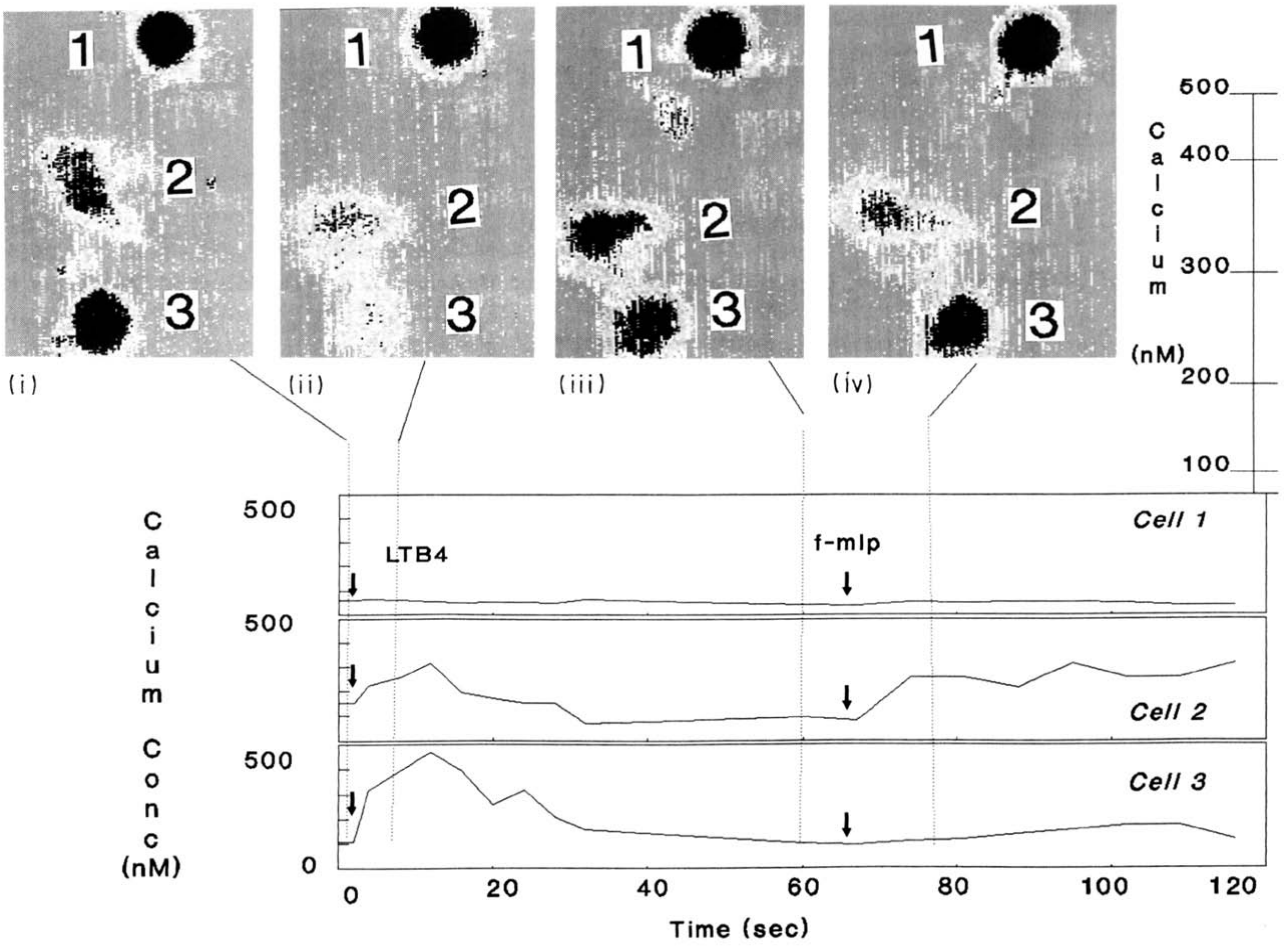

Fig. 1. Cytosolic free Ca^{2+} changes imaged in individual neutrophils, showing the responses of three individual cells to stimulation with LTB_4 (100 nM) and f-met-leu-phe (100 nM). (a) Cytosolic free Ca^{2+} within 3 neutrophils is shown as pseudo-grey images (i) at rest, (ii) 4 s after addition of LTB_4, (iii) 60 s after LTB_4, immediately before addition of f-met-leu-phe and (iv) 7 s after f-met-leu-phe. The level of grey within each cell represents the cytosolic free Ca^{2+} concentration given by the scale on the right. (b) The time course of the Ca^{2+} changes in these 3 neutrophils.

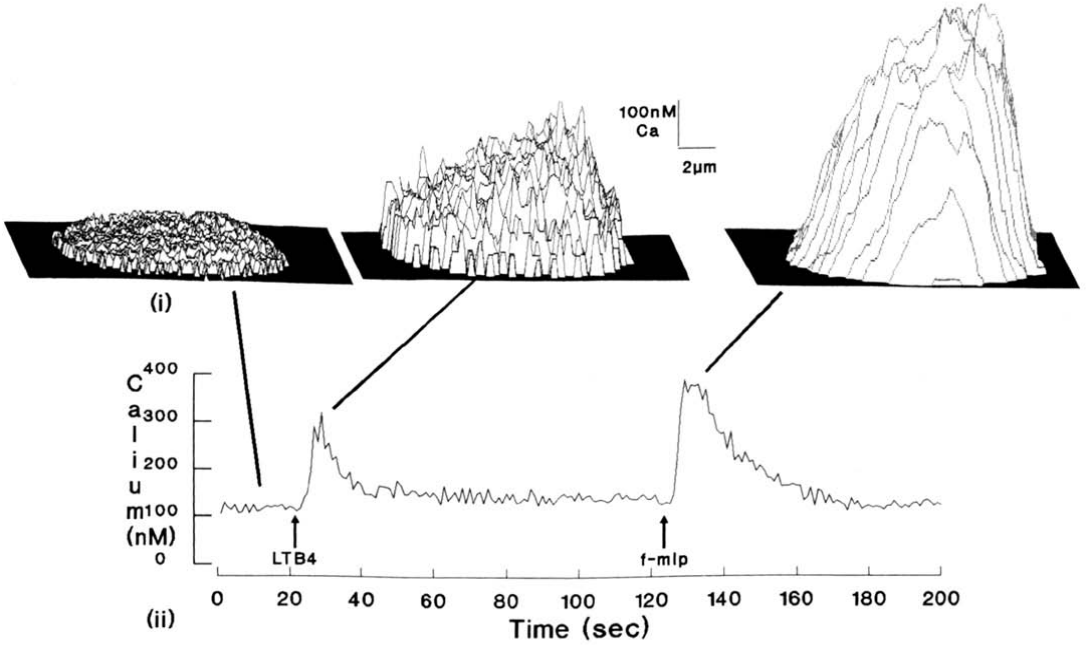

Fig. 2. (i) The intracellular distribution of cytosolic free Ca^{2+} within an individual neutrophil shown as a pseudo-3d plot, (a) at rest, (b) 4 s after LTB_4 (100 nM) and (c) 14 s after stimulation with f-met-leu-phe (100 nM). (ii) Cytosolic free Ca^{2+} measured within the neutrophil population during the same sequence of stimuli.

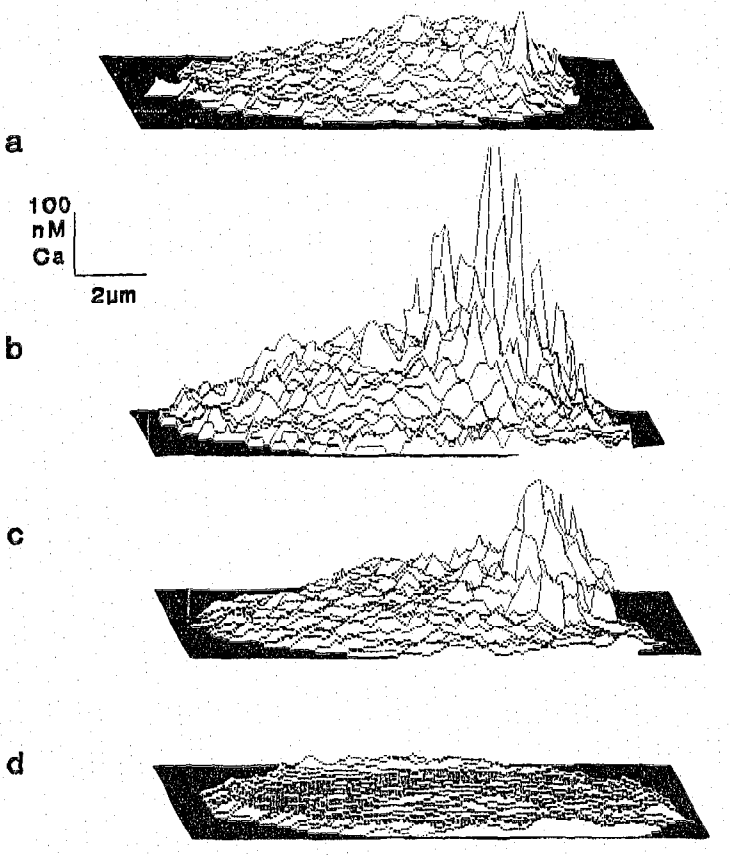

Fig. 3. Release of a localised cytosolic free Ca^{2+} 'cloud' by LTB_4 in the absence of extracellular Ca^{2+} (1 mM EGTA). Pseudo-3d plots of the cytosolic free Ca^{2+} distribution within an individual neutrophil (a) before addition of LTB_4 (100 nM), (b) 4 s, (c) 12 s and (d) 60 s after addition of LTB_4.

of extracellular Ca^{2+}, a highly localised transient 'cloud' of elevated cytosolic free Ca^{2+} was observed in some cells, which presumably originates from release from an intracellular Ca^{2+} store (Fig. 3).

3.2. *A population of neutrophils responds to LTB_4 but not f-met-leu-phe*

Individual neutrophils which responded immediately to LTB_4, with either transient or oscillatory Ca^{2+} responses, when exposed subsequently to f-met-leu-phe produced either changes in cytosolic free Ca^{2+} which were immediate (17%), oscillating (16%) or delayed (25%), or the cell failed to respond (42%). There was no correlation between the magnitude of the Ca^{2+} elevation within an individual neutrophil to either stimulus, nor was there consistency in whether the response was transient or sustained (Fig. 4). All neutrophils which failed to respond to LTB_4 (25%) also failed to respond to f-met-leu-phe (Fig. 4).

4. DISCUSSION

The results described in this paper show that the Ca^{2+} signals generated within individual neutrophils in response to LTB_4 and f-met-leu-phe are qualitatively different. Firstly, although delays before the onset of the Ca^{2+} signal were a striking feature of the response triggered by f-met-leu-phe, no delays in the onset of the cytosolic free Ca^{2+} rise in response to LTB_4 were observed. Secondly, Ca^{2+} signals in response to LTB_4 could be generated in neutrophils which were subsequently shown to be unresponsive to f-met-leu-phe. Thirdly, a sub-population of neutrophils was demonstrated, which failed to respond to either agonist.

These data are therefore consistent with a role for LTB_4 as an intercellular regulator in this system. The delays, between the addition of f-met-leu-phe and the Ca^{2+} signal, may thus arise from an extracellular indirect route of activation. The sub-population of neutrophils which fail to respond directly to f-met-leu-phe may subsequently be stimulated by the release of LTB_4 from neutrophils which were stimulated directly. A similar route has been demonstrated for the slow Ca^{2+} signal observed in neutrophil populations in response to zymosan, where it has been shown that it is PAF generated by the cells which indirectly triggers this response [8]. Intercellular signalling may also play a role in generating fluctuations and oscillations in the Ca^{2+} signals within neutrophils [9–11]. In other cell types, where similar delays in the Ca^{2+} response have been detected [12,13], it is not yet established whether the same mechanism with intercellular messengers is involved.

The findings we report here have an important implication for the mechanism of activation of neutrophils

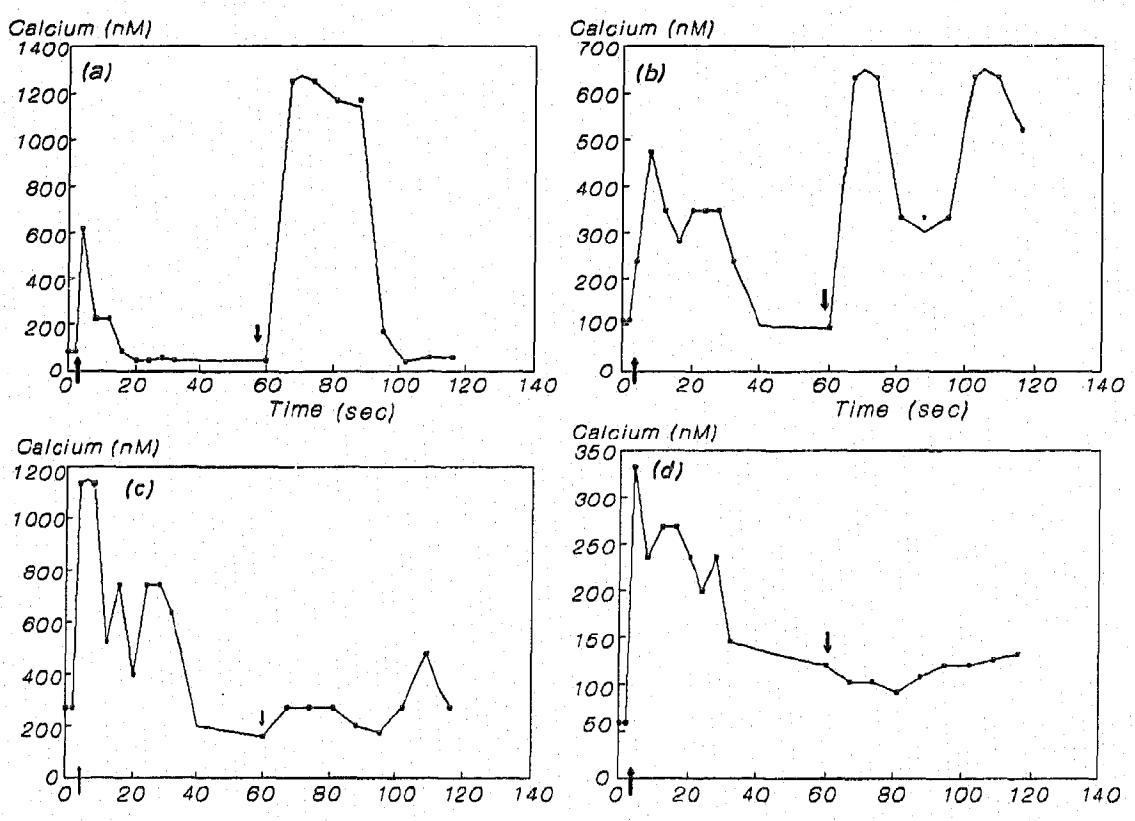

Fig. 4. Changes in cytosolic free Ca^{2+} concentration in individual neutrophils, following stimulation with LTB_4 (100 nM), at the first upward arrow, and f-met-leu-phe (100 nM) at the second downward arrow. The responses shown are representative of LTB_4-responsive neutrophils which subsequently responded to f-met-leu-phe by (a) an immediate transient Ca^{2+} signal, (b) a sustained and oscillating Ca^{2+} signal, (c) a delayed Ca^{2+} signal and (d) no change in cytosolic free Ca^{2+} concentration.

in inflammation. The existence of a sub-population of neutrophils which respond indirectly to intercellular messengers generated by other neutrophils, means that the magnitude of the whole population response increases not only with the initial stimulus, but also with the density of neutrophils within a defined area. Thus, as accumulation of neutrophils continues, a critical point may be reached when all neutrophils in the population become activated either directly or indirectly. A similar mechanism of intercellular messengers is responsible for the abrupt induction of light emission in colonies of luminous bacteria [14].

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