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Dissociation of store release from transmembrane influx of calcium in human neutrophils

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Release of Ca^{2+} from intracellular stores was visualised in individual neutrophils in the presence of the Mn^{2+} or SKF 96365. Influx of Mn^{2+} quenched fura-2 close to the plasma membrane but did not quench fura-2 at the site of store release. The size and location of the 'cloud' of elevated Ca^{2+} was unaffected by the channel blocker SKF 96365. Furthermore, the size and location was unaffected by the presence of extracellular Ca^{2+}. This dissociation of transmembrane influx from store release demonstrates that the entry of Ca^{2+} into the cytosol of neutrophils occurs directly into the cytosol and not via the store site.

Neutrophil; Ca^{2+} store; Ca^{2+} cloud; Ratio imaging

1. INTRODUCTION

Cell imaging has revealed that f-met-leu-phe (fmlp) produces an increase in free Ca^{2+} throughout the cytosol of human neutrophils [1]. Two hypotheses have been proposed; firstly that the entry of extracellular Ca^{2+} occurs directly into the cytosol via Ca^{2+} channels on the plasma membrane [2], and secondly that Ca^{2+} entry into the cytosol occurs only from intracellular stores depleted by IP_3 [3].

In neutrophils, two features support the latter hypothesis. First, in the absence of extracellular Ca^{2+}, a 'cloud' of elevated cytosolic Ca^{2+} is observed, presumably as a result of release from intracellular stores [4]. Second, in the presence of extracellular Ca^{2+}, a 'hotspot' of locally higher Ca^{2+} is not observed at this site [1,4], consistent with entry of Ca^{2+} occurring via the emptied store. However, the inability to detect store release, which is swamped by transmembrane influx [4,5], has prevented the testing of these hypotheses further.

Here, we report two strategies which enable internal store release of Ca^{2+} to be dissociated from transmembrane influx in the presence of extracellular Ca^{2+}. Release of Ca^{2+} from the store was visualised in the presence of the fura-2 quenching ion, Mn^{2+} [6] or by blocking the receptor-mediated Ca^{2+} channel with SKF 96365 [7]. Our data does not support the hypothesis that entry of extracellular Ca^{2+} occurs via the store. Furthermore, under either condition, extracellular Ca^{2+} did not significantly influence the characteristics of the cytosolic Ca^{2+} 'cloud'. Thus, transmembrane influx of Ca^{2+} in neutrophils played no significant role in the generation, location or magnitude of the fmlp-provoked Ca^{2+} release from the intracellular store.

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2. MATERIALS AND METHODS

2.1. *Materials*

Fura-2/AM and pluronic F-127 were purchased from Molecular Probes, Oregon, USA; fmlp, EGTA and manganese chloride were from Sigma Chemicals, Poole, Dorset. SKF 96365 was a kind gift from Dr. P. England, Smith Kline Beecham Pharmaceuticals, Welwyn, Herts.

2.2. *Neutrophil isolation*

Neutrophils were isolated from heparinized blood of healthy volunteers as described previously [1] and 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 were loaded with fura-2 and population and imaging measurements performed as previously described [1,4,5,8]. Excitation wavelengths were selected using monochromators (Spex, Glen Spectra, Stanmore, UK).

3. RESULTS

3.1. *Intracellular store release after SKF 96365*

In order to visualise Ca^{2+} release from stores in the presence of extracellular Ca^{2+}, influx of divalent ions via receptor-operated Ca^{2+} channels was blocked by SKF 96365 [7]. Mn^{2+} in the extracellular medium enabled entry of divalent ions to be monitored by the fura-2 quenching [6]. Blocking of transmembrane influx of divalent ions was demonstrated by prevention of Mn^{2+} quenching (Fig. 1A), yet an elevation of cytosolic free

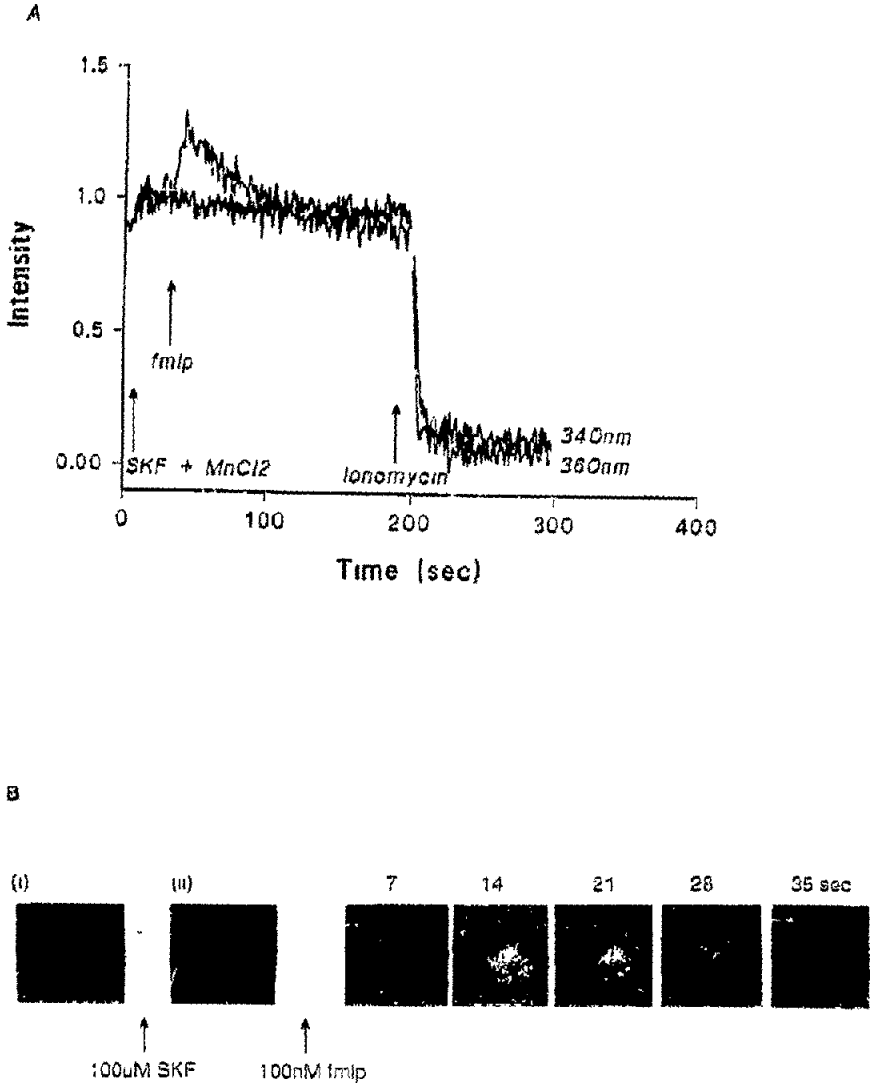

Fig. 1. Store release after SKF 96365. (A) Ca^{2+} (340 nm) and Mn^{2+} (360 nm) sensitive fluorescence form neutrophil populations. (B) Cytosolic free Ca^{2+} imaged in an individual neutrophil in the presence of SKF 96365 (100 μM). (i) at rest, (ii) 90 s after addition of SKF 96365, and 7 s, 14 s, 21 s, 28 s and 35 s after fmlp as indicated.

Ca^{2+} remained, indicating release from intracellular stores (Fig. 1A), in agreement with a previous report [7].

Imaging of the neutrophils, under these conditions revealed a discrete and localised 'cloud' of elevated Ca^{2+} (Fig. 1B) with similar spatial characteristics to those observed under other conditions (Table I). In 37% of neutrophils examined, SKF 96365 itself provoked a 'cloud' of elevated Ca^{2+}. Subsequent stimulation with fmlp released Ca^{2+} from the same site, suggesting an interaction of SKF 96365 with the fmlp releasable store. This interaction may account for the small inhibition and the slowing of fmlp-induced release observed in the presence of SKF 96365 (Fig. 2B). However, the kinetics of release of Ca^{2+} from the store and removal from the cytosol were unaffected by the absence of extracellular Ca^{2+} (Fig. 2A). This indicated that no entry of Ca^{2+} into

Table I

The % area of the cell occupied by the Ca^{2+} 'cloud' and the mean maximum cytosolic free Ca^{2+} concentration within the 'cloud'

	% Area	Ca^{2+} (nM)
+Ca+SKF	31 ± 10	472 ± 25
−Ca+SK+EGTA	19 ± 16	469 ± 91
+Ca+$MnCl_2$	39 ± 9	529 ± 42
−Ca+EGTA	22 ± 11	463 ± 44

Mean ± S.E., $n = 8$, are shown. For this analysis a 'cloud' was defined as the area in the cell with a Ca^{2+} concentration greater than 250 μM.

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Fig. 3. (A) Fura-2 fluorescence (340 nm and 360 nm). (B) The intracellular distribution of cytosolic free Ca^{2+} within an individual neutrophil under the same conditions shown as pseudo 3D plots: (i) at rest, (ii) 7 s, (iii) 14 s and (iv) 21 s after fmlp, and (v) 7 s and (vi) 14 s after ionomycin.

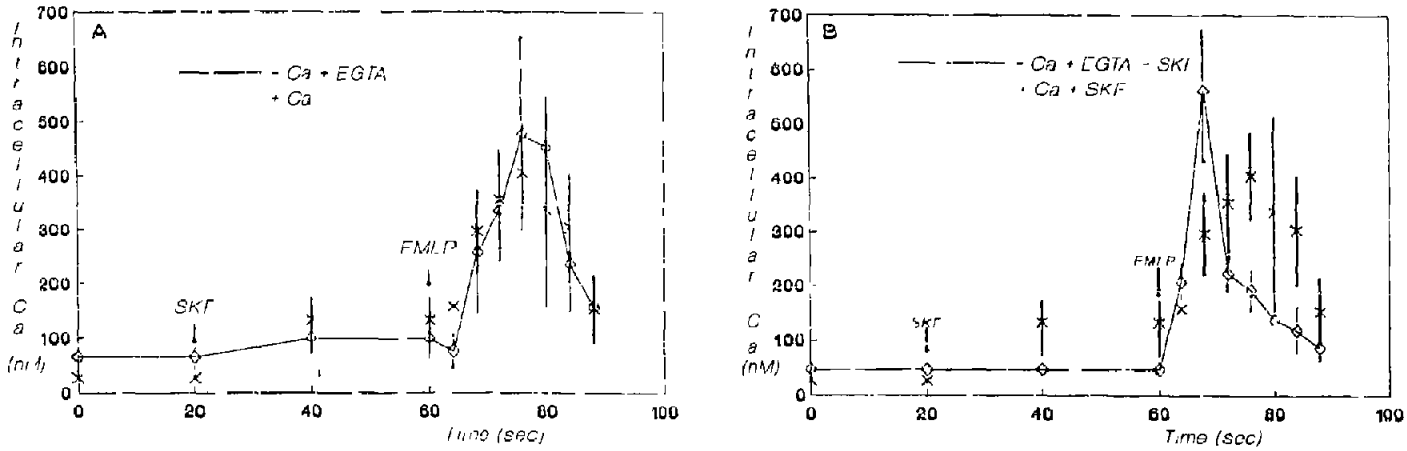
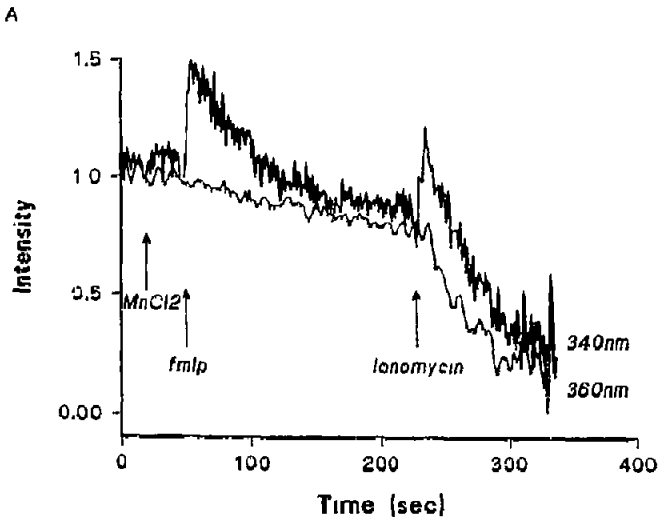
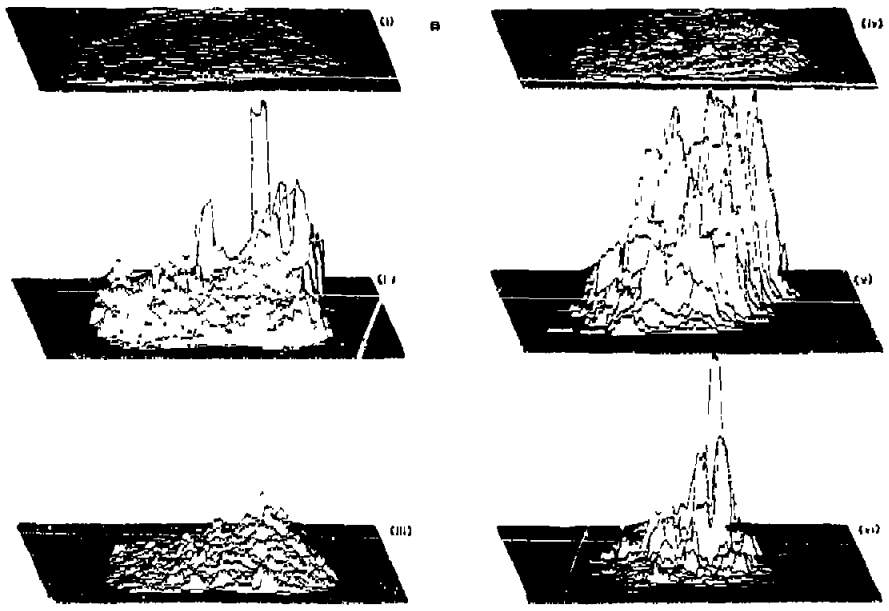


Fig. 2. (A) Time course of release from intracellular Ca^{2+} store in individual neutrophils (n = 8, ± S.E.) following incubation with SKF 96365 (50 μM) in the presence and absence of extracellular Ca^{2+}. (B) In absence of extracellular Ca^{2+} and SKF 96365.





the cytosol (or store) occurred other than via channels inhibited by SKF 96365.

3.2. *Visualisation of Mn^{2+} influx and intracellular Ca^{2+} release from stores*

Since both Ca^{2+} and Mn^{2+} entry occurred via channels which were inhibitable by SKF 96365, the route of divalent ion entry was visualised using Mn^{2+} quenching of fura-2. Under these conditions, a transient localised 'cloud' of elevation in 350/380 nm ratio was observed, indicating that a rise in cytosolic free Ca^{2+} occurred (Fig. 3A,B). No quenching by Mn^{2+} was observed at the site of elevated Ca^{2+}. Quenching of the fluorescence by Mn^{2+} was however visualised as a decrease in cell size, as the intensity of fura-2 at the cell edges fell below the ratioing threshold. The magnitude, kinetics (Fig. 4) and spatial characteristics were similar to those found previously in the absence of extracellular Ca^{2+} [4,5] (Table I). Due to the decrease in apparent cell size, the % area occupied by the 'cloud' in the presence of Mn^{2+} may have been overestimated. The addition of ionomycin produced a smaller rise in cytosolic free Ca^{2+} concentration at the same site (Fig. 3A,B), before measurement was prevented by total quenching of the fura-2 signal by Mn^{2+}.

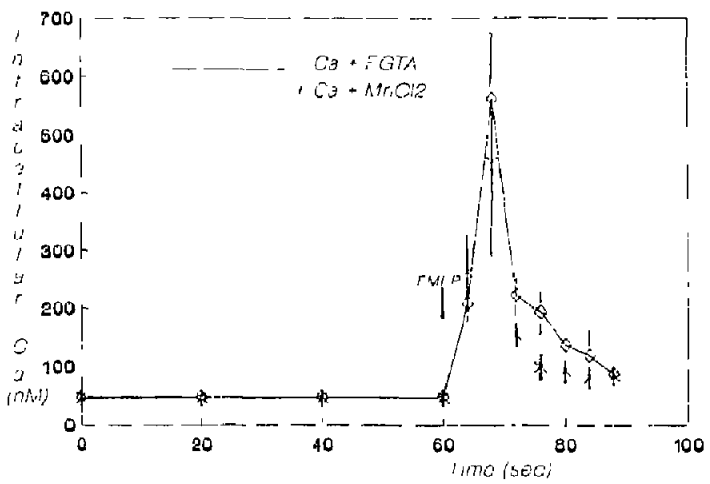


Fig. 4. Release of fmlp-stimulated intracellular Ca^{2+} store in individual neutrophils in the presence and absence of extracellular Ca^{2+}, and Mn^{2+}. The figure shows the Ca^{2+} concentration within the Ca^{2+} 'cloud' measured within a series of neutrophils (n = 8, ± S.E.).

4. DISCUSSION

The data presented in this paper demonstrate that transmembrane influx of divalent ions into neutrophils occurs directly into the cytosol, via channels which are inhibited by SKF 96365, and not via an internal store. The entry of both Ca^{2+} and Mn^{2+} ions into the cytosol, detected by fura-2 fluorescence enhancement and quenching respectively, was inhibited by SKF 96365. The failure of Mn^{2+} to quench the signal generated by release of Ca^{2+} from the internal store, demonstrates that Mn^{2+} does not enter the cytosol via the organelle from which Ca^{2+} was released. Since the presence of extracellular Ca^{2+} did not influence the magnitude and spatial characteristics of the cloud generated in the presence of SKF 96365, it was concluded that Ca^{2+} influx into the cytosol occurred exclusively through channels inhibited by the agent. Therefore, as no evidence for Mn^{2+} entry via SKF 96365-inhibited channels into the cytosol via the store site was found, it was also concluded that Ca^{2+} entry into neutrophils similarly does not proceed via the intracellular store. Furthermore, extracellular Ca^{2+} played no role in the kinetics or in determining the magnitude of released Ca^{2+} from the store. These results thus do not support the original model proposed by Putney [3], or the modified model proposed by Rink and Merritt [9], and Irvine [10]. They are, however, more in keeping with the recent proposal of Putney [11] where transmembrane influx and emptying of the intracellular Ca^{2+} store are not physically linked and refilling of the store occurs from the cytosol rather than the external medium. In this latter model it is the empty store which signals the opening of Ca^{2+} channels on the plasma membrane.

Localised elevations in Ca^{2+} have been observed in a number of cell types, including adrenal medulla cells in response to IP_3-generating agonists [12,13], dendritic spines [14,15], and in fibroblasts [16], in neutrophils and fibroblasts both being located to a site near the nucleus which stains with $DiOC_6(3)$ [4,16]. The storage and handling of intracellular Ca^{2+} in these cells may thus be similar. In some cell types, a second Ca^{2+} store whose release is regulated by cytosolic free Ca^{2+} concentration also exists [12,17–19]. However, here the release of Ca^{2+} from the store remained localised, suggesting the absence of Ca^{2+}-induced Ca^{2+} release sites in neutrophils. Several possibilities for the role of store release in neutrophils exist. Emptying the store may signal opening of channels on the plasma membrane, as suggested by the association of transmembrane influx to emptying of the intracellular stores by thapsigargin, 2,5-di-(*tert*-butyl)-1,4-hydroquinone and cyclopiazonic acid [20]. In contrast, it is possible that some physiological stimuli only release Ca^{2+} from this store providing a mechanism of localising the response to one part of the cell, such as may occur during chemotaxis and phagocytosis [4]. Further progress in establishing the role and regulation of the intracellular store may be made with methods for monitoring the Ca^{2+} within the stores of living cells, using endoplasmic reticulum targetted Ca^{2+}-activated photoprotein [22]. Since changes in the amount of releasable Ca^{2+} in the store may have profound effects on cell behaviour, the finding that neutrophils from inflammatory sites have an increased Ca^{2+} cloud [21] may therefore be important for an understanding of the behaviour of neutrophils during the inflammatory process.

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