Use of Manganese to Discriminate between Calcium Influx and Mobilization from Internal Stores in Stimulated Human Neutrophils*

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Stimulation of human neutrophils with f-met-leu-phe, platelet-activating factor, or leukotriene B4 resulted in an increase in [Ca\(^{2+}\)]. The [Ca\(^{2+}\)]\text{r}, rise was greater in the presence than absence of external Ca\(^{2+}\); the component that was dependent on external Ca\(^{2+}\) was blocked by Ni\(^{2+}\), or could be reconstituted by addition of external Ca\(^{2+}\) following discharge of the internal Ca\(^{2+}\) store. These measurements of [Ca\(^{2+}\)]\text{r} provide only indirect evidence for agonist-stimulated Ca\(^{2+}\) entry, and here we have used an alternative approach to demonstrate directly agonist-stimulated divalent cation entry. In the presence of extracellular Mn\(^{2+}\), f-met-leu-phe, leukotriene B4, and platelet-activating factor stimulate a quench in fluorescence of fura-2-loaded human neutrophils. This quench was due to stimulated Mn\(^{2+}\) influx and was blocked by Ni\(^{2+}\). When Mn\(^{2+}\) was added in the continued presence of agonist, after discharge of the internal store of Ca\(^{2+}\), a stimulated quench was seen; this result shows that an elevated [Ca\(^{2+}\)]\text{r} is not needed for the stimulation of Mn\(^{2+}\) entry. Depolarization by high [K\(^{+}\)] or addition of the L-type Ca\(^{2+}\) channel agonist, BAY-R-5417, had little or no effect on either [Ca\(^{2+}\)]\text{r} or Mn\(^{2+}\) entry. These results show that agonists stimulate divalent cation entry (Ca\(^{2+}\) or Mn\(^{2+}\)) by a mechanism independent of changes in [Ca\(^{2+}\)]\text{r}, and unrelated to voltage-dependent Ca\(^{2+}\) channels.

Stimulation of neutrophils (human, rabbit, or bovine) with chemotactic peptide (fMLP), PAF, or LTB\(_4\) results in an increase in [Ca\(^{2+}\)]\text{r}, as measured with the fluorescent Ca\(^{2+}\) indicator dyes, quin2, fura-2 or indo-1 (1–7). Responses of human peripheral neutrophils have been characterized in more detail in attempts to assess the source of the Ca\(^{2+}\) (2). These studies have shown that fMLP stimulates a rise in [Ca\(^{2+}\)]\text{r} in the absence of external Ca\(^{2+}\), presumably due to release of Ca\(^{2+}\) from internal stores. However, the fMLP-stimulated rise in [Ca\(^{2+}\)]\text{r} is greater in the presence than absence of external Ca\(^{2+}\), and the component of the [Ca\(^{2+}\)]\text{r}, rise that is dependent on external Ca\(^{2+}\) is blocked by inorganic Ca\(^{2+}\) antagonists such as La\(^{3+}\) or Co\(^{2+}\), suggesting that it is due to fMLP-stimulated Ca\(^{2+}\) entry. Many of these experiments (1–6) have been carried out using quin2-loaded neutrophils where stimulation causes significant changes in autofluorescence (due to NADPH oxidation) and the amounts of quin2 required to obtain a sufficient signal tend to buffer changes in [Ca\(^{2+}\)]\text{r} (1). Furthermore, the use of intracellular dyes to measure [Ca\(^{2+}\)]\text{r}, provides only indirect evidence for a stimulated Ca\(^{2+}\) influx. Alternative explanations for the larger rise in [Ca\(^{2+}\)]\text{r}, seen in the presence of external Ca\(^{2+}\) could include an extracellular Ca\(^{2+}\) requirement for more effective signal transduction or for more complete discharge of Ca\(^{2+}\) from intracellular stores. Studies of "Ca\(^{2+}\)" uptake into human neutrophils have provided independent evidence for fMLP-stimulated Ca\(^{2+}\) influx (8). Here, we have used an alternative approach to demonstrate agonist-stimulated divalent cation entry into fura-2-loaded human neutrophils. When Mn\(^{2+}\) binds to fura-2 (or quin2), its fluorescence is quenched. In quin2-loaded human blood platelets, agonists have been found to stimulate a quench in intracellular dye fluorescence in the presence of extracellular Mn\(^{2+}\), from which it can be concluded that agonists can stimulate Mn\(^{2+}\) entry (9). There have been previous reports that fMLP stimulates Mn\(^{2+}\) influx into human neutrophils (2, 7), but the evidence was not convincing. The rate of quench, measured at a single wavelength with a Ca\(^{2+}\)-sensitive component superimposed, was very slow, and the quenching below base line was scarcely detectable in the presence of the high cytosolic buffering.

Here, we have used fura-2 at two excitation wavelengths to allow simultaneous monitoring of changes in [Ca\(^{2+}\)]\text{r}, and quenching by Mn\(^{2+}\) independently of changes in [Ca\(^{2+}\)]\text{r}. We have used human neutrophils loaded lightly with fura-2 so that the buffering of [Ca\(^{2+}\)]\text{r}, is minimal but where agonist-stimulated changes in autofluorescence are insignificant relative to the fura-2 signal. We have studied the effect of fMLP, PAF, and LT\(_B\) on [Ca\(^{2+}\)]\text{r}; and their dependence on external Ca\(^{2+}\), and have used Mn\(^{2+}\) to confirm the existence of a divalent cation entry mechanism.

MATERIALS AND METHODS

Percoll and dextran T500 were from Pharmacia Ltd. (Uppsala, Sweden); fura-2 acetoxyxymethylester was from Molecular Probes Inc. (Eugene, OR); Hepes (ultrapure), PAF, and ionomycin were from Behring Diagnostics; fMLP and DTPA were from Sigma; LT\(_B\), was supplied by Smith, Kline and French Laboratories Ltd. (Philadelphia, PA); BAY-R-5417 was a gift from Bayer (Koln, West Germany).

Neutrophils were prepared from the blood of normal healthy volunteers by modifications of previously described methods (10, 11). Blood (27 ml) was collected into anticoagulant (4.5 ml of acid citrate dextrose: 1.5% citric acid, 2.5% trisodium citrate, 2% glucose). Dextran T500 (400 mg in 12 ml 0.9% NaCl) was added, and the erythrocytes were allowed to sediment at unit gravity for 45 min. The resultant leukocyte-rich plasma was removed, centrifuged 5 min at 300 × g, and the cell pellet was resuspended in 8 ml of the supernatant. This concentrated cell suspension was layered over a discontinuous density gradient of Percoll. (The different densities of Percoll were

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† The abbreviations used are: fMLP, formyl-methionyl-leucyl-phenylalanine; [Ca\(^{2+}\)]\text{r}, cytosolic free calcium concentration; DTPA, diethylenetriaminepentaacetic acid; LT\(_B\), leukotriene B\(_4\); PAF, platelet-activating factor; (L-α-leucin, β-acetyl, γ-O-alkyl); Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; EGTA, ethylenbis(oxyethylenenitrilo)tetraacetic acid.
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preparing by mixing isosmotic Percoll and 0.9% NaCl to obtain the required densities. The gradient was prepared by layering 2 ml of the Percoll mixture at a density of 1.110 g/ml below a 2-ml layer at 1.088 g/ml. Following centrifugation for 20 min at 600 \( \times \) g, the neutrophils band at the interface between the two layers of Percoll, with erythrocytes below the Percoll and lymphocytes/monocytes at the Percoll-plasma interface. The neutrophil band was carefully removed (after discarding the upper layers), and washed by dilution in buffer and centrifugation for 5 min at 300 \( \times \) g. This method results in a preparation containing >95% neutrophils with a viability of >99% (assessed by trypan blue exclusion).

The buffer for these experiments contains 145 mM NaCl, 5 mM KCl, 1 mM MgCl\(_2\), 10 mM Hepes (pH 7.4 at 37 °C), 10 mM glucose. For loading cells with fura-2, this buffer was supplemented with 1 mM CaCl\(_2\) and 1% bovine serum albumin. Cells (approximately 6.10\(^6\)/ml) were incubated with 0.5 \( \mu \)M fura-2 AM for 30 min at room temperature, then centrifuged (5 min at 300 \( \times \) g), resuspended (cell count approximately 2.5.10\(^6\)/ml) in a buffer supplemented with 1 mM CaCl\(_2\), and kept at room temperature until use. This procedure resulted in a cytosolic dye concentration of 50-100 \( \mu \)M, and there was no significant loss of dye over at least 6 h. The size of the fura-2 signal from aliquots of the cell suspension remained constant over this time period. The lack of dye leakage in individual experiments is clearly shown in Fig. 3.

For fura-2 fluorescence measurements, aliquots (0.7 ml) of cell suspension were centrifuged and the cells resuspended in medium with no CaCl\(_2\) or albumin. Fluorescence (500 nm) emission was measured at 37 °C in a dual excitation wavelength fluorimeter (Spex) with additions as indicated in the figures. (Agonist-stimulated changes in autofluorescence were insignificant relative to the fura-2 signal.) In Figs. 1 and 7, [Ca\(^{2+}\)] was calculated from the ratio of fluorescence at two excitation wavelengths (340 and 380 nm), as described by Grynkiewicz et al. (12). All the other figures show raw fluorescence traces with excitation wavelengths of 340 and 360 nm.

All the records are typical of at least three others obtained from different cell preparations.

RESULTS

Fig. 1 shows the effect of maximally effective concentrations of fMLP (10 nM), PAF (10 nM), and LTB\(_4\) (10 nM) on [Ca\(^{2+}\)], in fura-2-loaded human neutrophils in the presence or absence of extracellular Ca\(^{2+}\) (1 mM of CaCl\(_2\) or 1 mM EGTA added, as indicated). In the presence of external Ca\(^{2+}\), all three agonists caused a rapid rise in [Ca\(^{2+}\)], from a resting level of around 80 nM to transient peak values in excess of 1 \( \mu \)M. In each case, [Ca\(^{2+}\)], then began to decline, although the shapes of the transient [Ca\(^{2+}\)], rise differed for each agonist. Subsequent removal of external Ca\(^{2+}\) by addition of excess (2.5 mM) EGTA curtailed the responses and caused a rapid decline in [Ca\(^{2+}\)], to base line (results not shown). In the absence of external Ca\(^{2+}\), fMLP, PAF, and LTB\(_4\), stimulated a rapid rise in [Ca\(^{2+}\)], to a peak (smaller than that seen in the presence of external Ca\(^{2+}\)), which then declined back to base line. The responses seen in the absence of external Ca\(^{2+}\) appear similar for each agonist. When CaCl\(_2\) (in excess of EGTA) was added in the continuous presence of agonist after [Ca\(^{2+}\)], has returned to base line, a further rapid rise in [Ca\(^{2+}\)], was seen. This rise in [Ca\(^{2+}\)], was transient and declined again to a level which remained above the prestimulated basal level. The shape and extent of the rise seems to be dependent on the agonist and resembles the response seen in the continued presence of external Ca\(^{2+}\). The response to the addition of Ca\(^{2+}\) in the presence of LTB\(_4\) was considerably smaller than that seen with PAF or fMLP and corresponds to the smaller

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**Fig. 1.** Effects of fMLP, PAF, and LTB\(_4\) on [Ca\(^{2+}\)], in fura-2-loaded neutrophils. [Ca\(^{2+}\)], was calculated from the ratio of fura-2 fluorescence at two excitation wavelengths. The arrows indicate the times of addition of CaCl\(_2\) (Ca, 1 or 2.5 mM after EGTA), EGTA (1 mM), NiCl\(_2\) (Ni, 5 mM), fMLP (10 nM), PAF (10 nM), and LTB\(_4\) (10 nM).
maintained rise in \([\text{Ca}^{2+}]\), seen with LTB4. Since removal of external \(\text{Ca}^{2+}\) may have additional effects other than solely removing the extracellular supply of \(\text{Ca}^{2+}\) for the rise in \([\text{Ca}^{2+}]\), we compared the agonist responses produced in the presence of \(\text{Ni}^{2+}\) (5 mM) and external \(\text{Ca}^{2+}\) (1 mM) with that in the presence of EGTA. Fig. 1 (lower panels) show that addition of \(\text{Ni}^{2+}\) in the presence of \(\text{Ca}^{2+}\) resulted in agonist-stimulated \([\text{Ca}^{2+}]\), transients similar to those seen in the absence of external \(\text{Ca}^{2+}\).

Fig. 2 shows raw fluorescence traces (500 nm emission) at two excitation wavelengths (340 and 360 nm) from suspensions of fura-2-loaded neutrophils. At an excitation wavelength of 340 nm, fura-2 fluorescence increases with increasing \([\text{Ca}^{2+}]\), while the fluorescence at 360 nm excitation is insensitive to changes in \([\text{Ca}^{2+}]\). However, the fluorescence at both excitation wavelengths is quenched by \(\text{Mn}^{2+}\). The top panel of Fig. 2 shows the effect of fMLP in the absence of added \(\text{Ca}^{2+}\) or \(\text{Mn}^{2+}\). Addition of fMLP (10 nM) and subsequent addition of the \(\text{Ca}^{2+}\) ionophore, ionomycin (2 \(\mu\)M), caused increases in fluorescence at 340 nm (showing the increase in \([\text{Ca}^{2+}]\)), while the fluorescence at 360 nm remained unaltered. Ionomycin can translocate \(\text{Mn}^{2+}\) as well as \(\text{Ca}^{2+}\), and addition of \(\text{MnCl}_2\) (100 \(\mu\)M) in the presence of ionomycin resulted in a rapid quench in fluorescence at both excitation wavelengths. The middle panel of Fig. 2 shows the effect of adding \(\text{Mn}^{2+}\) (100 \(\mu\)M) before fMLP (10 nM). Addition of \(\text{Mn}^{2+}\), alone, caused a slow decline in fluorescence at both excitation wavelengths (this is shown over a longer period in the lower panel), presumably due to a slow basal leak of \(\text{Mn}^{2+}\) into the cells. Addition of fMLP then caused a transient increase in fluorescence at 340 nm (due to the stimulated rise in \([\text{Ca}^{2+}]\), caused by discharge of \(\text{Ca}^{2+}\) from intracellular stores) which then declined to well below the baseline. At 360 nm excitation, where the fluorescence is unaffected by changes in \([\text{Ca}^{2+}]\), a rapid decrease in fluorescence intensity was observed. This result is consistent with a stimulated uptake of \(\text{Mn}^{2+}\) into the cells. Addition of ionomycin now has little further effect, presumably because fMLP has already stimulated uptake of \(\text{Mn}^{2+}\) across the plasma membrane to quench the intracellular dye. An alternative interpretation, however, could be that stimulation has caused increased leakage of dye from the cells, which could then be quenched by the extracellular \(\text{Mn}^{2+}\). Fig. 3 confirms that fMLP does not
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Fig. 4. Effect of Ni²⁺ on the fMLP-stimulated quenching of fura-2 fluorescence. The excitation wavelengths were 340 nm (solid lines) and 360 nm (broken lines). fMLP (10 nM), ionomycin (iono, 2 μM), digitonin (dig, 50 μM), MnCl₂ (Mn, 100 μM), and NiCl₂ (Ni, 5 mM) were added at the times shown.

cause increased leakage of fura-2 from the neutrophils. The top panel of Fig. 3 shows that, in the presence of Mn²⁺ (100 μM), fMLP (10 nM) stimulated a quench in fura-2 fluorescence. Following the stimulated quench, the membrane-impermeant heavy metal chelator, DTPA (200 μM), was added. If the Mn²⁺-quenched dye was extracellular, chelation of extracellular Mn²⁺ should allow the dye to bind Ca²⁺ and cause an increase in fluorescence. However, it is clear (Fig. 3, top panel) that addition of DTPA had very little effect on the fluorescence signal, confirming that most of the Mn²⁺-quenched dye was intracellular. The lower panel of Fig. 3 confirms that sufficient DTPA was added to chelate the extracellular Mn²⁺. When Mn²⁺ was removed by addition of DTPA before stimulation with fMLP, no subsequent fMLP-stimulated quench was observed.

Fig. 4 compares the effects of Mn²⁺ and Ni²⁺, alone and in combination, on fura-2 fluorescence at 340 and 360 nm excitation. The top panel shows the fMLP-stimulated Mn²⁺ entry into neutrophils. Fura-2 fluorescence is also quenched upon binding Ni²⁺, and the middle panel of Fig. 4 shows the effect of adding Ni²⁺ (5 mM) before fMLP. No appreciable change in fluorescence at 360 nm was seen, which shows that fMLP is unable to stimulate entry of Ni²⁺ into neutrophils. Addition of ionomycin (2 μM) then caused a slow quenching of fluorescence presumably due to a slow translocation of Ni²⁺; complete quenching often required permeabilization of the cells with digitonin (50 μM). The lower panel of Fig. 4 shows that Ni²⁺ (5 mM) blocked the fMLP-stimulated entry of Mn²⁺ into neutrophils. No change in fluorescence at 360 nm was seen when fMLP (10 nM) was added in the presence of both Ni²⁺ (5 mM) and Mn²⁺ (100 μM); the fluorescence was only quenched upon addition of ionomycin (2 μM).

Fig. 5 shows the effect of adding Mn²⁺ (100 μM) at various times after fMLP (10 nM). The top trace in each panel shows a single 340 nm trace reporting changes in [Ca²⁺]. The lower
FIG. 6. Effect of Mn$^{2+}$ on fura-2 fluorescence of neutrophils stimulated with fMLP, PAF, and LTB$_4$. Fluorescence was monitored at 340 nm (solid lines) and 360 nm (broken lines) excitation. The arrows indicate the times of addition of MnCl$_2$ (Mn, 100 µM), fMLP (10 nM), PAF (10 nM), LTB$_4$ (10 nM), and ionomycin (iono, 2 µM).

FIG. 7. Effects of high [K$^+$] and BAY-R-5417 on [Ca$^{2+}$], and on Mn$^{2+}$ influx in neutrophils. [Ca$^{2+}$], was calculated from the ratio of fluorescence at 340 and 380 nm excitation. The experiments with Mn$^{2+}$ show raw fluorescence at 340 nm (solid lines) and 360 nm (broken lines) excitation. CaCl$_2$ (Ca, 1 mM), MnCl$_2$ (Mn, 100 µM), KCl (K, 50 mM), and BAY-R-5417 (BAYR, 100 nM) were added at the times shown.

part of each panel shows a number of superimposed 360 nm traces. The rate of fMLP-stimulated quenching of the 360 nm response appears similar when Mn$^{2+}$ was added 10 s before fMLP or at various times (10 s to 5 min) after fMLP, although the initial rate of quenching appears slower during the first 20 s of stimulation. This result shows that the rate of fMLP-stimulated Mn$^{2+}$ entry is independent of the [Ca$^{2+}$] over the range evoked by stimulation with fMLP.

Fig. 6 shows that PAF (10 nM) and LTB$_4$ (10 nM), in addition to fMLP (10 nM), can stimulate Mn$^{2+}$ entry. In each case, Mn$^{2+}$ entry was stimulated by the agonists when Mn$^{2+}$ was added before the agonist, or in the continued presence of agonist after [Ca$^{2+}$]; had returned to near base line. This stimulated Mn$^{2+}$ entry was also blocked by 5 mM Ni$^{2+}$ (results not shown). The agonist-stimulated entry of Mn$^{2+}$ after [Ca$^{2+}$], has returned to base line parallels the results shown in Fig. 1 where extracellular Ca$^{2+}$ was added back at a similar time after agonist; LTB$_4$ was less effective than fMLP or PAF at stimulating Mn$^{2+}$ influx or a further rise in [Ca$^{2+}$], at this time.

Fig. 7 shows that depolarization of cells with 50 mM K$^+$ and/or addition of the enantiomerically pure L-type Ca$^{2+}$ channel agonist, BAY-R-5417 (100 nM), had no effect on Mn$^{2+}$ influx, again confirming the absence of voltage-dependent Ca$^{2+}$ channels in neutrophils. The lack of effect of high [K$^+$] or BAY-R-5417 (100 nM) on [Ca$^{2+}$], is also shown in Fig. 7. However, addition of BAY-R-5417 to cells already depolarized by high [K$^+$] caused a very small rise in [Ca$^{2+}$], from 80 to 120 nM.

DISCUSSION

We have shown that three different agonists, fMLP, PAF, and LTB$_4$, cause increases in [Ca$^{2+}$], in fura-2-loaded human neutrophils. There have been a number of previous reports of agonist-stimulated rises in [Ca$^{2+}$], in quin2-loaded neutrophils (1–6). The fluorescence intensity of quin2 is lower than fura-2, so higher dye concentrations are required, which results in buffering and slowing of changes in [Ca$^{2+}$]. Previous studies have generally shown slower changes in [Ca$^{2+}$], than we report here. The fluorescence levels measured with quin2 are considerably lower than those with fura-2, so agonist-stimulated changes in autofluorescence due to NADPH oxidation (associated with O$_2$ formation) will have significant effects on the quin2 signal (1). Since the fluorescence yield of fura-2 is higher, agonist-stimulated changes in autofluorescence become insignificant relative to the fura-2 fluorescence.

In the absence of extracellular Ca$^{2+}$, the transient rise in...
have been obtained with a number of cell-types, including mediated Caz+ entry mechanism. Such results and conclusions on extracellular Ca2+ are not due to Ca2+ entry through L-type voltage-dependent Ca2+ channels or to a Ca'+-activated times after fMLP, when [Ca'+]i was at different levels, the Ca2+ entry, the alternative is an, as yet undefined, receptor-stimulated by fMLP. cells (19, 20).

rate of stimulated quenching (Mn2+ entry) remained constant already been reported for indo-1-loaded neutrophils stimulated with fMLP (7). This rise in [Ca2+]i cannot be due to stimulated Ca2+ entry. Similar results have been discharged and [Ca'+]i had returned to base line, Mn2+ base line, and addition of extracellular Ca2+ in the absence of agonist has very little effect on [Ca'+]i (Fig. 1). Analogously, when Mn2+ was added at various agonist-stimulated rise in [Ca2+],. Thus, following agonist-stimulation, Ca'+ and Mn'+ appear to mobilization of Ca2+ from internal stores by inositol (1,4,5)-trisphosphate. Both fMLP and LTB4 have been shown to activate phospholipase C to increase the formation of inositol (1,4,5)-trisphosphate in human neutrophils (3, 13, 14). The additional rise in [Ca2+]; that was seen in the presence of external Ca2+ was inhibited by Ni2+ (a Ca2+-channel antagonist), a result consistent with this part of the response being due to stimulated Ca2+ entry (see also Refs. 2 and 6). We have confirmed this by using Mn2+, which we know can only appear in the cytosol as a consequence of entry from outside the cell. The correlation between stimulated Mn2+ entry and the external Ca2+-sensitive component of the rise in [Ca2+]; is strengthened by the observation that the Mn2+ entry is also blocked by Ni2+ (5 mM). Anderson et al. (2) have shown that the external Ca2+-sensitive component is blocked by the inorganic Ca2+ antagonists, Co2+ and La3+, while the organic Ca2+ antagonists, which are known to block L-type voltage-dependent Ca2+ channels, are relatively ineffective (partial inhibition is observed at very high concentrations). This is consistent with our observations that (a) depolarization with high [K+] has no effect on [Ca2+]; and (b) the enantiotERICALLY pure L-type Ca2+ channel agonist, BAY-R-5417, at a concentration well in excess of that required to open L-type Ca2+ channels was ineffective in elevating [Ca2+]i when added alone, and had very little effect when added to K+-depolarized cells. Mn2+ entry, like Ca2+ entry, was not stimulated by high [K+] or by BAY-R-5417, further strengthening the correlation between agonist-stimulated Mn2+ entry and the external Ca2+-sensitive component of the agonist-stimulated rise in [Ca2+];. Thus, following agonist-stimulation, Ca2+ and Mn2+ appear to enter neutrophils by the same mechanism, which is not a L-type voltage-dependent channel.

Patch-clamp studies of human neutrophil membranes have suggested that a [Ca2+]i-activated Ca2+ entry mechanism may be involved (5). However, our results argue against such a mechanism. When neutrophils are stimulated with agonist in the absence of external Ca2+, [Ca2+]i rises transiently and then declines to base line, presumably due to discharge followed by depletion of the internal store of Ca2+. If extracellular Ca2+ is then added, in the continued presence of agonist, after discharge of the intracellular store when [Ca2+]; has returned to base line, a stimulated increase in [Ca2+]; is observed; this is presumably due to stimulated Ca2+ entry. Similar results have already been reported for indo-1-loaded neutrophils stimulated with fMLP (7). This rise in [Ca2+]; cannot be due to [Ca2+]i-activated Ca2+ entry because [Ca2+];, has returned to base line, and addition of extracellular Ca2+ in the absence of agonist has very little effect on [Ca2+]; (Fig. 1). Analogously, when Mn2+ was added in the continued presence of agonist (fMLP, PAF, and LTB4) after the internal Ca2+ store had been discharged and [Ca2+];, had returned to base line, Mn2+ entry was still stimulated. When Mn2+ was added at various times after fMLP, when [Ca2+]; was at different levels, the rate of stimulated quenching (Mn2+ entry) remained constant after the first 20 s, which suggests that the rate of stimulated divalent cation entry is independent of [Ca2+]; over the range stimulated by fMLP.

Since agonist-stimulated rises in [Ca2+]; that are dependent on extracellular Ca2+ are not due to Ca2+ entry through L-type voltage-dependent Ca2+ channels or to a Ca2+-activated Ca2+ entry, the alternative is an, as yet undefined, receptor-mediated Ca2+ entry mechanism. Such results and conclusions have been obtained with a number of cell-types, including endothelial cells (15, 16), platelets (17, 18), and parotid acinar cells (19, 20).

Various agonists have been found to stimulate Mn2+ entry into human platelets (9, 21) and human endothelial cells (22). However, agonists were unable to stimulate Mn2+ entry into rat parotid acinar cells (21), although there is evidence for a stimulated Ca2+ influx (19, 20). The relative timings of stimulation of internal release and influx vary according to cell type and agonist. In ADP-stimulated platelets, Mn2+ or Ca2+ influx was found to precede release of Ca2+ from intracellular stores (23, 24), and we found the converse in thrombin-stimulated endothelial cells, where internal release preceded Mn2+ influx.2 In contrast to both of these observations, we were unable to separate, in neutrophils, the times for release of internal Ca2+ and influx of Mn2+, suggesting that they both occur simultaneously. This result is consistent with a report on quin2-loaded neutrophils, where the time delay before [Ca2+]; rises in response to fMLP is the same in the presence or absence of external Ca2+ (5). Similarly, in parotid cells the lag times for an agonist-stimulated rise in [Ca2+]; are no different in the presence or absence of external Ca2+ (25). These detailed studies of divalent cation fluxes, using fura-2 and Mn2+, not only clarify the contribution of Ca2+ entry to agonist-stimulated changes in [Ca2+];, but are also useful in exploring the diversity of Ca2+ (and Mn2+) entry mechanisms in different cell types.

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REFERENCES

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