Agonist-induced Ca^{2+} influx in human neutrophils is secondary to the emptying of intracellular calcium stores

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Emptying of the intracellular calcium stores of human neutrophils, by prolonged incubation in Ca^{2+} -free medium, by treatment with low concentrations of the Ca^{2+} ionophore ionomycin, or by activation with cell agonists, increased the plasma-membrane permeability to Ca^{2+} and Mn^{2+} . The chemotactic peptide formylmethionyl-leucyl-phenylalanine and the natural agonists platelet-activating factor and leukotriene B_4 released different amounts of calcium from the stores and induced Ca^{2+} (Mn^{2+}) uptake, the rate of which correlated inversely with the amount of calcium left in the stores. The increased Mn^{2+} uptake induced by these agonists was persistent in cells incubated in Ca^{2+} -free medium, but returned to basal levels in cells incubated in Ca^{2+} -containing medium, with the same time course as the refilling of the calcium stores. The calcium-stores-regulated Mn^{2+} influx, including that induced by agonists, was prevented by cytochrome *P*-450 inhibitors. We propose that agonist-induced Ca^{2+} (Mn^{2+}) influx in human neutrophils is secondary to the emptying of the intracellular stores which, in turn, activates plasma-membrane Ca^{2+} channels by a mechanism involving microsomal cytochrome *P*-450, similar to that described previously in thymocytes [Alvarez, Montero & Garcia-Sancho (1991) *Biochem. J.* 274, 193–197].

INTRODUCTION

Stimulation of human neutrophils with several agonists induces an increase in the cytoplasmic free Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$) both by releasing Ca^{2+} from the intracellular calcium stores and by increasing the plasma-membrane permeability to Ca^{2+} [1–6]. There is strong evidence that, under physiological conditions, most of the intracellular Ca^{2+} is located in a non-mitochondrial pool and that $\text{Ins}(1,4,5)\text{P}_3$ mediates the agonist-induced release of Ca^{2+} from this pool [7,8]. On the contrary, the mechanism of the agonist-induced increase in the plasma-membrane Ca^{2+} -permeability is unclear. It has been proposed, in the first place, that increased Ca^{2+} influx in human neutrophils takes place through non-selective cation channels activated by the initial rise in $[\text{Ca}^{2+}]_i$ [3]. A second hypothesis proposes the involvement of $\text{Ins}(1,3,4,5)\text{P}_4$ or a concerted action of both $\text{Ins}(1,3,4,5)\text{P}_4$ and $\text{Ins}(1,4,5)\text{P}_3$ [9–11]. In the third place, it has been suggested in several cell lines, including neutrophils, that the decrease in calcium content of the intracellular calcium stores can be sensed and produces by itself an increase in the plasma-membrane Ca^{2+} -permeability [12–19]. We have shown previously that, in the absence of cell agonists, manoeuvres that empty the Ca^{2+} stores increase the plasma-membrane Ca^{2+} -permeability in rat thymocytes 10–20-fold [18]. On the basis of the inhibition pattern of this effect, we have proposed that cytochrome *P*-450 may be the link between the state of filling of the calcium stores and the plasma-membrane Ca^{2+} -permeability [19].

We have studied here the effects of emptying the intracellular calcium stores on the plasma-membrane Ca^{2+} -permeability of human neutrophils. Plasma-membrane Ca^{2+} influx was monitored in fura-2-loaded neutrophils exposed to either Ca^{2+} or Mn^{2+} , a Ca^{2+} surrogate for calcium channels [18–21]. The calcium stores were emptied by prolonged incubation in Ca^{2+} -free medium, by treatment with low concentrations of the Ca^{2+} ionophore ionomycin, or by the use of agonists such as formylmethionyl-leucyl-phenylalanine (fMLP), platelet-

activating factor (PAF) and leukotriene B_4 (LTB_4). In all the cases the emptying of the intracellular calcium stores activated Ca^{2+} (Mn^{2+}) influx. The entry of Ca^{2+} (Mn^{2+}) induced by fMLP, PAF or LTB_4 was not the result of a direct effect of the agonists on the plasma membrane, but was secondary to the emptying of the intracellular stores. The entry of Ca^{2+} induced by emptying the intracellular calcium stores was, in all the cases, prevented by cytochrome *P*-450 inhibitors.

METHODS

Human neutrophils were obtained from blood of normal volunteers, which was mixed with 6 vol. of acid citrate–dextrose. Dextran (T500; Pharmacia) was then added to give a final concentration of 1.3%. After 45 min at room temperature, the upper phase containing no red cells was removed and centrifuged (300 *g*, 10 min). The cell pellet was resuspended, layered on a Ficoll gradient (lymphocyte separation medium; Flow Laboratories, Irvine, Scotland, U.K.), and centrifuged for 20 min at 400 *g*. The cells were resuspended, and contaminating red cells were disrupted by hypo-osmotic lysis [22]. Neutrophils were finally suspended at 1–2% cytocrit in standard medium containing (in mM): NaCl, 145; KCl, 5; MgCl_2 , 1; CaCl_2 , 0.2; sodium Hepes, 10; glucose, 10; pH 7.4.

Neutrophils were loaded with fura-2 by incubation with 2–4 μM -fura-2/AM for 30 min at room temperature in standard medium. Cells were then washed twice and resuspended at 0.5–1% cytocrit in standard medium. In some experiments cells were loaded with fura-2 and resuspended in nominally Ca^{2+} -free medium in order to deplete the intracellular Ca^{2+} stores. $[\text{Ca}^{2+}]_i$ was measured at 37 °C under magnetic stirring in a fluorescence spectrophotometer constructed by Cairn Research Ltd. (Newnham, Sittingbourne, Kent, U.K.), which allows simultaneous excitation of fluorescence at 340, 360 and 380 nm. Fluorescence emission was set at 530 nm. Fluorescence readings were integrated at 1 s intervals, and $[\text{Ca}^{2+}]_i$ was calculated from

Abbreviations used: $[\text{Ca}^{2+}]_i$, cytoplasmic free calcium concentration; ETYA, 5,8,11,14-eicosatetraynoic acid; NDGA, nordihydroguaiaretic acid; PAF, platelet-activating factor (1-*O*-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine); LTB_4 , leukotriene B_4 ; fMLP, formylmethionyl-leucyl-phenylalanine.

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Fig. 1. Refilling of intracellular Ca^{2+} stores after different periods of incubation with 1 mM- Ca^{2+} in Ca^{2+} -depleted human neutrophils (a) and comparison of the time course of the refilling of the stores and the increase in $[Ca^{2+}]_i$ after Ca^{2+} addition (b)

Cells were depleted of Ca^{2+} by incubation in nominally Ca^{2+} -free medium for 3 h at room temperature. In (a) these cells were incubated at 37 °C with 1 mM- Ca^{2+} for different time periods (A, none; B, 15 s; C, 30 s; D, 1 min; E, 2 min; F, 5 min). Then 5 mM-EGTA and 100 nM-ionomycin were added ($t = 0$ in the Figure). In (b) the time course of $[Ca^{2+}]_i$ increase (continuous trace, scale at left) and the filling of the intracellular stores (● and dotted lines, scale at right) after Ca^{2+} addition are shown. Store filling was estimated from the increase of $[Ca^{2+}]_i$ over the basal level at the peak after ionomycin addition in the experiments in (a).

Fig. 2. Uptake of Mn^{2+} by fura-2-loaded Ca^{2+} -depleted neutrophils

Depletion of the Ca^{2+} stores was as in Fig. 1. In curve A, 1 mM- $MnCl_2$ was added at zero time. In curve B, 1 mM- $MnCl_2$ and 1 mM- $CaCl_2$ were added simultaneously at $t = 0$. In curve C, 1 mM- $MnCl_2$ was added at $t = 0$ to cells first incubated for 2 min with 4 μ M-econazole. In curve D, 1 mM- $MnCl_2$ was added at $t = 0$ to cells first incubated with 1 mM- $CaCl_2$ for 2 min. Fluorescent emission excited at 360 nm (F_{360}) is shown on the ordinate. The values are normalized to 100 % just after Mn^{2+} addition ($t = 0$ in the Figure).

Fig. 3. Delayed acceleration of Mn^{2+} uptake by 1 nM-ionomycin in human neutrophils with filled Ca^{2+} stores

$MnCl_2$ (1 mM) was added to cells incubated in standard medium (containing 0.2 mM- $CaCl_2$). In curve A, 1 nM-ionomycin (Iono) was added at $t = 0$. In curve B, 1 nM-ionomycin was added at $t = 0$ to cells first incubated with 4 μ M-econazole for 2 min. In curve C, 5 mM- $NiCl_2$ and 1 nM-ionomycin were added at $t = 0$. The insert shows the $[Ca^{2+}]_i$, calculated from the ratio of fluorescences excited at 340 and 380 nm, obtained from the same experiment as curve A.

the ratio of the fluorescences excited at 340 and 380 nm [23]. Mn^{2+} uptake was monitored simultaneously by the quenching of the fluorescence excited at 360 nm, which is insensitive to variations in $[Ca^{2+}]_i$ [18–21].

Fura-2/AM was obtained from Molecular Probes, Eugene, OR, U.S.A. PAF and ionomycin were purchased from Calbiochem. Econazole, miconazole, clotrimazole, ketoconazole, fMLP and nordihydroguaiaretic acid (NDGA) were obtained from Sigma (London) Chemical Co. LTB_4 and eicosatetraynoic acid (ETYA) were purchased from Biomol Research Laboratories, Plymouth Meeting, PA, U.S.A. Other chemicals were obtained either from Sigma (London) or Merck (Darmstadt, Germany).

RESULTS

Human neutrophils incubated for about 3 h at room temperature in a nominally Ca^{2+} -free medium have a low $[Ca^{2+}]_i$ (about 30 nM). The calcium content of the intracellular stores can be assessed by releasing it to the cytoplasm with ionomycin in cells suspended in Ca^{2+} -free (EGTA-containing) medium. Under these conditions, the magnitude of the $[Ca^{2+}]_i$ peak after ionomycin reflects the degree of filling of the intracellular stores [18,19]. Fig. 1(a) shows the results of such a manoeuvre in cells that had been first incubated in Ca^{2+} -free medium for 3 h (trace A) and in the same cells after different periods (15 s to 5 min) of incubation with 1 mM- Ca^{2+} at 37 °C (traces B–F). The increase in the $[Ca^{2+}]_i$ peak after ionomycin is evidence of the refilling of the intracellular stores on incubation in Ca^{2+} -containing medium. In Fig. 1(b) the increase in $[Ca^{2+}]_i$ at the peak after ionomycin is plotted as a function of the time of incubation with 1 mM- Ca^{2+} (●, dotted lines). This time course shows that half-maximal refilling requires about 30 s of incubation in Ca^{2+} -containing medium. We have superimposed the time course of $[Ca^{2+}]_i$ changes on addition of extracellular Ca^{2+} (1 mM) to the same batch of cells. On addition of Ca^{2+} , $[Ca^{2+}]_i$ increases from the low values found in Ca^{2+} -depleted cells (30 nM) to 60–70 nM with a half-time of 9 ± 1 s (mean $\pm$ S.E.M.; $n = 4$), indicating that the increase in $[Ca^{2+}]_i$ observed in Ca^{2+} -depleted cells on addition of extracellular Ca^{2+} precedes the refilling of the calcium stores.

Next we compared the Ca^{2+} influx through the plasma membrane in cells with either empty or refilled calcium stores.

Fig. 4. Effects of agonist-induced emptying of the intracellular stores on Ca^{2+} uptake by human neutrophils

(a) Release of Ca^{2+} from intracellular stores by 1 nM-ionomycin (Iono), PAF, fMLP and LTB_4 . First, 1 mM-EGTA (first arrow) was added to cells suspended in standard medium (containing 0.2 mM- CaCl_2). Then, from top to bottom, 1 nM-ionomycin, 10 ng of PAF/ml, 10 nM-fMLP, or 10 nM- LTB_4 was added. Then 3 min later, 100 nM-ionomycin was added to release the residual Ca^{2+} present in the stores. In the bottom trace the first (agonist) addition was omitted. (b) Increase in $[\text{Ca}^{2+}]_i$ induced by re-addition of external Ca^{2+} to cells first treated with 1 nM-ionomycin, PAF, fMLP or LTB_4 . The experiments were performed as in (a), except that 2 mM- CaCl_2 was added instead of 100 nM-ionomycin after the agonists. In the top panel, a trace in which EGTA was replaced by 5 mM- NiCl_2 is included.

Mn^{2+} , a Ca^{2+} surrogate for calcium channels, is more suitable than Ca^{2+} itself for influx measurements, since, not being a substrate for the Ca^{2+} pump, the interfering back-flux through this transport system is avoided. Fig. 2 shows that Mn^{2+} influx in cells with emptied calcium stores (trace A) was about 10 times faster than in cells with refilled stores (trace D, the same cells incubated with 1 mM- Ca^{2+} for 2 min before addition of Mn^{2+}). It could be argued that the effect was due to interference of the extracellular Ca^{2+} with the uptake of Mn^{2+} . Trace B in Fig. 2 shows that this was not the case, since, when Ca^{2+} and Mn^{2+} were added simultaneously, the initial rate of Mn^{2+} uptake was high (compare the first 15–30 s of traces A and B). The delayed inhibition seen later fits nicely with the time course for the filling of the intracellular stores described above (Fig. 1). Fig. 2 also shows that econazole, a cytochrome *P*-450 inhibitor that antagonizes store-regulated Mn^{2+} influx in thymocytes [19], also prevents Mn^{2+} uptake by neutrophils (trace C).

Depletion of the intracellular Ca^{2+} stores can also be achieved by addition of the calcium ionophore ionomycin to cells with filled Ca^{2+} stores [19]. Fig. 3 shows that addition of 1 nM-ionomycin to human neutrophils suspended in medium containing 1 mM- Mn^{2+} induces an uptake of Mn^{2+} which is delayed by about 15 s with respect to the addition of the ionophore. The insert shows that this delay corresponds to the time necessary for $[\text{Ca}^{2+}]_i$ to rise. Since the rise in $[\text{Ca}^{2+}]_i$ is due to the release of calcium from the intracellular stores effected by the ionophore, its time course also reflects that of the emptying of the calcium stores. The increase in Mn^{2+} influx then coincides in time with the emptying of the calcium stores. It is clear that the influx of Mn^{2+} is not mediated directly by ionomycin, since: (i) it is delayed; (ii) it is inhibited by Ni^{2+} (curve C), which does not prevent transport through ionomycin; (iii) it is prevented by the cytochrome *P*-450 inhibitor econazole (curve B). Ionomycin is able to transport Mn^{2+} , but the low concentration used in the experiments of Fig.

Fig. 5. Uptake of Mn^{2+} induced by treatment with 1 nM-ionomycin (Iono), PAF, fMLP or LTB_4 .

The experiments were performed as in Fig. 4(b), except that 2 mM- MnCl_2 was added instead of 2 mM- CaCl_2 and fluorescence excited at 360 nm (F_{360}) was recorded. Fluorescence was normalized to 100% just after addition of MnCl_2 ($t = 0$ in the Figure). Records are from the same batch of cells as in Fig. 4.

Fig. 6. Correlation between the calcium content of the intracellular stores and the uptake of Ca^{2+} (a) or of Mn^{2+} (b).

The calcium contents of the intracellular stores were estimated from the increase in $[\text{Ca}^{2+}]_i$ (peak $[\text{Ca}^{2+}]_i$ – basal $[\text{Ca}^{2+}]_i$) after addition of 100 nM-ionomycin in two series of experiments similar to those shown in Fig. 4(a). The Ca^{2+} uptake was estimated from the increase in $[\text{Ca}^{2+}]_i$ (peak $[\text{Ca}^{2+}]_i$ – basal $[\text{Ca}^{2+}]_i$) obtained after addition of 2 mM- CaCl_2 in two series of experiments similar to those shown in Fig. 4(b). The Mn^{2+} uptake was estimated from the rate of fluorescence quenching obtained after addition of 2 mM- MnCl_2 in two series of experiments similar to those shown in Fig. 5. Controls (○, ●); LTB_4 (△, ▲); fMLP (□, ■); PAF (▽, ▼); 1 nM-ionomycin (◆, ◇).

3 is not enough to produce by itself a detectable increase in Mn^{2+} influx, even though it is able to release Ca^{2+} effectively from the intracellular stores. We have previously reported this apparent selectivity of calcium ionophores for endomembranes over plasma membranes [24]. In any case, Figs. 1–3 clearly indicate the existence of a mechanism which regulates the plasma-

membrane Ca^{2+} (Mn^{2+})-permeability according to the filling state of the intracellular calcium stores in human neutrophils. The characteristics of this mechanism are very similar to that reported previously in rat thymocytes [18,19].

The following experiments explore the possible contribution of the store-regulated plasma-membrane Ca^{2+} pathway to the effects of the chemotactic peptide fMLP and the physiological agonists PAF and LTB_4 . Figs. 4–6 document the correlation between the ability of these agonists to release Ca^{2+} from the intracellular stores and the Ca^{2+} or Mn^{2+} influx that they induce. In Fig. 4(a) the extent of the release of Ca^{2+} from the intracellular stores by different agonists is compared. Ionomycin (1 nM), PAF (10 ng/ml), fMLP (10 nM) or LTB_4 (10 nM) was added to cells suspended in Ca^{2+} -free (EGTA-containing) medium, and the peak resulting from the release of Ca^{2+} was recorded. Then 3 min later 100 nM-ionomycin was added in order to release the residual Ca^{2+} still contained within the stores, and the height of the $[\text{Ca}^{2+}]_i$ peak obtained was used to estimate the content of Ca^{2+} in the stores. This method has the shortcoming that the height of the $[\text{Ca}^{2+}]_i$ peak depends both on the release of Ca^{2+} from the stores and on the efflux of Ca^{2+} to the extracellular space. Therefore, we should have in mind that, although higher $[\text{Ca}^{2+}]_i$ peaks should correspond to higher Ca^{2+} contents in the stores, the relationship between both parameters may not be linear. In the bottom panel of Fig. 4, the first (agonist) addition was omitted in order to have a control in which 100 nM-ionomycin was releasing the full store contents. Note that, although all three agonists produced a $[\text{Ca}^{2+}]_i$ peak of similar height, they emptied the intracellular stores to different extents, as revealed by the peak obtained after the addition of the high (100 nM) concentration of ionomycin. According to this last criterion (the amount of calcium left in the stores), the potency to release Ca^{2+} increased from bottom to top of Fig. 4 in the order: control < LTB_4 < fMLP < PAF < 1 nM-ionomycin. The area below the $[\text{Ca}^{2+}]_i$ peak induced by every agonist also increased in this order, suggesting that this may be a better parameter than the height of the peak in order to estimate the amount of Ca^{2+} released. Fig. 4(b) shows that re-addition of Ca^{2+} to the extracellular medium 3 min after the agonist produced an increase in $[\text{Ca}^{2+}]_i$ whose magnitude correlated well with the extent of the agonist-induced emptying of the intracellular stores [estimated either from the residual Ca^{2+} revealed by the addition of the high concentration of ionomycin in Fig. 4(a) or from the area under the $[\text{Ca}^{2+}]_i$ peak obtained after agonist addition]. In agreement with the above, Fig. 5 shows that addition of Mn^{2+} 3 min after every agonist also initiated a Mn^{2+} uptake whose rate correlated with the extent of the emptying of the intracellular stores (1 nM-ionomycin > PAF > fMLP > LTB_4 > control). In all the cases the acceleration of Mn^{2+} uptake was prevented by Ni^{2+} (only shown for ionomycin in Fig. 5). Fig. 6 summarizes the correlation between the degree of filling of the intracellular stores in neutrophils treated with different agonists and the uptake of Ca^{2+} (Fig. 6a) or of Mn^{2+} (Fig. 6b). The curves obtained were very similar to that reported previously for rat thymocytes, in which different degrees of store-filling were obtained by incubation of Ca^{2+} -depleted cells with different Ca^{2+} concentrations, i.e. in the absence of agonists [19].

It has been reported recently that the rate of Mn^{2+} uptake induced by fMLP in neutrophils incubated in Ca^{2+} -free medium remains constant over a period of at least 7 min after addition of the agonist and that it is independent of $[\text{Ca}^{2+}]_i$ [6]. Fig. 7(a) shows a very similar result obtained with PAF. Fig. 7(b) shows that, when the same experiment was performed in Ca^{2+} -containing medium, the rate of Mn^{2+} uptake obtained just after addition of PAF was the same as in Ca^{2+} -free medium, but it then decreased with time up to a value similar to that before PAF addition. Similar results were obtained with fMLP as the agonist

Fig. 7. Effect of PAF on $[\text{Ca}^{2+}]_i$ and on the uptake of Mn^{2+} in the presence and in the absence of external Ca^{2+}

In (a) 1 mM-EGTA was added to cells suspended in standard medium, and then 10 ng of PAF/ml was added at $t = 0$. The upper trace shows the record of $[\text{Ca}^{2+}]_i$. The lower traces show Mn^{2+} -uptake records obtained by addition of Mn^{2+} either before PAF or 0.5, 1, 2 or 5 min after addition of PAF. The arrows with asterisks indicate the times at which 2 mM- Mn^{2+} was added. In (b), 0.8 mM- CaCl_2 was added instead of EGTA and the experiment was done in the same way as in (a). The arrows with asterisks indicate the points at which 1 mM- Mn^{2+} was added.

Fig. 8. Effect of econazole on the uptake of Mn^{2+} induced by PAF, fMLP and LTB_4

MnCl_2 (1 mM) was added to cells suspended in standard medium. At zero time, 10 ng of PAF/ml (a), 10 nM-fMLP (b) or 10 nM- LTB_4 (c) was added. Fluorescence was recorded simultaneously at 340, 360 and 380 nm excitation. The upper curves represent the $[\text{Ca}^{2+}]_i$ obtained from the ratio of fluorescence excited at 340 and 380 nm. The lower curves represent the Mn^{2+} uptakes obtained from the quenching of fluorescence excited at 360 nm. The continuous line is the control, and the dotted line corresponds to cells first incubated with 2 μM -econazole for 2 min.

(not shown). The results of Fig. 7 are consistent both with the effect of PAF on the plasma membrane being secondary to the emptying of the intracellular calcium stores and with the kinetics of store refilling documented in Fig. 1. The transient effect of PAF on the stores could only be evidenced in the experiments of

Fig. 7 when extracellular Ca^{2+} is available for refilling the calcium stores. When the stores are filled again, the plasma-membrane permeability to Mn^{2+} returns to basal levels. The different time courses of inactivation of Mn^{2+} influx obtained in Ca^{2+} -free and in Ca^{2+} -containing medium would be hard to

Table 1. K_i values of several drugs for inhibition of the delayed Mn^{2+} uptake induced by 1 nM-ionomycin in human neutrophils

Experiments were performed as in Fig. 3.

Inhibitor	K_i (μM)
Econazole	1
Miconazole	1
Clotrimazole	5
Ketoconazole	25
NDGA	8
ETYA	10

explain on the basis of either a direct action of PAF on plasma-membrane channels or a second messenger whose synthesis is directly induced by PAF.

The plasma-membrane pathway activated by emptying the intracellular Ca^{2+} stores that we have described previously in thymocytes could not be open in the presence of cytochrome *P*-450 inhibitors [19]. We have tested the effects of several such inhibitors on the agonist-induced Mn^{2+} uptake by human neutrophils. Fig. 8 shows that 2 μM -econazole prevented the uptake of Mn^{2+} induced by PAF, fMLP and LTB_4 (lower traces in panels). Note that the $[Ca^{2+}]_i$ peak, which always preceded by 15–30 s the uptake of Mn^{2+} , was not modified by econazole (upper traces in panels in Fig. 8). The inorganic channel blocker Ni^{2+} had the same effects as econazole, i.e. prevented Mn^{2+} uptake without modifying the $[Ca^{2+}]_i$ peak (results not shown). The same results were obtained with other known inhibitors of cytochrome *P*-450. Table 1 summarizes the K_i values obtained for inhibition of the Mn^{2+} uptake induced by 1 nM-ionomycin.

DISCUSSION

This paper analyses the kinetics of calcium redistribution between the cytoplasm and the intracellular calcium stores in human neutrophils, the effects of the degree of filling of the stores on the plasma-membrane permeability to Ca^{2+} , and the possible involvement of the last mechanism in the Ca^{2+} influx induced by several agonists. Once the intracellular stores have been depleted of calcium by incubation in Ca^{2+} -free medium, addition of Ca^{2+} to the extracellular medium produces a $[Ca^{2+}]_i$ rise ($t_1 = 9$ s), followed by refilling of the intracellular calcium stores ($t_2 = 30$ s). These findings are qualitatively similar to those reported previously for rat thymocytes and Ehrlich ascites-tumour cells, where the rise in $[Ca^{2+}]_i$ was also 3–10-fold faster than the refilling of the intracellular stores [18]. These results suggest that, at least in these three cell types, the filling of the stores takes place through the cytoplasm and not directly from the extracellular medium, as was recently proposed [25–27].

We have shown previously in rat thymocytes that the filling state of the intracellular calcium stores regulates the plasma-membrane influx of Mn^{2+} , used as a Ca^{2+} surrogate for Ca^{2+} channels [18,19]. We demonstrate here the operation of a similar mechanism in human neutrophils. When the stores are emptied by prolonged incubation in Ca^{2+} -free medium (Fig. 2), by ionomycin (Fig. 3) or by several cell agonists (Figs. 5, 7 and 8), the plasma-membrane permeability to Mn^{2+} increases. Additionally, the rate of both Mn^{2+} and Ca^{2+} uptake is correlated with the extent of store depletion (Fig. 6), and refilling of the stores restores a low plasma-membrane permeability to Mn^{2+} (Figs. 2 and 7). The internal consistency between Ca^{2+} and Mn^{2+} uptakes confirms that Mn^{2+} is a good tracer for Ca^{2+} influx in human neutrophils. As reported previously in thymocytes [18,19], the

high Mn^{2+} permeability can be seen in neutrophils with either low or high $[Ca^{2+}]_i$ (compare Figs. 2, 3 and 7). This excludes the possibility that a $[Ca^{2+}]_i$ -activated non-specific cation channel reported previously ([3]; see also [11]) was the main pathway for Mn^{2+} influx in our experiments.

The increased Mn^{2+} uptake observed in Ca^{2+} -depleted cells cannot be attributed to second messengers such as $Ins(1,4,5)P_3$ or $Ins(1,3,4,5)P_4$, and it is unlikely that such messengers could mediate the delayed Mn^{2+} influx induced by ionomycin (Fig. 3). Certainly, it has been shown that fMLP induces release of inositol phosphates in human neutrophils [28]. Moreover, in the closely related differentiated HL60 cell, it has also been shown that both fMLP and LTB_4 induce release of inositol phosphates [29]. However, if these messengers had to mediate the effect on the plasma membrane, it would be hard to explain the different time courses of inactivation of agonist-induced Mn^{2+} influx observed in Ca^{2+} -containing and in Ca^{2+} -free medium (Fig. 7). On the other hand we have been able to show a time lag for activation of the plasma-membrane permeability which closely corresponds to the time required to empty the calcium stores (Figs. 3, 7 and 8). Finally, the acceleration of Mn^{2+} influx induced by any of the store-emptying manoeuvres, including agonists, was prevented by econazole and the other cytochrome *P*-450 inhibitors.

We have proposed that microsomal cytochrome *P*-450 could be the link between the plasma-membrane Ca^{2+} pathway and the calcium stores in rat thymocytes [19]. Imidazole antimycotics are considered to be specific inhibitors of cytochrome *P*-450 [30,31], and the lipooxygenase inhibitors NDGA and ETYA have also been found to antagonize this system [30]. The K_i values for inhibition of store-dependent Mn^{2+} influx for imidazole antimycotics, NDGA and ETYA found in neutrophils (Table 1) were similar to those previously reported in thymocytes [19], suggesting that the action mechanism may be the same. The *N*-substituted imidazole group is the key factor for the inhibitory interaction of imidazole antimycotics with the haem group of cytochrome *P*-450 [31–33]. It has been reported recently that compound SK&F 96365 also inhibits agonist-induced Mn^{2+} influx in neutrophils without affecting calcium release from the intracellular stores [34]. Since this compound contains a *N*-substituted imidazole group, we suggest that it could be acting by inhibiting cytochrome *P*-450.

Agonist-induced discharge of the intracellular Ca^{2+} stores is thought to be mediated by $Ins(1,4,5)P_3$ [7,8]. It has been reported in differentiated HL60 cells that fMLP generates more $Ins(1,4,5)P_3$ than does LTB_4 , even though both agonists raised $[Ca^{2+}]_i$ to similar peak levels [29]. Peak levels of the same height were also found in a recent study in human neutrophils with LTB_4 , fMLP and PAF [6]. Our results agree with these findings, but a close inspection of the $[Ca^{2+}]_i$ peaks suggested that the amount of Ca^{2+} released can be better related to the area below the peak than to its height. Direct estimations of the amount of Ca^{2+} left in the stores by addition of ionomycin after the agonist confirmed this view (Fig. 4): fMLP, which produces more $Ins(1,4,5)P_3$ and during more time than LTB_4 [29], was in fact releasing more Ca^{2+} from the stores. This evidences the relevance of the Ca^{2+} -extruding mechanisms in limiting the height of the $[Ca^{2+}]_i$ peaks.

The final outcome of this work is that neutrophil agonists activate 'receptor-operated' plasma-membrane Ca^{2+} channels by a rather indirect mechanism, involving neither direct interaction with the channel nor even direct generation of a channel-activating second messenger. The mechanism itself has, however, plenty of physiological sense. It reinforces the $[Ca^{2+}]_i$ signal of the agonist and, at the same time, introduces a time lag between the ends of the effects on the stores and on the plasma membrane.

This lag allows refilling of the intracellular stores, with extracellular Ca^{2+} entering through the plasma membrane in order to get the cell ready to respond to a new stimulus. Current literature is full of examples of delayed agonist-induced Ca^{2+} influx, which is compatible with the mechanism proposed here for neutrophils. Direct evidence of such mechanism has been obtained for the Ca^{2+} (Mn^{2+}) influx induced by histamine in endothelial cells [16] and by thrombin in platelets (M. T. Alonso, unpublished work). Further work will be needed to assess how general is this store-regulated Ca^{2+} influx and how it relates or overlap with receptor-operated and second-messenger-operated Ca^{2+} channels.

This work was supported by a grant from DGICYT (PB89/0359). We thank Mr. Jesús Fernández for excellent technical assistance.

REFERENCES

- Pozzan, T., Lew, D. P., Wollheim, C. B. & Tsien, R. Y. (1983) *Science* **221**, 1413–1415
- Andersson, T., Dahlgren, C., Pozzan, T., Stendahl, O. & Lew, D. P. (1986) *Mol. Pharmacol.* **30**, 437–443
- Von Tscharner, V., Prod'homme, B., Gaggiolini, M. & Reuter, H. (1986) *Nature (London)* **324**, 369–372
- Nasmyth, P. E. & Grinstein, S. (1987) *FEBS Lett.* **221**, 95–100
- Korchak, H. M., Rutherford, L. E. & Weissmann, G. (1984) *J. Biol. Chem.* **259**, 4070–4075
- Merritt, J. E., Jacob, J. & Hallam, T. J. (1989) *J. Biol. Chem.* **264**, 1522–1527
- Prentki, M., Wollheim, C. B. & Lew, P. D. (1984) *J. Biol. Chem.* **259**, 13777–13782
- Hamachi, T., Hirata, M. & Koga, T. (1986) *Biochim. Biophys. Acta* **889**, 136–148
- Irvine, R. F. & Moor, R. M. (1986) *Biochem. J.* **240**, 917–920
- Irvine, R. F. & Moor, R. M. (1987) *Biochem. Biophys. Res. Commun.* **146**, 284–290
- Pittet, D., Lew, D. P., Mayr, G. W., Monod, A. & Schlegel, W. (1989) *J. Biol. Chem.* **264**, 7251–7261
- Pandolfi, S. J., Schoeffield, M. S., Fimmel, C. J. & Muallem, S. (1987) *J. Biol. Chem.* **262**, 16963–16968
- Takemura, H. & Putney, J. W., Jr. (1989) *Biochem. J.* **258**, 409–412
- Sage, S. O., Reast, R. & Rink, T. J. (1990) *Biochem. J.* **265**, 675–680
- Sage, S. O., Pintado, E., Mahaut-Smith, M. P. & Merritt, J. E. (1990) *Biochem. J.* **265**, 915–918
- Jacob, R. (1990) *J. Physiol. (London)* **421**, 55–77
- Takemura, H., Hughes, A. R., Thastrup, O. & Putney, J. W., Jr. (1989) *J. Biol. Chem.* **264**, 12266–12271
- Montero, M., Alvarez, J. & Garcia-Sancho, J. (1990) *Biochem. J.* **271**, 535–540
- Alvarez, J., Montero, M. & Garcia-Sancho, J. (1991) *Biochem. J.* **274**, 193–197
- Alonso, M. T., Sanchez, A. & Garcia-Sancho, J. (1989) *Biochem. Biophys. Res. Commun.* **162**, 24–29
- Garcia-Sancho, J., Alonso, M. T. & Sanchez, A. (1989) *Biochem. Soc. Trans.* **17**, 980–982
- Goldstein, I. M., Brai, M., Oster, A. G. & Weissmann, G. (1973) *J. Immunol.* **111**, 33–37
- Gryniewicz, G., Poenie, M. & Tsien, R. (1985) *J. Biol. Chem.* **260**, 3440–3450
- Artalejo, A. R. & Garcia-Sancho, J. (1988) *Biochim. Biophys. Acta* **941**, 48–54
- Merritt, J. E. & Rink, T. J. (1987) *J. Biol. Chem.* **262**, 17362–17369
- Putney, J. W., Jr (1986) *Cell Calcium* **7**, 1–12
- Ghosh, T. K., Mullaney, J. M., Tarazi, F. I. & Gill, D. L. (1989) *Nature (London)* **340**, 236–239
- Di Virgilio, F., Vicentini, L. M., Treves, S., Riz, G. & Pozzan, T. (1985) *Biochem. J.* **229**, 361–367
- Andersson, T., Schlegel, W., Monod, A., Krause, K.-H., Stendahl, O. & Lew, D. P. (1986) *Biochem. J.* **240**, 333–340
- Gapdevila, J., Gil, L., Orellana, M., Marnett, L. J., Mason, J. I., Yadagiri, P. & Falk, J. R. (1988) *Arch. Biochem. Biophys.* **261**, 257–263
- Rodrigues, A. D., Gibson, G. G., Ioannides, C. & Parke, D. V. (1987) *Biochem. Pharmacol.* **36**, 4277–4281
- Ballard, S. A., Lodola, A. & Tarbit, M. H. (1988) *Biochem. Pharmacol.* **37**, 4643–4651
- Yoshida, Y. & Aoyama, Y. (1987) *Biochem. Pharmacol.* **36**, 229–235
- Merritt, J. E., Armstrong, P., Benham, C. D., Hallam, T. J., Jacob, R., Jaxa-Chamiec, A., Leigh, B. K., McCarthy, S. A., Moores, K. E. & Rink, T. J. (1990) *Biochem. J.* **271**, 515–522

Received 27 December 1990/11 February 1991; accepted 1 March 1991