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# Rapid kinetics of agonist-evoked changes in cytosolic free $Ca^{2+}$ concentration in fura-2-loaded human neutrophils

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The initial kinetics of agonist-evoked rises in the cytosolic $Ca^{2+}$ concentration $[Ca^{2+}]_i$ were investigated in fura-2-loaded human neutrophils by stopped-flow fluorimetry. The rises in $[Ca^{2+}]_i$ evoked by chemotactic peptide (fMet-Leu-Phe), platelet-activating factor and ADP all lagged behind agonist addition by 1–1.3 s. Lag times were not significantly different in the presence and in the absence of external $Ca^{2+}$. Stimulation of the cells in the presence of extracellular $Mn^{2+}$ resulted in a quench of fluorescence with a similar lag time to $[Ca^{2+}]_i$ rise. The delay in onset of the rise in $[Ca^{2+}]_i$ evoked by fMet-Leu-Phe was dependent on concentration, becoming longer at lower concentrations of agonist. These results indicate that both the agonist-evoked discharge of the intracellular $Ca^{2+}$ stores and the generation of bivalent-cation influx lag behind agonist–receptor binding in neutrophils. Both pathways thus appear to be mediated by indirect mechanisms, rather than by a directly coupled process such as a receptor-operated channel. The temporal coincidence of the onset of store discharge with the commencement of bivalent-cation influx suggests that the two events may be causally linked.

## INTRODUCTION

Stimulation of human neutrophils with various agonists including chemotactic peptide (fMet-Leu-Phe), platelet-activating factor (PAF) and ATP results in an increase in the cytosolic $Ca^{2+}$ concentration $[Ca^{2+}]_i$ as measured by the use of fluorescent $Ca^{2+}$ indicator dyes [1–8]. Agonist-evoked rises in $[Ca^{2+}]_i$ are due to both $Ca^{2+}$ release from intracellular stores and $Ca^{2+}$ entry from the extracellular medium. The release of $Ca^{2+}$ from intracellular stores is indicated by the ability of agonists to elevate $[Ca^{2+}]_i$ in the absence of extracellular $Ca^{2+}$ [1–8]. An additional influx component is apparent from the following studies. Firstly, agonist-evoked increases in $[Ca^{2+}]_i$ are larger in the presence than in the absence of extracellular $Ca^{2+}$ [1–6]. The additional component may be blocked by using inorganic $Ca^{2+}$ antagonists such as $La^{3+}$ [8] and $Ni^{2+}$ [6] as well as SK&F 96365, a blocker of receptor-mediated $Ca^{2+}$ entry [9]. Secondly, agonists evoke $^{45}Ca^{2+}$ uptake [10], and, thirdly, agonists can stimulate $Mn^{2+}$ entry [6].This last-mentioned technique makes use of the ability of $Mn^{2+}$ to bind and quench the fluorescence of fura-2. If the dye is excited at a wavelength of 360 nm, at which its fluorescence is insensitive to changes in $[Ca^{2+}]_i$ [11], stimulated quenching of the cytosolic fluorescence by $Mn^{2+}$ indicates bivalent-cation entry [6,12]. Thus the stimulation of cells in the presence of extracellular $Mn^{2+}$ allows the influx of bivalent ions to be distinguished from the release of $Ca^{2+}$ from intracellular stores.

Agonist-evoked discharge of the intracellular $Ca^{2+}$ store is believed to be mediated by the diffusible second messenger Ins(1,4,5)$P_3$ [13]. However, the mechanisms by which agonists evoke $Ca^{2+}$ entry in neutrophils remain uncertain. Previous studies have shown that agonist-evoked increases in $[Ca^{2+}]_i$ commence at the same time in the presence and in the absence of external $Ca^{2+}$ [2] and that stimulated $Mn^{2+}$ entry coincides with the onset of rise in $[Ca^{2+}]_i$ [6]. However, these studies used suspensions of cells in cuvettes, and lacked accurate resolution of the initial changes in fluorescence.

We have now investigated agonist-evoked increases in $[Ca^{2+}]_i$ and stimulated $Mn^{2+}$ entry in fura-2-loaded human neutrophils by using a stopped-flow technique [12,14,15]. Such studies with platelets and parotid acinar cells have yielded considerable information that could not have been obtained by conventional fluorimetry. For example, important differences between agonists and cell types, indicative of different mechanisms of $Ca^{2+}$ entry, have been described [12,14,15].

## EXPERIMENTAL

Neutrophils were prepared from freshly drawn human blood essentially as described previously [6]. Briefly, blood was collected into citrate anticoagulant, centrifuged for 5 min at 1500 *g* and the platelet-rich plasma removed. Dextran T500 was then added to sediment the erythrocytes at unit gravity. The resultant leucocyte-rich plasma was concentrated and the neutrophils were purified by centrifugation through a discontinuous density gradient of Percoll. This method results in a preparation containing > 95% neutrophils with a viability of > 99% (assessed by Trypan Blue exclusion).

The cells were loaded with fura-2 by incubation in Hepes-buffered saline (145 mM-NaCl/5 mM-KCl/1 mM-$MgCl_2$/10 mM-Hepes/NaOH buffer, pH 7.4 at 37 °C)

Abbreviations used: $[Ca^{2+}]_i$, cytosolic free $Ca^{2+}$ concentration; PAF, platelet-activating factor (1-*O*-alkyl-2-*O*-acetylglycero-3-phosphocholine).

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supplemented with 1 mM-$CaCl_2$, 1% (w/v) BSA and 0.3 $\mu$M-fura-2 acetoxymethyl ester for 30 min at 37 °C, collected by centrifugation at 300 *g* for 5 min and resuspended in Hepes-buffered saline at a density of approx. $2 \times 10^6$ cells/ml.

In cuvette experiments, measurements were made on 0.8 ml of cell suspension placed in a cylindrical cuvette mounted in a thermostatically controlled holder in a Perkin–Elmer MPF 44A spectrophotometer. The suspension could be stirred by means of a magnetic stir bar. Fura-2 fluorescence, with excitation at 340 nm and emission at 500 nm, was recorded on a chart recorder and calibrated in terms of $[Ca^{2+}]_i$ by using a method previously described for platelets [16].

For measurement of subsecond kinetics, the fluorescence from fura-2-loaded neutrophils was recorded as previously described for platelets [14] by using a Hi-Tech Scientific SFA-II Rapid Kinetic Accessory mounted in the spectrophotometer and thermostatically maintained at 37 °C. Dye-loaded cells were injected through one port and a solution of the agonist in Hepes-buffered saline, with $CaCl_2$ or $MnCl_2$ added as required, through the other. Traces show the averages of eight scans recorded at 15 s intervals. With some preparations, a slow basal leak of $Mn^{2+}$ into the cells was observed. In such cases, agonist-free controls were subtracted from the experimental records.

Fura-2 acetoxymethyl ester was from Molecular Probes, Eugene, OR, U.S.A.; fMet-Leu-Phe was from Sigma Chemical Co., Poole, Dorset, U.K.; ATP, PAF and Hepes were from Calbiochem, Cambridge Bioscience, Cambridge, U.K. Percoll and dextran T500 were from Pharmacia, Uppsala, Sweden. All other chemicals were of analytical grade.

## RESULTS AND DISCUSSION

Fig. 1 shows responses evoked by a maximally effective concentration of fMet-Leu-Phe (100 nM) on $[Ca^{2+}]_i$ in fura-2-loaded neutrophils in the presence and in the absence of external $Ca^{2+}$. The initial rises in fluorescence are similar under both conditions. In the presence of 1 mM external $Ca^{2+}$, $[Ca^{2+}]_i$ was elevated from a basal level of $110 \pm 8$ nM to a peak of $1032 \pm 153$ nM (means ± S.E.M., $n = 10$). In the absence of added $Ca^{2+}$, $[Ca^{2+}]_i$ rose from a basal level of $105 \pm 9$ nM to a peak of $817 \pm 138$ nM (means ± S.E.M., $n = 6$). In the presence of external $Ca^{2+}$, $[Ca^{2+}]_i$ declined from the initial fMet-Leu-Phe-evoked peak to a plateau well above the basal level of $[Ca^{2+}]_i$. This maintained rise in $[Ca^{2+}]_i$ is attributable to $Ca^{2+}$ entry. In contrast, in the absence of added $Ca^{2+}$, $[Ca^{2+}]_i$ declined from its slightly lower peak back to basal levels. This response is assumed to be due to discharge of intracellular $Ca^{2+}$ stores, which then become depleted. Traces such as those shown in Fig. 1 give little information about the early kinetics of the elevation in $[Ca^{2+}]_i$, such detail being obscured by unknown mixing delays and the instrumental response time. These problems are overcome by using the stopped-flow technique, in which the mixing time is less than 30 ms and recording by computer is immediately triggered on agonist addition. Typical traces of the first 5 s of stimulation are shown in Fig. 2.

When the cells were stimulated with 100 nM-fMet-Leu-Phe, the onset of fluorescence rise in the presence of external $Ca^{2+}$ (Fig. 2*a*) was delayed by $1020 \pm 40$ ms

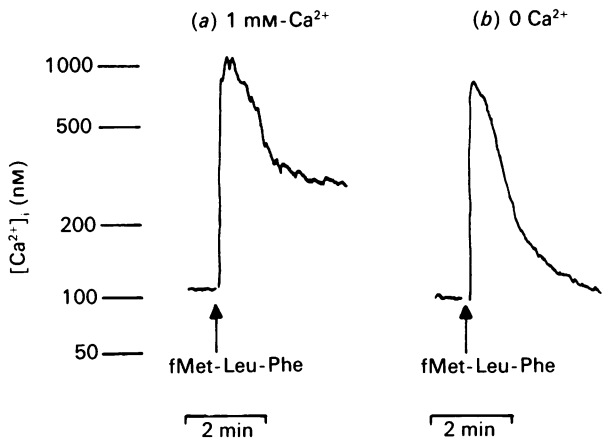


**Fig. 1. Effect of fMet-Leu-Phe on $[Ca^{2+}]_i$ in fura-2-loaded neutrophils**

Traces show the effect of 100 nM-fMet-Leu-Phe on neutrophil $[Ca^{2+}]_i$ in the presence of 1 mM external $Ca^{2+}$ (*a*) and in the absence of added $Ca^{2+}$ (*b*).

(mean ± S.E.M., $n = 18$). In the absence of external $Ca^{2+}$ (Fig. 2*b*) the delay was $1080 \pm 40$ ms ($n = 18$). The difference was not significant (Student's *t* test, difference means, $0.5 > P > 0.1$). The onset of fMet-Leu-Phe-evoked $Mn^{2+}$ quench (Fig. 2*c*) lagged behind agonist addition by $1160 \pm 60$ ms ($n = 19$). This lag time was not significantly different from that in the onsets of fluorescence rise in the presence and in the absence of external $Ca^{2+}$ ($0.1 > P > 0.5$). When the cells were stimulated with 10 nM-PAF (Figs. 2*d*–2*f*) the lag times in the onset of a measurable response were $1070 \pm 60$ ms ($n = 9$) in the presence of external $Ca^{2+}$, $1070 \pm 30$ ms ($n = 8$) in the absence of external $Ca^{2+}$ and $1100 \pm 80$ ($n = 8$) in the presence of external $Mn^{2+}$. With 100 $\mu$M-ATP as the agonist (Figs. 2*g*–2*i*) the lag times were $1340 \pm 40$ ms ($n = 10$), $1350 \pm 40$ ms ($n = 10$) and $1380 \pm 100$ ms ($n = 12$) under the same conditions.

Fig. 3 shows the concentration-dependence of the lag between agonist addition and onset of detectable rise in $[Ca^{2+}]_i$ for fMet-Leu-Phe in the presence of 1 mM external $Ca^{2+}$. The curve shows that the lag time falls with increasing agonist concentration, even though the lowest dose of fMet-Leu-Phe used (3 nM) evoked a maximal rise in $[Ca^{2+}]_i$ (results not shown). At high concentrations the results indicate an irreducible delay in the onset of response of approx. 900 ms.

The delays in the onset of responses evoked by fMet-Leu-Phe, PAF and ATP in the absence of external $Ca^{2+}$, where the elevation in $[Ca^{2+}]_i$ is the result of the release of $Ca^{2+}$ from intracellular stores, are as expected for a process that relies upon the generation of a diffusible second messenger, Ins(1,4,5)$P_3$ [13]. The delay in the responses evoked by all three agonists in the presence of external $Ca^{2+}$, and the irreducible nature of the delay demonstrated with fMet-Leu-Phe, suggests that the generation of $Ca^{2+}$ influx in neutrophils is also dependent on a sequence of biochemical events (e.g. the generation of a diffusible messenger or activation of a G-protein), rather than on a process directly coupled to receptor occupation.

The lack of significant temporal separation between the onset of agonist-evoked responses in the presence

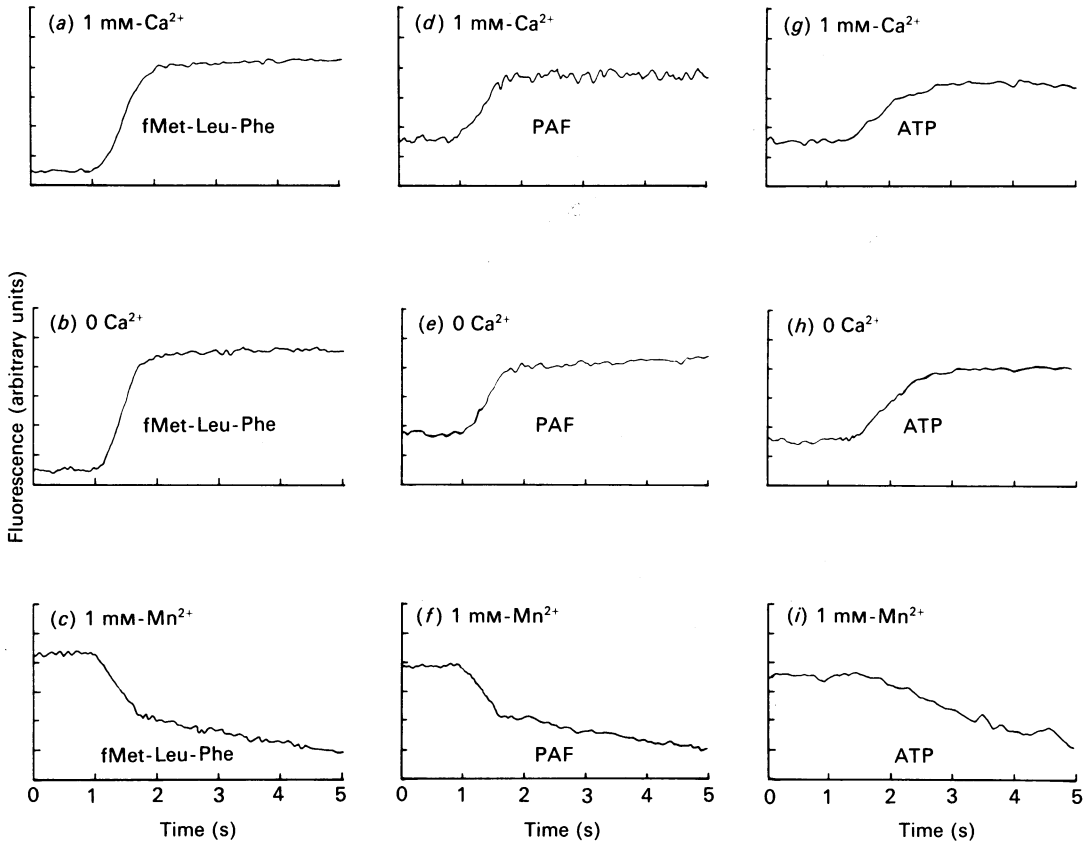


**Fig. 2. Stopped-flow fluorimetry of fura-2-loaded neutrophils stimulated with (*a–c*) fMet-Leu-Phe (100 nM), (*d–f*) PAF (10 nM) or (*g–i*) ATP (100 μM)**

Neutrophils suspended in nominally $Ca^{2+}$-free medium were mixed with agonist solution at zero time. $CaCl_2$ (*a*, *d* and *g*) or $MnCl_2$ (*c*, *f* and *i*) was introduced with the agonist to attain a final concentration of 1 mM. Traces (*b*), (*e*) and (*h*) were obtained in the absence of added bivalent cations. Fura-2 fluorescence was recorded at an excitation wavelength of 340 nm (*a*, *b*, *d*, *e*, *g* and *h*) or 360 nm (*c*, *f* and *i*); emission was 500 nm in each case. Controls, with $Mn^{2+}$ but no agonist, were subtracted digitally from the stimulated $Mn^{2+}$ records (*c*, *f* and *i*). Each record is the average of eight scans recorded at 15 s intervals.

and in the absence of external $Ca^{2+}$ might be because no $Ca^{2+}$ entry occurs during the early stages of neutrophil activation. The rises in fluorescence were very similar in the presence and in the absence of external $Ca^{2+}$ (Fig. 2), but reference to Fig. 1 shows that very small changes in fluorescence in the region of the peak of the signal equate to large changes in $[Ca^{2+}]_i$. This is due to the non-linear response of fura-2 to changes in $[Ca^{2+}]$ [11]. The similarity in fluorescence traces in the presence and in the absence of external $Ca^{2+}$ cannot therefore rule out a major role for $Ca^{2+}$ entry at early times. In any case, fura-2 measures only steady-state $[Ca^{2+}]_i$ and gives no direct measure of flux. The use of external $Mn^{2+}$ provides a direct measure of stimulated bivalent-cation influx [6,12,17,18], and thus gives a clear demonstration of an early stimulated influx component. Such a stimulated entry of $Mn^{2+}$ (monitored as quenching of fura-2 fluorescence) was demonstrated to commence at a similar time to the onset of rises in $[Ca^{2+}]_i$ evoked by all three neutrophil agonists, results clearly showing that bivalent-cation entry does commence at the same time as release from $Ca^{2+}$ stores in these cells.

It has been suggested that $Ca^{2+}$ influx in neutrophils is conducted via $Ca^{2+}$-activated cation channels in the plasma membrane, which open following the discharge

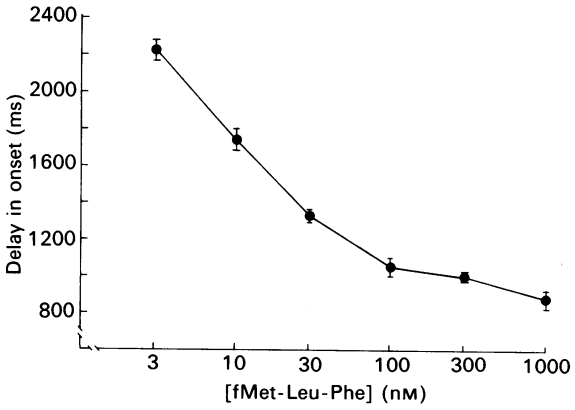


**Fig. 3. Effect of concentration of fMet-Leu-Phe on delay in onset of rise in $[Ca^{2+}]_i$**

Neutrophils suspended in nominally $Ca^{2+}$-free medium were mixed with fMet-Leu-Phe solutions of various concentrations at zero time. $CaCl_2$ was present at a final concentration of 1 mM. The delay in onset of fluorescence rise was measured from traces obtained at 340 nm as in Fig. 2. Each point is the mean ± S.E.M. for five to seven determinations.

of the intracellular $Ca^{2+}$ stores and the resultant elevation of $[Ca^{2+}]_i$ [2]. Our results are not easily reconciled with this model, since $Mn^{2+}$, and therefore we believe $Ca^{2+}$, entry commences at a time when $[Ca^{2+}]_i$ has risen little, if at all. In addition, we and others have shown that a rise in $[Ca^{2+}]_i$ above basal levels is not a requirement for agonist-evoked $Ca^{2+}$ or $Mn^{2+}$ entry in neutrophils. Agonist-evoked $Ca^{2+}$ or $Mn^{2+}$ entry is not prevented when rises in $[Ca^{2+}]_i$ are greatly buffered by loading the cells with the $Ca^{2+}$ chelator 1,2-bis-(2-aminophenoxy)-ethane-*NNN'N'*-tetra-acetic acid ('BAPTA') [5]. Similarly, the addition of $Ca^{2+}$ or $Mn^{2+}$ after $[Ca^{2+}]_i$ has returned to basal levels following the addition of agonist in the absence of extracellular bivalent cation results in stimulated entry [6].

Our rapid kinetic data showing simultaneous discharge of $Ca^{2+}$ from intracellular stores (the rise in fura-2 fluorescence in the absence of extracellular $Ca^{2+}$) and bivalent-cation entry (responses in the presence of extracellular $Ca^{2+}$ or $Mn^{2+}$) are compatible with models for the generation of $Ca^{2+}$ influx in neutrophils in which discharge of the intracellular $Ca^{2+}$ stores and the opening of a bivalent-cation-entry pathway are closely associated.

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## REFERENCES

1. Pozzan, T., Lew, D. P., Wollheim, C. B. & Tsien, R. Y. (1983) Science **221**, 1413–1415
2. von Tscharner, V., Prod'hom, B., Baggiolini, M. & Reuter, H. (1986) Nature (London) **324**, 369–372
3. von Tscharner, V., Deranleau, D. A. & Baggiolini, M. (1986) J. Biol. Chem. **261**, 10163–10168
4. Lew, P. D., Monod, A., Waldvogel, F. A. & Pozzan, T. (1987) Eur. J. Biochem. **162**, 161–168
5. Nasmith, P. E. & Grinstein, S. (1987) FEBS Lett. **221**, 95–100
6. Merritt, J. E., Jacob, R. & Hallam, T. J. (1989) J. Biol. Chem. **264**, 1522–1527
7. Merritt, J. E. (1989) Br. J. Pharmacol. **97**, 438*P*
8. Andersson, T., Dahlgren, C., Pozzan, T., Stendahl, O. & Lew, D. (1986) Mol. Pharmacol. **30**, 437–443
9. Merritt, J. E., Armstrong, W. P., Hallam, T. J., Jaxa-Chamiec, A. A., Leigh, B. K., Moores, K. E. & Rink, T. J. (1989) Br. J. Pharmacol. **98**, 674*P*
10. Korchak, H. M., Rutherford, L. E. & Weissmann, G. (1984) J. Biol. Chem. **259**, 4070–4075
11. Grynkiewicz, G., Poenie, M. & Tsien, R. Y. (1985) J. Biol. Chem. **260**, 3340–3350
12. Sage, S. O., Merritt, J. E., Hallam, T. J. & Rink, T. J. (1989) Biochem. J. **258**, 923–926
13. Prentki, M., Wollheim, C. B. & Lew, P. D. (1984) J. Biol. Chem. **259**, 13777–13782
14. Sage, S. O. & Rink, T. J. (1987) J. Biol. Chem. **262**, 16364–16369
15. Merritt, J. E. & Rink, T. J. (1987) J. Biol. Chem. **262**, 4958–4960
16. Pollock, W. K., Rink, T. J. & Irvine, R. F. (1986) Biochem. J. **235**, 869–877
17. Hallam, T. J., Jacob, R. & Merritt, J. E. (1989) Biochem. J. **259**, 125–129
18. Sage, S. O., Reast, R. & Rink, T. J. (1990) Biochem. J. **265**, 675–680

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