# Characterization of a complement-fragment-C5a-stimulated calcium-influx mechanism in U937 monocytic cells

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The mechanism by which complement fragment C5a elevates intracellular $Ca^{2+}$ ($[Ca^{2+}]_i$) levels in two cell types, a monocytic cell line, U937, and neutrophils, has been investigated by the use of fluorometric and radiometric techniques. In U937 cells the influx of extracellular $Ca^{2+}$ can be distinguished from the release of intracellular $Ca^{2+}$ stores in terms of dose-responsiveness to C5a and sensitivity to pertussis-toxin poisoning. This suggests that the mechanism of $Ca^{2+}$ influx in these cells is at least partially independent of both the production of inositol phosphates and elevation of internal $Ca^{2+}$ concentration. The C5a-stimulated influx of $^{45}Ca^{2+}$ into U937 cells is inhibited by a series of metal ions ($Zn^{2+} > Co^{2+} > Mn^{2+} > Sr^{2+} \cong Ni^{2+} > La^{3+}$). The stimulated influx of $Ca^{2+}$ into neutrophils is inhibited differently ($Ni^{2} \gg Co^{2+} > Zn^{2+} \cong La^{3+} > Mn^{2+} \cong Sr^{2+}$), is less sensitive to C5a and both the influx of extracellular $Ca^{2+}$ and the release of intracellular stores are equally sensitive to pertussis toxin treatment. Taken together these results indicate that $[Ca^{2+}]_i$ is controlled in U937 monocytes by mechanisms distinct from those which appear to operate in other myeloid cells, such as neutrophils, stimulated with C5a and formylpeptide.

## INTRODUCTION

The elevation of intracellular $Ca^{2+}$ ($[Ca^{2+}]_i$) levels is an important element in the signal-transduction network activated following the binding of complement fragment C5a to cell-surface receptors on monocyte-like dibutyryl cyclic AMP (db-cAMP)-differentiated U937 cells (Monk and Banks 1991a,b). Several responses to chemoattractant, including the production of reactive oxygen intermediates (ROI), are at least partially dependent on this increase in $[Ca^{2+}]_i$. A number of other responses, however, including actin polymerization and actin nucleation, do not require changes in $[Ca^{2+}]_i$. Similarly, there are large differences in the sensitivity to pertussis-toxin poisoning of C5a-stimulated responses (Monk and Banks, 1991b).

C5a stimulates the hydrolysis of inositol phospholipids in U937 cells (Monk and Banks, 1991a), which presumably leads to the release of intracellular stores of $Ca^{2+}$ by the activation of receptors for Ins(1,4,5)$P_3$ (Streb et al., 1983). The presence of extracellular $Ca^{2+}$ is also required for maximal ROI production, probably by prolonging rises in intracellular $Ca^{2+}$ through an influx mechanism. The nature of this C5a-stimulated influx mechanism in db-cAMP-U937 cells is unknown. In neutrophils and related cells (e.g. differentiated HL-60) the activation of several mechanisms has been described. The chemoattractant *N*-formylmethionyl-leucylphenylalanine (fMLP) apparently modulates the activity of an amiloride- and lanthanum ion ($La^{3+}$)-sensitive $Na^+/Ca^{2+}$ exchanger (Simchowitz et al., 1990), leading to elevated $[Ca^{2+}]_i$, and the production of ROI (Simchowitz and Cragoe, 1990). A $Ca^{2+}$-stimulated $Ca^{2+}$ channel has also been described in fMLP-stimulated neutrophils that is dependent on the release of intracellular $Ca^{2+}$ stores prior to the influx of extracellular $Ca^{2+}$ (Von Tscharner et al., 1986). A $Ca^{2+}$-insensitive non-selective cation channel is also activated by fMLP in HL-60 cells and neutrophils (Krautwurst et al., 1992; Schumann et al., 1992). In neutrophils, dimethyl sulphoxide-differentiated HL60 and phorbol ester-differentiated U937 cells influx is considered to be dependent on the emptying of internal $Ca^{2+}$ stores (Putney, 1990; Alonso-Torre et al., 1993), although influx does not appear to occur via the organelle which contains the $Ca^{2+}$ store (Davies et al., 1992). The elevation of $[Ca^{2+}]_i$ in fMLP-stimulated neutrophils appears to be entirely dependent on pertussis-toxin-sensitive G-proteins (Goldman et al., 1985).

Here we show that human monocyte-like U937 cells possess a $Ca^{2+}$-influx mechanism that differs from those described in human neutrophils and that additional systems are present for the modulation of $[Ca^{2+}]_i$. The influx of extracellular $Ca^{2+}$ stimulated by C5a does not appear to be absolutely dependent on the prior release of intracellular $Ca^{2+}$ and is insensitive to pertussis-toxin treatment and 1 mM $NiCl_2$ and $LaCl_3$, all of which are inhibitory in neutrophils.

## MATERIALS AND METHODS

### Preparation of neutrophils and differentiated U937 cells

U937 cells were stimulated to differentiate by treatment with 1 mM db-cAMP (Sigma) for 48 h as described previously (Banks et al., 1988). Cells were washed and resuspended in buffered salts solution (BSS; 150 mM NaCl, 5 mM KCl, 10 mM D-sorbitol, 13 mM $K_2HPO_4$,$3H_2O$, 1 mM $KH_2PO_4$ and 10 mM Hepes, pH 7.4). Treatment with pertussis toxin (Sigma and Calbiochem) was for 4 h at 37 °C in differentiation medium. Viability, as determined by Trypan Blue dye exclusion, was not significantly affected at the concentrations of pertussis toxin used.

Human neutrophils were prepared from fresh peripheral blood by the method of Barnett-Foster et al. (1978).

### Measurement of ROI production

ROI production was assessed as maximal luminol-dependent chemiluminescence in the presence of 1 mM $CaCl_2$ as described previously (Monk and Banks, 1991a). Background chemiluminescence was subtracted from all results.

Abbreviations used: db-cAMP, dibutyryl cyclic AMP; ROI, reactive oxygen intermediates; fMLP, *N*-formylmethionyl-leucylphenylalanine; Fura2-am, BAPTA-am and Fluo3-am, acetoxymethyl esters of Fura2, BAPTA and Fluo3; $[Ca^{2+}]_i$, intracellular $Ca^{2+}$ concentration; BSS, buffered salts solution (for composition, see the text).

### Measurement of intracellular $Ca^{2+}$ levels with Fluo3

Intracellular $Ca^{2+}$ levels were monitored according to the method of Omann and Porasik-Lowes (1991). Briefly, cells [(1–5) × $10^6$/ml] were loaded with $Ca^{2+}$ indicator Fluo3 acetoxymethyl ester (Fluo3-am; Molecular Probes; 1 $\mu$M) at room temperature for 30 min in BSS + 0.1 % (w/v) BSA. After washing once, the cells were resuspended in fresh BSS + 0.1 % BSA/1 mM $CaCl_2$. The cells were stored on ice prior to use, then warmed to the incubation temperature for 2 min before the addition of recombinant human C5a (Sigma). In some experiments, $CaCl_2$ was omitted and 1 mM EGTA added immediately prior to cytometry. Under these conditions basal fluorescence in both U937 cells and neutrophils was reduced by 42 % on average. The fluorescence analysis was performed on an Orthocyte flow cytometer, analysing 2000 cells for each reading with an acquisition time of 5 s.

### Measurement of intracellular $Ca^{2+}$ levels with Fura2

Intracellular $Ca^{2+}$ levels were measured using the $Ca^{2+}$ indicator Fura2 acetoxymethyl ester (Fura2-am; Sigma). Cells were washed twice with BSS before resuspension in BSS + 1 mM $CaCl_2$/0.5 mM $MgCl_2$ at 2 × $10^6$ cells/ml and incubation with 2 $\mu$M Fura2-am for 20 min at 37 °C. After two more washes in BSS, cells were resuspended in BSS + 0.1 % (w/v) BSA at 3 × $10^6$ cells/ml and stored on ice prior to use. For determination of $[Ca^{2+}]_i$, 1 ml of this cell suspension was added to 2 ml of BSS + 0.1 % (w/v) BSA, with either 1 mM $CaCl_2$ or 1 mM EGTA, and warmed to room temperature. $Ca^{2+}$ concentrations were calibrated by the addition of digitonin and $MnCl_2$, as described previously (Grynkiewicz et al., 1985). Fluorometric measurements were performed at $\lambda_{excitation}$ = 340 nm, $\lambda_{emission}$ = 505 nm in a Perkin–Elmer LS5B spectrofluorimeter modified to allow stirring of cuvette contents.

### Measurement of $Ca^{2+}$ influx with $^{45}Ca^{2+}$

Neutrophils and U937 cells were resuspended at 1 × $10^7$/ml in BSS + 0.1 % BSA and incubated with 10 $\mu$M BAPTA-AM (Molecular Probes) for 30 min at 37 °C. After washing, cells were resuspended at (2–3) × $10^7$/ml in BSS + 0.1 % BSA/1 mM $CaCl_2$ and stored on ice prior to use. The cells were preincubated at 37 °C for 5 min before the addition of $^{45}Ca^{2+}$ (2.5 $\mu$Ci/ml; Amersham International). Samples of the cells (50 $\mu$l) were withdrawn and added to equal volumes of 5 mM EGTA in BSS on ice. Separation of cells from the radiolabelled $Ca^{2+}$ solution was achieved by centrifugation on 100 $\mu$l of Versilube silicone fluid (General Electric) at 12000 *g* for 1 min. Cell pellets were added to SafeFluor S (Lumac, Groningen, The Netherlands) scintillant and allowed to dissolve overnight prior to the measurement of radioactivity. Control experiments were performed in the presence of EGTA to assess radioactivity trapped in the volume of the cell pellet but not taken up by cells. This was subtracted from all results.

## RESULTS

### Flow cytometric analysis of intracellular $Ca^{2+}$

High (nanomolar) concentrations of C5a stimulated an increase in $[Ca^{2+}]_i$ levels in differentiated U937 cells and neutrophils loaded with the fluorescent $Ca^{2+}$ indicator Fluo3-am. In the presence of free extracellular $Ca^{2+}$, $[Ca^{2+}]_i$ was maximal and ≅ 30 s in U937 cells and ≅ 45 s in neutrophils and remained elevated over basal levels for > 120 s. If extracellular $Ca^{2+}$ was chelated by the addition of 1 mM EGTA, control levels were regained within 90 s–120 s, and the maximal rise in $[Ca^{2+}]_i$ (from a lower basal level) was reduced by 64 ± 13 % (± S.D., $n$ = 6) and 76 ± 9 % (± S.D., $n$ = 3) in U937 cells and neutrophils respectively. The time courses of these changes in $[Ca^{2+}]_i$ were not markedly different when measured at either 37 °C or room temperature, but were prolonged at 4 °C (results not shown). However, the dose-responsiveness to C5a differed markedly between the two cell types (Figures 1a and 1b). The U937 cells showed a clear difference in sensitivity between total $Ca^{2+}$ mobilization (i.e. measured in the presence of 1 mM $CaCl_2$) and the release of intracellular stores alone (i.e. measured in the

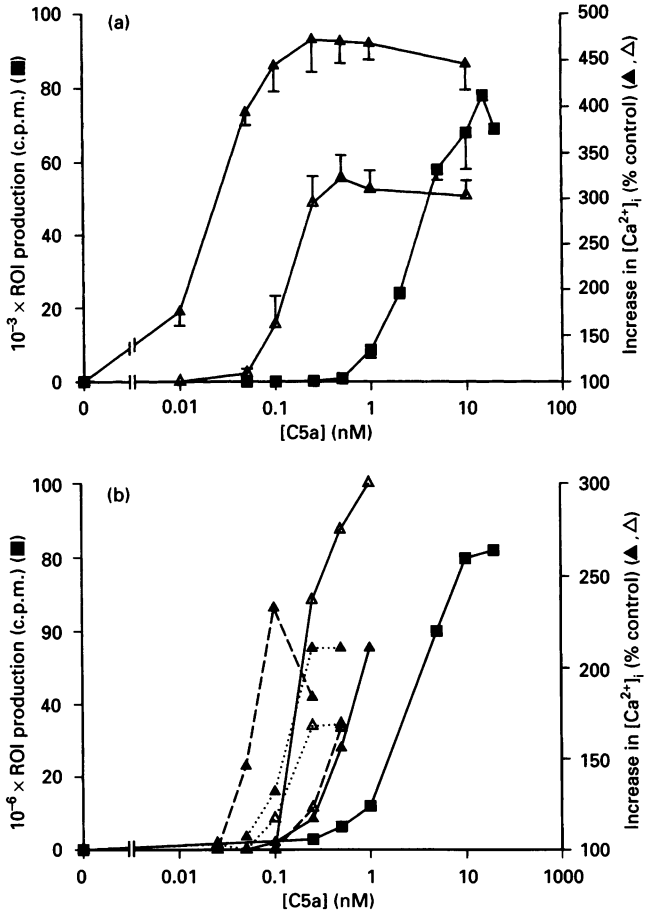


**Figure 1 C5a stimulates elevation of intracellular $Ca^{2+}$ and the production of ROI in a dose-dependent manner**

(**a**) Peak intracellular $Ca^{2+}$ levels in Fluo3-loaded db-cAMP-treated U937 cells were measured using flow cytometry, in the presence of either 1 mM $CaCl_2$ (▲) or 1 mM EGTA (△), after the addition of the stated concentration of C5a. Results shown are the means ± S.E.M. for three experiments performed in duplicate. ROI production (■) was assessed as maximum luminol-dependent chemiluminescence. Results are the means ± S.D. for two separate experiments. (**b**) Peak intracellular $Ca^{2+}$ levels in Fluo3-loaded human neutrophils were measured using flow cytometry, in the presence of either 1 mM $CaCl_2$ (▲) or 1 mM EGTA (△), after the addition of the stated concentration of C5a. Results shown are the means of three experiments performed in duplicate. Neutrophils from three different donors were used, as shown by the different line types. ROI production (■) was assessed as maximum luminol-dependent chemiluminescence. Results are the means from a single experiment performed in duplicate and are typical of three performed.

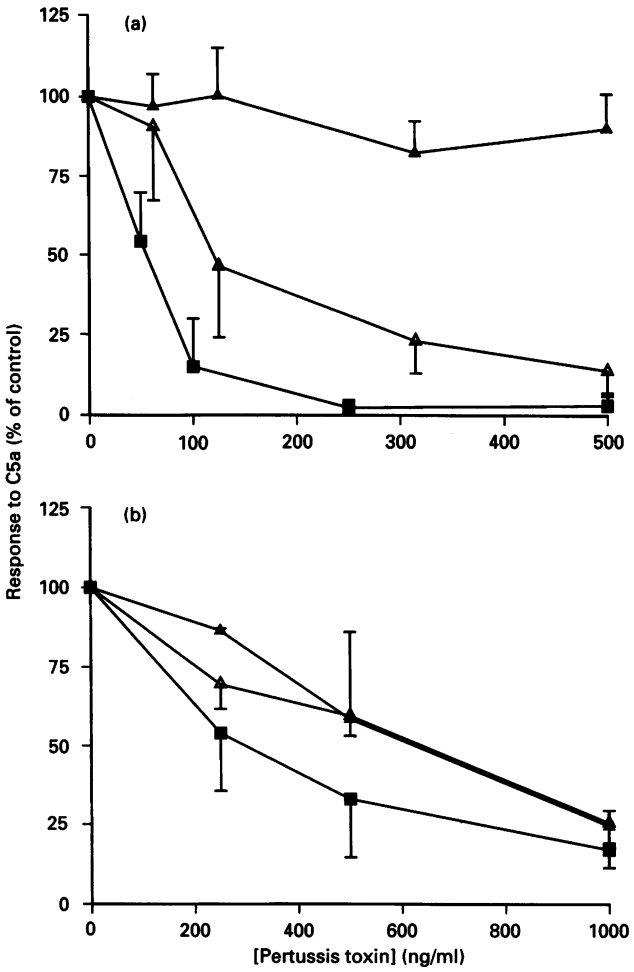


**Figure 2 Treatment with pertussis toxin inhibits the C5a-stimulated influx of extracellular $Ca^{2+}$ into neutrophils but not U937 cells**

(**a**) U937 cells were treated with the stated concentrations of pertussis toxin for 4 h at 37 °C prior to assessment of intracellular $Ca^{2+}$ levels by flow cytometry. The cells were stimulated for 30 s with 1 nM C5a. Results are shown as the means ± S.E.M. for four experiments performed in duplicate. ROI production was stimulated with 10 nM C5a and the results shown are the means ± S.D. for two separate experiments. The symbols used are the same as those in Figure 1. (**b**) Human neutrophils were treated with the stated concentrations for 2 h at 37 °C prior to stimulation for 45 s with 1 nM C5a for the assessment of intracellular $Ca^{2+}$ levels by flow cytometry and with 10 nM C5a for determination of ROI production. Results are shown as the means ± S.D. for one experiment performed in duplicate. The symbols used are the same as those in Figure 1.

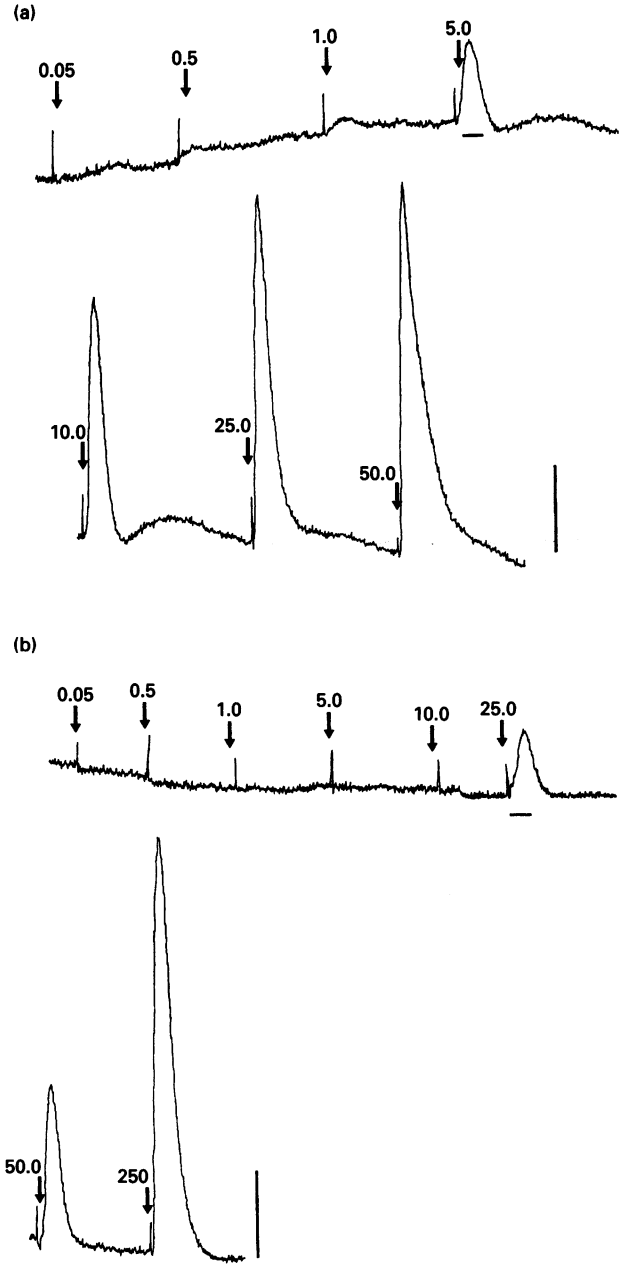


**Figure 3 C5a stimulates distinctly different patterns of increased $[Ca^{2+}]_i$ in Fura2-loaded U937 cells in the presence or absence of extracellular $Ca^{2+}$**

(**a**) Changes in $[Ca^{2+}]_i$ were monitored in U937 cells loaded with Fura2 in the presence of 1 mM $CaCl_2$ after consecutive additions of C5a (0.05–50 pM), as indicated by arrows. (**b**) Changes in $[Ca^{2+}]_i$ were monitored in U937 cells loaded with Fura2 in the presence of 1 mM EGTA after consecutive additions of C5a (0.05–250 pM). In both (**a**) and (**b**) the horizontal bar represents a time interval of 1 min and the vertical bar a change in $[Ca^{2+}]_i$ of 646 nM. The traces shown are typical of those obtained in three similar experiments.

presence of 1 mM EGTA). In the presence of extracellular $Ca^{2+}$, the $EC_{50}$ for C5a is 25 pM. However, when EGTA is added the $EC_{50}$ rises to 200 pM. At concentrations of C5a between 1 pM and 25 pM substantial increases in $[Ca^{2+}]_i$ levels occur, apparently by influx of extracellular $Ca^{2+}$, in the absence of a detectable release of $Ca^{2+}$ from internal stores. In neutrophils the presence of either EGTA or $CaCl_2$ makes little difference to the sensitivity to C5a, although the chelation of extracellular $Ca^{2+}$ generally diminishes the size of the response, indicating that influx is stimulated by C5a (Figure 1b). However, substantial differences in sensitivity to C5a were observed between donors; particularly in the presence of extracellular $Ca^{2+}$. The stimulation of ROI production in U937 cells and neutrophils by C5a has an $EC_{50}$ of 5 nM (Figures 1a and 1b), substantially higher than that for the release of internal $Ca^{2+}$ stores (100–200 pM).

The treatment of U937 cells for 4 h with pertussis toxin inhibited the C5a-stimulated elevation in $[Ca^{2+}]_i$ in the presence of EGTA (Figure 2a). The concentration of toxin is 2–3-fold higher than that required to inhibit ROI production. However, at all concentrations of pertussis toxin used here (0–500 ng/ml), no significant inhibition of the elevation in $[Ca^{2+}]_i$ in the presence of $CaCl_2$ was observed. In contrast, changes in neutrophil $[Ca^{2+}]_i$ under both sets of conditions were equally sensitive to toxin

treatment, although at higher concentrations than those necessary to inhibit U937 responsiveness. The inhibition of C5a-stimulated production of ROI also required higher PT concentrations; at 1000 ng of pertussis toxin/ml some 20% of stimulated ROI production remained. As with U937 cells, ROI production in neutrophils was 2–3-fold more sensitive to pertussis intoxication than the release of internal $Ca^{2+}$ stores.

### Spectrofluorometric analysis of intracellular $Ca^{2+}$

In the absence of data at early time points from flow-cytometric experiments we cannot exclude the possibility that the release of intracellular stores at low C5a concentrations occurs too rapidly to be detected. This problem was addressed by the use of conventional spectrofluorimetry, using cells loaded with the $Ca^{2+}$ indicator Fura2-am, which has the additional advantage of being more sensitive than Fluo3 to changes in $[Ca^{2+}]_i$. The C5a-stimulated changes in $[Ca^{2+}]_i$ in U937 cells were complex. In the presence of extracellular $Ca^{2+}$ (Figure 3a) very low [C5a] (0.05–0.5 pM) stimulated a small increase in $[Ca^{2+}]_i$ after a lag of $\cong$ 60 s which was maintained for several minutes. At higher [C5a] (> 1–250 pM) a rapid, transient 'spike' or elevated $[Ca^{2+}]_i$ was seen, followed immediately by a second, more sustained, rise (Figure 3a). After 5–6 min, $[Ca^{2+}]_i$ returned to resting levels. In the presence of EGTA, no changes in $[Ca^{2+}]_i$ were observed until the addition of 25–50 pM C5a (Figure 3b). Single spikes of elevated $[Ca^{2+}]_i$ were produced with no subsequent sustained rises. However, at 250 pM, the peak level of $[Ca^{2+}]_i$ was similar to the spike observed in the presence of extracellular $Ca^{2+}$ at 50 pM C5a.

Neutrophils displayed a different set of responses (Figure 4). The dose response to C5a was similar to the presence in either EGTA or $Ca^{2+}$; no response was seen until the addition of 25 pM C5a, and the increases in $[Ca^{2+}]_i$ were considerably smaller than those observed in U937 cells. In the presence of $Ca^{2+}$ basal levels of $[Ca^{2+}]_i$ were restored more slowly than in the presence of EGTA, but no secondary elevations of $[Ca^{2+}]_i$ appeared to occur.

### Inhibition of $^{45}Ca^{2+}$ influx by bivalent metal ions

The mechanism of $Ca^{2+}$ influx has also been characterized in terms of sensitivity to bivalent cations in both U937 cells and neutrophils using $^{45}Ca^{2+}$. The intracellular $Ca^{2+}$ chelator BAPTA was loaded into the cells in order to increase the sensitivity of the assay to allow the detection of small $Ca^{2+}$ transients. However, the technique appeared to be relatively insensitive; no influx was detectable after an incubation period of 120 s, at C5a concentrations below 100 pM (U937) or 250 pM (neutrophils) (results not shown). At higher concentrations, C5a stimulated the influx of $^{45}Ca^{2+}$ into both cell types with slightly different time courses (Figures 5a and 5b). The major difference between the cell types is the sensitivity to the presence of 1 mM $NiCl_2$. This completely inhibits $Ca^{2+}$ influx into neutrophils, but has little effect on U937 cells, which are more strongly inhibited by $ZnCl_2$. The effects of $LaCl_3$ on influx were also examined using this system. However, 1 mM $LaCl_3$ appeared to stimulate a rapid increase in the association of $^{45}Ca^{2+}$ with the cell pellet in the absence of C5a (results not shown). In addition of $LaCl_3$ to U937 cells loaded with Fura2 also caused an apparent increase in Fura2 fluorescence (results not shown), although it was not clear whether this was due to the formation of high-affinity $La^{2+}$–Fura2 complexes, which are strongly fluorescent, or to the influx of $Ca^{2+}$. This effect was not observed in cells loaded with Fluo3 for analysis by flow cytometry. Using Fluo3 we observed that 1 mM $LaCl_3$ inhibited the influx of $Ca^{2+}$ stimulated by 10 nM C5a into neutrophils by 64 ± 3% (mean ± S.D., $n = 2$), but had no effect on U937 cells.

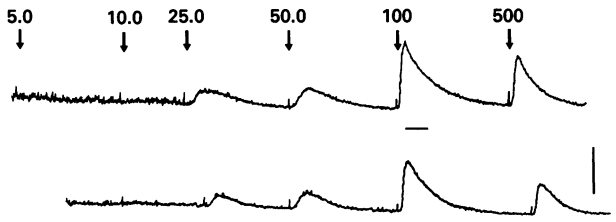


**Figure 4 C5a stimulates similar changes in $[Ca^{2+}]_i$ in Fura2-loaded neutrophils in the presence on absence of extracellular $Ca^{2+}$**

Neutrophils were loaded with Fura2 and the changes in $[Ca^{2+}]_i$ were monitored after consecutive additions of C5a (5–500 pM), indicated by arrows. The upper and lower traces show experiments performed in the presence of 1 mM $CaCl_2$ and 1 mM EGTA respectively. The horizontal bar indicates a time interval of 1 min. The vertical bar represents a change in $[Ca^{2+}]_i$ of 450 nM.

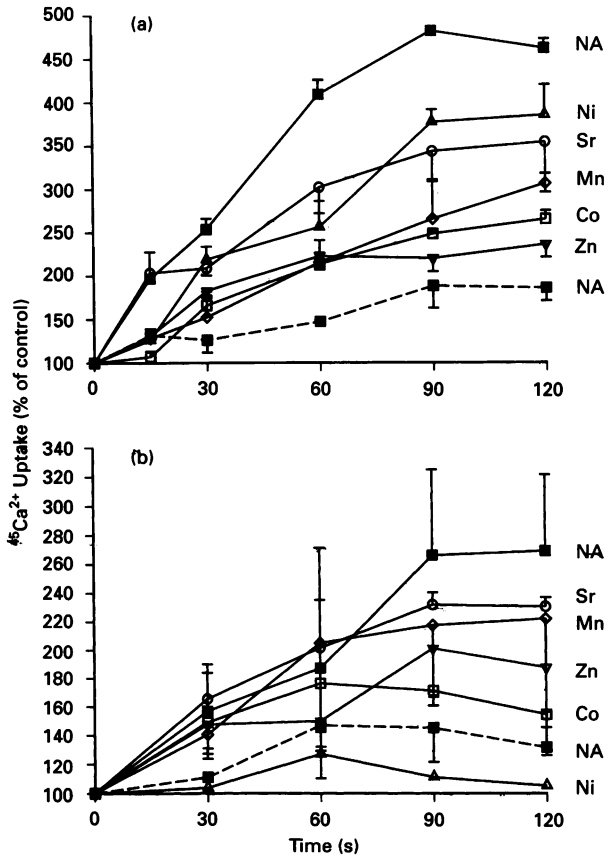


**Figure 5 Neutrophils and U937 cells show different patterns of inhibition of the C5a-stimulated uptake of $^{45}Ca^{2+}$ by bivalent metal ions**

(**a**) U937 cells were loaded with BAPTA and incubated for 1 min at 37 °C with 1 mM of the stated metal chloride before addition of 250 pM C5a. Samples were quenched with excess EGTA and separated from free $^{45}Ca^{2+}$ by centrifugation in silicone oil. Results shown are the means ± S.E. for three separate experiments. The broken line shows uptake in the absence of C5a. (**b**) Neutrophils, from three different donors, were treated as for U937 cells, except 500 pM C5a was used. Results shown are the means ± S.E. for four separate experiments. The broken line shows uptake in the absence of C5a.

## DISCUSSION

In the present study we have characterized a complex system for the control of $[Ca^{2+}]_i$ in C5a-stimulated db-cAMP-differentiated U937 cells that has features distinct from mechanisms reported in other leucocytic cell types such as neutrophils, dimethyl sulphoxide-differentiated U937 cells and db-cAMP-differentiated HL60 cells (Von Tscharner et al., 1986; Simchowitz et al., 1990; Krautwurst et al., 1992).

The C5a dose–response relationships for flow cytometrically measured $Ca^{2+}$ influx into Fluo3-loaded U937 cells and the release of intracellular stores are markedly different and suggest that release of internally sequestered $Ca^{2+}$ may not be a prerequisite for influx. In the absence of data at early time points (< 30 s) we cannot totally exclude the possibility that, at low concentrations of C5a, elevation of cytosolic $Ca^{2+}$ by release from internal stores is occurring and returning to basal levels too rapidly to be measured by this flow-cytometric technique. However, the results from experiments on Fura2-loaded U937 cells performed by conventional spectrofluorimetry indicate that $[Ca^{2+}]_i$ is maximal at times > 30 s following stimulation in the presence and absence of extracellular $Ca^{2+}$. These more detailed experiments also indicate that several different mechanisms for elevating $[Ca^{2+}]_i$ are activated by C5a in U937 cells. At very low doses of C5a (0.05–0.5 pM) in the presence of extracellular $Ca^{2+}$, small persistent increases in $[Ca^{2+}]_i$ occur; at higher doses (0.5–25 pM) a transient increase in $[Ca^{2+}]_i$ is seen, with a rapid return to near basal levels. For C5a concentrations above 5–10 pM, these transient increases are followed almost immediately by a second, more prolonged, rise. In the absence of free extracellular $Ca^{2+}$ only the transient increase in $[Ca^{2+}]_i$ occurs, at C5a concentrations $\geqslant$ 25 pM. However, the maximum increases in $[Ca^{2+}]_i$ (after the addition of 250 pM C5a) are similar to the $Ca^{2+}$ spikes obtained in the presence of extracellular $Ca^{2+}$ at much lower C5a concentrations ($\sim$ 50 pM).

The absence of detectable spikes at low C5a concentrations (0–10 pM) and their diminution at intermediate concentrations (25–250 pM) in the presence of EGTA may be due to the lack of a concurrent influx of $Ca^{2+}$ into U937 cells, leaving only the release of intracellular stores. Alternatively, incubation with EGTA may cause the depletion of a specific pool of stored intracellular $Ca^{2+}$ which is not therefore able to contribute to a rise in $[Ca^{2+}]_i$. This argument is partially supported by the observation that the fluorescence of Fluo3-loaded cells diminishes rapidly after the addition of EGTA and reaches a lower steady-state level (the subsequent addition of C5a still causes an elevation in $[Ca^{2+}]_i$, suggesting that depletion is only partial). The lack of significant levels of influx of $^{45}Ca^{2+}$ in response to low concentrations of C5a (< 100 pM) in U937 cells may also be explained in this way, but may also be due to the insensitivity of this technique. It is possible that different pools of stored $Ca^{2+}$ are released at different C5a concentrations; this would explain the apparent increase in the sensitivity of Fluo3-loaded U937 cells to C5a in the presence of extracellular $Ca^{2+}$, without invoking a separate influx mechanism. However, the similar maximum levels (albeit at different C5a concentrations) of the $Ca^{2+}$ spike measured using Fura2 in the presence and absence of EGTA suggest that internal stores are not substantially depleted. It is likely therefore that the spikes observed in the presence of extracellular $Ca^{2+}$ are at least partly due to an influx mechanism.

The dose–response curve for the release of 'EGTA-insensitive' internal $Ca^{2+}$ stores (measured by flow cytometry), is similar to one portion of the roughly biphasic response observed for the production of inositol phosphates [$EC_{50} \cong$ 300 pM and 5 nM (Monk and Banks, 1991a)], indicating that this release is controlled by polyphosphoinositide hydrolysis. However, increases in $[Ca^{2+}]_i$ are also observed at C5a concentrations substantially below those required to stimulate detectable levels of inositol phosphate production (i.e. < 50 pM). Therefore it seems likely that $Ca^{2+}$ influx is occurring (or an intracellular store highly dependent on external $[Ca^{2+}]$ is being released) in an inositol phosphate-independent manner at these low C5a concentrations.

The lack of a dependency of changes in $[Ca^{2+}]_i$ in the presence of extracellular $Ca^{2+}$ on inositol phosphate production can also be observed in U937 cells treated with pertussis toxin. The influx/release of 'EGTA-sensitive' stores is resistant to levels of pertussis intoxication that would almost completely inhibit the release of $Ca^{2+}$ from 'EGTA-insensitive' intracellular stores, ROI production and the formation of inositol phosphates ($InsP_1$ –$InsP_3$) in db-cAMP-U937 (the present results; Monk and Banks, 1991a).

The effects of C5a on Fluo3-loaded human neutrophils indicates that a different mechanism may be operating in these cells. Elevations in $[Ca^{2+}]_i$ are stimulated at similar C5a concentrations in the presence and absence of free $Ca^{2+}$, although there is a degree of variation between donors. The results suggest that the influx of extracellular $Ca^{2+}$ may be a consequence of the release of intracellular stores. This is also suggested by the results from Fura2-loaded neutrophils. In the presence or absence of extracellular $Ca^{2+}$, the dose response to C5a is identical, although the removal of extracellular $Ca^{2+}$ reduces the peak height of the $Ca^{2+}$ transient and causes basal levels to be more rapidly restored.

The effect of treating neutrophils with pertussis toxin is similar on both responses to C5a, which also suggests that influx is dependent on the prior release of internal $Ca^{2+}$ stores. We and others have observed that neutrophils treated with toxin formed two separate cell populations, detectable by flow cytometry. In one population, $Ca^{2+}$ responses were completely inhibited; in the other $Ca^{2+}$ responses to C5a were normal. The relative sizes of these populations were proportional to the degree of pertussis intoxication (P. N. Monk, unpublished work; Omann and Porasik-Lowes, 1991; Omann et al., 1992). We did not observe this phenomenon in U937 cells, and it does not occur in human blood monocytes (Lund-Johanssen and Olweus, 1992). Thus this 'all-or-nothing' effect may be unique to the neutrophil response to pertussis toxin.

Differences in the degree of pertussis-toxin poisoning of cells required to inhibit different responses may be due to differences in the levels of activated G-protein necessary to maximally stimulate these responses; i.e. pertussis toxin-sensitivity may be related to the ligand-sensitivity of the particular response. In U937 cells, the inhibition of the release of internal $Ca^{2+}$ stores requires 2–3 fold higher concentrations of toxin than the inhibition of ROI production. This may be due to quantitatively different G-protein requirements, reflecting the 10-fold difference in sensitivity to C5a. A similar difference is apparent in C5a-sensitivity between the $[Ca^{2+}]_i$ elevation mechanisms. However, the lack of any significant inhibition of $Ca^{2+}$ influx at 500 ng toxin/ml strongly suggests that the insensitivity to pertussis toxin is not a function of a lowered G protein requirement. Interestingly, when the release of 'EGTA-insensitive' internal $Ca^{2+}$ stores is almost fully inhibited by pertussis-toxin poisoning in U937 cells, the elevation of $Ca^{2+}$ levels by influx results in cytosolic $Ca^{2+}$ concentrations equivalent to those seen in the absence of toxin. This indicates that influx is controlled by a feedback mechanism which is dependent on $[Ca^{2+}]_i$. The similarity of the peak sizes of the $Ca^{2+}$ spikes in the presence and absence of EGTA also suggests this type of mechanism.

The influx of $Ca^{2+}$ into neutrophils is inhibited by high (mM) concentrations of $La^{3+}$, $Co^{2+}$ and $Ni^{2+}$ (the present results;

Boucek and Snyderman, 1976; Merritt et al., 1991). The influx of extracellular $Ca^{2+}$ into U937 cells could be partly inhibited by 1 mM $Zn^{2+}$, $Mn^{2+}$ and $Co^{2+}$, but appeared insensitive to both $La^{3+}$ and $Ni^{2+}$. This suggests that different influx mechanisms are stimulated by C5a in U937 cells and neutrophils.

Taken together, these results suggest that both the C5a-stimulated signal-transduction pathways leading to elevations of $[Ca^{2+}]_i$ in U937 cells are different to those found in C5a- and fMLP-stimulated neutrophils and also that the mechanism of $Ca^{2+}$ influx may be different. The simulation of $Ca^{2+}$ influx by leukotrienes $D_4$ and $E_4$ in dimethyl sulphoxide-differentiated U937 cells has some similarities to the C5a-activated pathway described above (Saussy et al., 1989; Pollock and Creba, 1990) in that influx is only partially sensitive to pertussis-toxin poisoning and is not dependent on inositol lipid hydrolysis. In contrast, the response to fMLP in these cells is totally inhibited by pertussis-toxin treatment (Pollock and Creba, 1990). It is likely that U937 cells differentiated with dimethyl sulphoxide are not identical with cells differentiated with db-cAMP. Differences in the expression of G-proteins could explain the dissimilar sensitivity to pertussis toxin, although we have previously observed that differentiation-linked changes in the levels of membrane-associated toxin substrates $G_{13\alpha}$ and $G_{12\alpha}$ in db-cAMP-U937 are similar to those observed in dimethyl sulphoxide-treated cells (Sheth et al., 1991; Pollock et al., 1990). The expression of other G-proteins that are known not to be substrates for pertussis toxin remains to be determined in differentiating U937 cells. The monocytic cell line THP-1 and the monocyte/granulocyte precursor cell line HL-60 have both been shown to express $G_{16\alpha}$, which is not ADP-ribosylated by pertussis toxin (Amatruda et al., 1991). Human kidney cells co-transfected with C5aR and the $G_{16\alpha}$ show pertussis-toxin-insensitive changes in $[Ca^{2+}]_i$ and in inositol lipid hydrolysis in response to C5a (Buhl et al., 1993), suggesting that coupling can occur between C5aR and G proteins other than $G_i$. The pertussis-toxin-insensitivity of the influx of extracellular $Ca^{2+}$ is not exclusive to the response to C5a; human blood monocytes stimulated with fMLP also show toxin-insensitive increases in $[Ca^{2+}]_i$ (Lund-Johansen and Olweus, 1992). Thus partial pertussis-toxin-insensitivity may be a characteristic feature of chemoattractant signalling in monocytic cells.

Here we have characterized a C5a-stimulated $Ca^{2+}$ influx mechanism in db-cAMP-differentiated U937 cells that differs in several respects to that observed in neutrophils and also in U937 cells differentiated with other agents. The results suggest that in U937 cells there is not a simple relationship between the emptying of internal $Ca^{2+}$ stores and the influx of extracellular $Ca^{2+}$. Chemoattractant receptors may couple to different G-proteins and effector systems in different cell types and this may result in the insensitivity to pertussis toxin of some responses of U937 monocytic cells to C5a.

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