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A SOLUBLE CELLULAR FACTOR DIRECTLY STIMULATES Ca^{2+} ENTRY IN NEUTROPHILS

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A soluble factor, extracted from neutrophils and P388D1 cells, stimulated a transient rise in cytosolic free Ca^{2+} and a small increase in the permeability to Mn^{2+} in fura2-loaded neutrophils. These effects were not prevented by blockade of formylated peptide receptors by t-boc-met-leu-phe. The rise in cytosolic free Ca^{2+} was partly attributed to transmembrane influx and partly due to store release. Ca^{2+} store release but not transmembrane influx was inhibited by the PLC inhibitor, U73122, demonstrating a direct effect of the factor on channel opening. It was concluded that the soluble cellular factor directly stimulated Ca^{2+} entry in neutrophils.

The chemotactic peptide, f-met-leu-phe, signals neutrophils by raising cytosolic free Ca^{2+} concentration [1-3]. This increase in free Ca^{2+} occurs throughout the cytosol and results from both entry of extracellular Ca^{2+} via Ca^{2+} channels and by release of Ca^{2+} from intracellular Ca^{2+} stores [2,3]. However, in neutrophils, extracellular Ca^{2+} is the major source of Ca^{2+} for this rise [2,3], and occurs directly into the cytosol and not via the Ca^{2+} store [4]. This raises the question of the function of the Ca^{2+} store in neutrophils.

Several lines of evidence suggests that, in neutrophils, a key signal for triggering the entry of Ca^{2+} into the cytosol is emptying of the Ca^{2+} store [5-8]. Recently Randriamapita and Tsien [9] and Parekh et al [10] have shown that, in several cell types, a diffusible messenger, released from Ca^{2+} stores, stimulates Ca^{2+} influx. The possibility, therefore, exists that this provides a mechanism by which Ca^{2+} channels opening is controlled by linkage to the Ca^{2+} store in neutrophils. The aims of the work presented here, therefore, were (i) to establish

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Abbreviations: CIF Ca^{2+}-influx factor; PLC, phospholipase C; f-met-leu-phe, N-formyl-methionyl-leucyl-phenylalanine.

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whether a similar soluble cellular factor opens Ca^{2+} channels in neutrophils and (ii) whether it had the neccessary characteristics to act as a messenger in f-met-leu-phe evoked Ca^{2+} signalling.

Materials and Methods

Materials

Fura-2/AM and pluronic F-127 were purchased from Molecular Probes, Oregon, U.S.A.; eonazole, t-boc-met-leu-phe and f-met-leu-phe from Sigma Chemicals, Poole, Dorset. SKF 96365 was a kind gift from Dr.P.England, SmithKline Beecham, Welwyn, Herts. U73122 and the inactive analogue, U73343 were purchased from Biomol (Plymouth, PA).

Preparation of neutrophils

Neutrophils were isolated from heparinized blood of healthy volunteers as described previously [2] 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).

Measurement of cytosolic free Ca^{2+} concentration

Neutrophils were loaded with fura-2 and population and imaging measurements performed as previously described [2,3,4,11]. Excitation wavelengths were selected using monochromators (Spex, Glen Spectra, Stanmore, UK).

Extraction of a calcium influx factor (CIF)

CIF was prepared from two sources, neutrophils and P388D1 macrophage cell line using the method described by Randriamapita and Tsien [9]. For the majority of experiments CIF was prepared from P388D1 macrophages as these cells provided a reliable and convienient source.

Results

Addition of CIF extracted from P388D1 macrophages to neutrophil populations resulted in a rise in cytosolic free Ca^{2+} which peaked at approximately at 300nM before reaching a plateau of approximately 200nM (figure 1a). This rise in intracellular Ca^{2+} was also observed in both neutrophils and P388D1 macrophages with CIF extracted from neutrophils. Pretreatment of the neutrophils with t-boc-met-leu-phe, an inhibitor which binds to f-met-leu-phe receptors, did not affect CIF-induced Ca^{2+} increases but severly decreased f-met-leu-phe stimulated Ca^{2+} responses, demonstrating that CIF did not act via f-met-leu-phe receptors (figure 1c and d). Removal of extracellular Ca^{2+} resulted in only a 37% reduction of the peak Ca^{2+} signal but total inhibition of the plateau phase. This result indicated that the CIF-induced Ca^{2+} rise was the result of both release of Ca^{2+} from intracellular Ca^{2+} stores and transmembrane Ca^{2+} influx (figure 1a,b).

The possibility that the Ca^{2+} channel opening was a consequence of CIF-induced Ca^{2+} store release, was excluded by pre-incubation with the PLC inhibitor, U73122 [13]. This

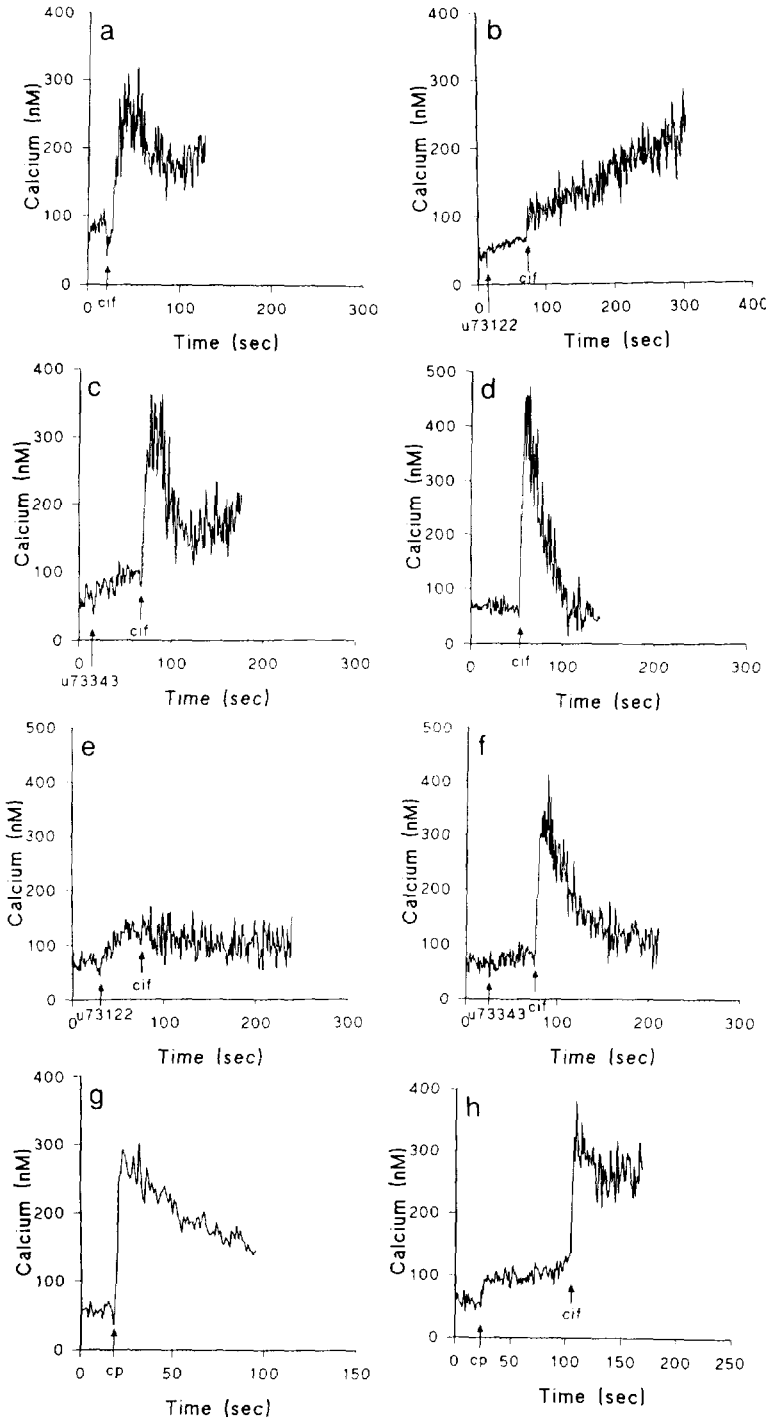

Figure 1.
CIF induced Ca^{2+} rises in neutrophil populations. CIF extract (1:4 dilution) (a) in Ca^{2+}-containing Krebs buffer, (b) in Ca^{2+}-free (EGTA 1mM) Krebs buffer, (c and d) after preincubation with the PLC inhibitor U73122 (10μM) in Ca^{2+}-containing and Ca^{2+}-free (EGTA 1mM) Krebs buffer, respectively, (e and f) after preincubation with the inactive analogue U73343 (10μM) in Ca^{2+}-containing and Ca^{2+}-free (EGTA 1mM) Krebs buffer, respectively, (g) f-met-leu-phe (10nM) alone and (h) f-met-leu-phe followed by CIF after pre-incubation with t-boc-met-leu-phe (50 μM) for 5 mins.

agent, but not an inactive anologue, U73343, totally inhibited the initial Ca^{2+} store release, without inhibiting the second transmembrane phase of the response (fig 1 e-h). The dissociation of transmembrane influx from store release demonstrated that CIF was not acting via a conventional route, but was acting directly on Ca^{2+} channels.

The Ca^{2+} response to CIF in the majority of individual neutrophils was transient (18/25 cells examined) (figure 2a). However, the addition of more CIF, following the initial Ca^{2+} transient, resulted in a second transient rise in intracellular Ca^{2+} of similar magnitude to the first (fig 2b), suggesting that the transient rise in Ca^{2+} was the result of consumption of CIF.

The question arose whether the factor played a physiological role in Ca^{2+} signalling via formylated petide receptors. Three lines of evidence suggest that it did not. Firstly, unlike f-met-leu-phe, CIF only weakly induced Mn^{2+} influx (fig 3a). Secondly, after the CIF-induced increase in Ca^{2+} or Mn^{2+} permeability, f-met-leu-phe evoked further increases in permeability to either ion (fig 3 b,c). Thirdly, SKF 96365, which blocks Ca^{2+} channels opened by f-met-

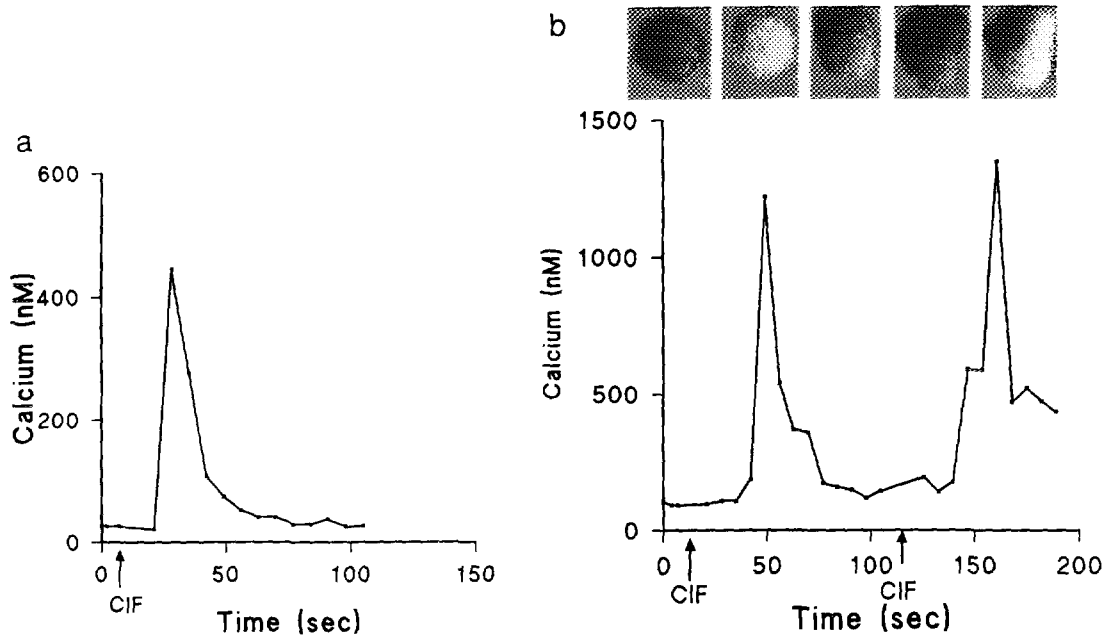

Figure 2.
CIF induced Ca^{2+} rises in individual neutrophils.
Ca^{2+} transients in response to (a) a single application of CIF (1:4 dilution) and (b) two applications of CIF (1:4). The pseudo-grey images above b show the Ca^{2+} changes in an individual neutrophil (i) at rest, (ii) 42 sec and (iii) 63sec after the first addition of CIF, and then (iv) 7 sec and (v) 35 sec after the second addition.

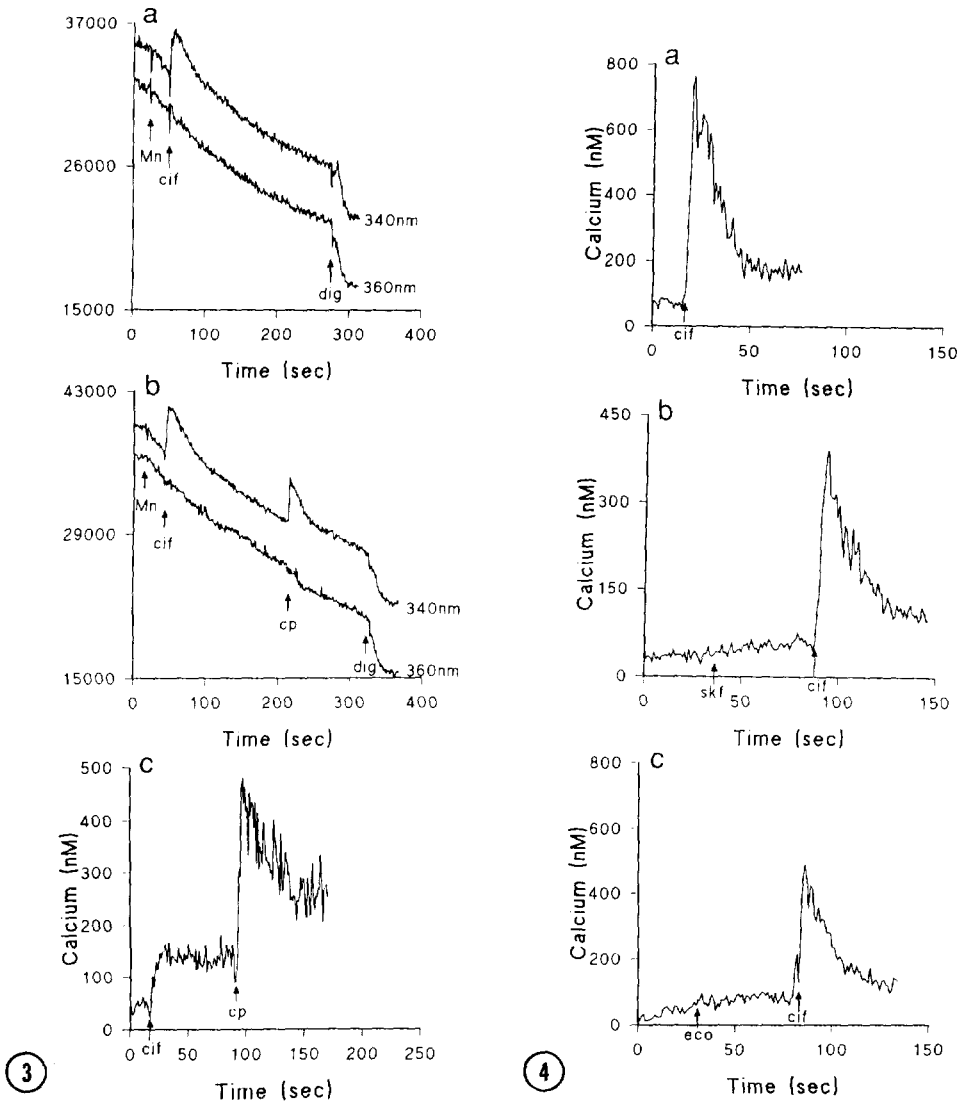

Figure 3.
Interaction between CIF and f-met-leu-phe. Fura2 intensity changes at 340nm (Ca^{2+} and Mn^{2+} sensitive) and 360nm (Mn^{2+} sensitive) in response to (a) CIF (1:4) alone and (b) CIF (1:4) then f-met-leu-phe (1μM). Ca^{2+} was measured in neutrophil populations stimulated by (c) CIF (1:4) then f-met-leu-phe (1μM).

Figure 4.
CIF induced Ca^{2+} changes. Cytosolic free Ca^{2+} changes were measured in response to CIF (1:4 dilution) (a) alone and (b) SKF 96365 (50μM) and (c) following incubation with econazole (5μM).

leu-phe [4,14], had no effect on CIF induced transmebrane Ca^{2+} influx (fig 4 a,b). Interestingly, a related compound, econazole, reported to inhibit the action of CIF [9] inhibited the Ca^{2+} transmembrane influx phase of the CIF response (fig 4c). This data together suggests that CIF and f-met-leu-phe operated on different Ca^{2+} permeability routes.

Discussion

The data presented in this paper demonstrate that an extract obtained from both P388D1 macrophages and neutrophils contained a factor which caused influx of Ca^{2+} in neutrophils. There were, however, two unexpected characteristics. The first was that release of store Ca^{2+} occured and the second was that the transmembrane influx was transient. Although we have no evidence for the mechanism of store release, the transient Ca^{2+} influx resulted from the apparent consumption of CIF and not from desensitisation to CIF. The possibility exists that under physiological conditions when CIF is released into the cytosol, it enters a cycle where once Ca^{2+} influx is activated, it is regenerated further channel opening. Using CIF extracellularly may not activate the regenerative process and thus consumption may result.

A further feature was that the factor caused Ca^{2+} entry mainly via Ca^{2+} selective channels, with only a small proportion of the Ca^{2+} entering via channels which were permeant to both Mn^{2+} and Ca^{2+}. Previous work on other cell types has also shown that there are non-specific Ca^{2+} channels present in the plasma membrane as well as those which show a selectivity for Mn^{2+} over Ca^{2+} [12,15,16]. In mast cells, the majority of Ca^{2+} influx occurs via I_{CRAC} channels which are selective for Ca^{2+} over Ba^{2+}, Sr^{2+} and Mn^{2+}, with a smaller proportion entring via non-specific, 50ps cation channels which are both Mn^{2+} and Ca^{2+} permeable [15]. This also appears to be true for hepatocytes, where two pathways are thought to be responsible for receptor mediated Ca^{2+} entry. Llopis et al [16] have reported that the capacitative pathway, which involves the Ca^{2+} level in the IP_3-sensitive store, is permeable to Ca^{2+} but impermeant to Mn^{2+}. They also reported a second pathway, dependent upon receptor occupation, which is Mn^{2+} permeant [16].

Physiological stimulation of neutrophils with f-met-leu-phe induces Ca^{2+} increases which occur via channels which are permeable to both Ca^{2+} and Mn^{2+} since SKF 96365 blocks influx of both ions [4,14]. Demaraux et al [17] have also described a Ca^{2+} influx pathway in neutrophils which is stimulated by intracellular Ca^{2+} store emptying which is poorly permeable to Mn^{2+} in comparison to Ca^{2+}. Although the data here suggests that a CIF-like messenger acts in a similar manner, opening channels which were permeant to Ca^{2+}, it is unlikely to account for transmembrane signalling by f-met-leu-phe route, because the pharmacological and permeability properties are different (figs 3 and 4). The possiblity, however, exists that CIF may be involved in other signalling pathways in neutrophils, which open this Ca^{2+} entry route.

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