Biochem. J. (1992) 288, 519–525 (Printed in Great Britain)

Control of plasma-membrane Ca^{2+} entry by the intracellular Ca^{2+} stores

Kinetic evidence for a short-lived mediator

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We have studied the correlation between the degree of filling of the intracellular Ca^{2+} stores and the plasma-membrane permeability to Mn^{2+}, a Ca^{2+} surrogate for plasma-membrane Ca^{2+} channels, in human neutrophils loaded with fura-2. Refilling of the stores of cells previously depleted of Ca^{2+} decreased the entry of Mn^{2+}, but the magnitude of this effect depended on the refilling protocol. When refilling was allowed to proceed to steady-state levels by a 3 min incubation with different external Ca^{2+} concentrations (0.05–1 mM), almost complete inhibition of Mn^{2+} entry was observed at 40% of maximum refilling. In contrast, when different degrees of store refilling were attained by incubation with 1 mM-Ca^{2+} for short periods (10–40 s), inhibition of Mn^{2+} entry was smaller at comparable degrees of refilling. When quick refilling was allowed to proceed up to 40% (about 20 s at 37 °C) and then stopped at this level by removal of external Ca^{2+}, the rate of Mn^{2+} uptake was high just after refilling and then decreased with time within the next few seconds (half-times $\sim$ 7 s at 37 °C and $\sim$ 20 s at 25 °C). We have proposed previously that the Ca^{2+} stores, when emptied of Ca^{2+}, may generate a second messenger able to open the plasma-membrane Ca^{2+} channels by a mechanism involving cytochrome *P*-450. The results here are consistent with the existence of such a messenger and suggest that it is cleared from the cytoplasm with a half-time of about 7 s at 37 °C. In addition, inhibition of Mn^{2+} entry in cells with empty Ca^{2+} stores by cytochrome *P*-450 inhibitors showed a time lag consistent with the clearance kinetics proposed above.

INTRODUCTION

Many cell agonists induce in human neutrophils a biphasic increase in the cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$), which first involves release of Ca^{2+} from the intracellular stores to the cytosol, followed by activation of Ca^{2+} entry through the plasma membrane [1–6]. The role of Ins(1,4,5)P_3 as the second messenger for the release of Ca^{2+} from the intracellular stores is well established [7.8], but the mechanism of the increase of the plasma-membrane Ca^{2+} permeability is still unclear. The Ca^{2+} entry has been proposed to be controlled by a variety of mechanisms, which may be different depending on the cell type and the nature of the receptor [9–14]. It has been shown in several cell lines that Ca^{2+} entry is a consequence of the emptying of the intracellular Ca^{2+} stores induced by Ins(1,4,5)P_3 [13–24]. The 'capacitative model' [14,25,26] proposes that depletion of the agonist-sensitive intracellular Ca^{2+} stores generates a secondary signal of unknown nature, which in turn activates Ca^{2+} entry through the plasma membrane. We have shown previously that this mechanism is operative in rat thymocytes [21,22], human neutrophils [23] and human platelets [24] and that it can account for receptor-activated Ca^{2+} entry in these cells. Additionally, on the basis of the effects of a series of highly selective inhibitors, we have proposed that a microsomal cytochrome *P*-450 could be the link between the state of filling of the intracellular Ca^{2+} stores and the plasma-membrane Ca^{2+} permeability [22–24,27].

If the opening of the plasma-membrane Ca^{2+} channels were due to the genesis of a second messenger by the Ca^{2+} stores, a delay between emptying or refilling of the stores and changes in plasma-membrane permeability should be observed. This delay would correspond to the time required to build up a cytoplasmic concentration of the mediator large enough to activate the plasma-membrane Ca^{2+} channels or to the time required to clear it from the cytoplasm, respectively. A time lag of 15–30 s between the emptying of the Ca^{2+} stores and the appearance of the increased plasma-membrane permeability has been documented before [22,23]. However, it was not clear in that study whether this lag corresponded to the time required to empty the stores, rather than to the synthesis of a mediator.

In this paper, we have studied the time course of the decrease in the plasma-membrane Mn^{2+} permeability of human neutrophils with empty intracellular Ca^{2+} stores which follows the quick refilling of the stores. We have shown previously that Mn^{2+} is a suitable surrogate of Ca^{2+} for the plasma-membrane Ca^{2+} channels which are activated by emptying the Ca^{2+} stores in human neutrophils [23], and new evidence for the adequacy of Mn^{2+} as a Ca^{2+} surrogate in these cells is presented in the present paper. Our results show that refilling of the Ca^{2+} stores was followed by a time- and temperature-dependent decay of the plasma-membrane Ca^{2+} (Mn^{2+}) permeability, which could reflect the clearance of the possible mediator.

METHODS

Human neutrophils were obtained from blood of healthy volunteers anticoagulated by mixing 6:1 (v/v) with acid citrate–dextrose. Dextran (T500; Pharmacia) was then added to obtain 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;

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

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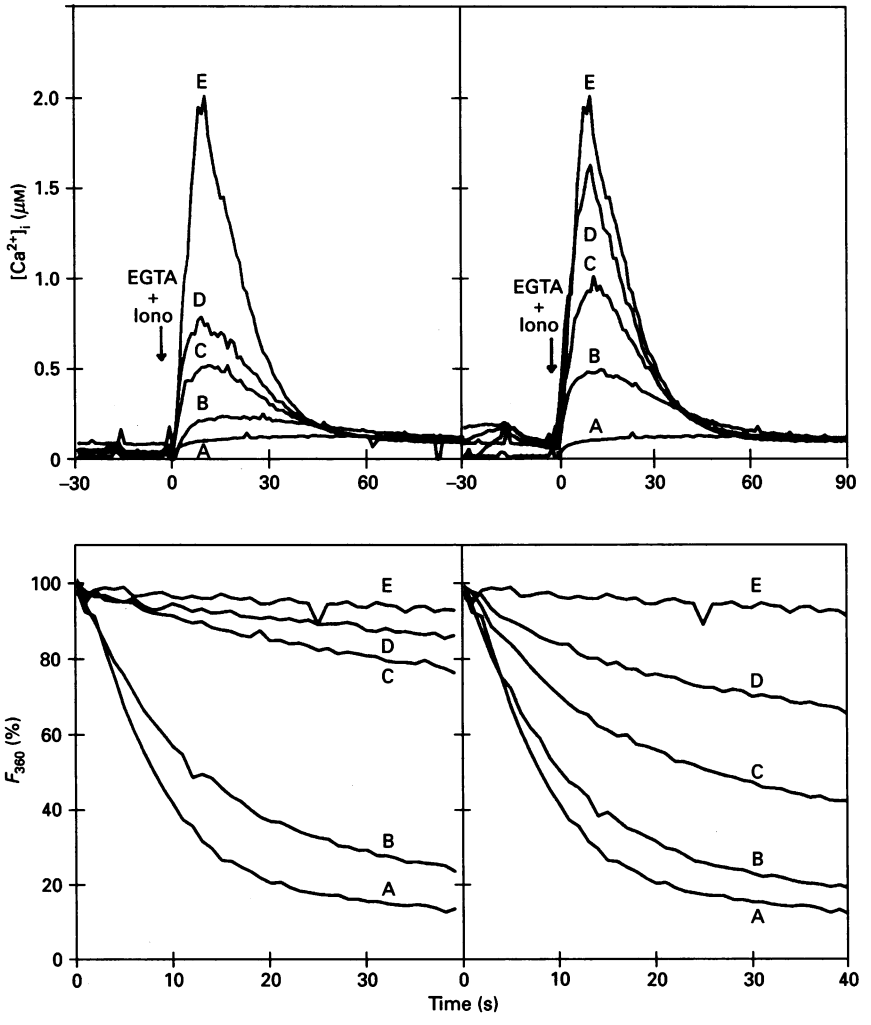

Fig. 1. Effect of the filling state of the intracellular Ca^{2+} stores on Mn^{2+} entry

In the left panels, Ca^{2+}-depleted cells were incubated for 3 min at 37 °C with different extracellular Ca^{2+} concentrations: A, no Ca^{2+}; B, 50 μM; C, 100 μM; D, 150 μM; E, 1 mM. In the right panels, cells were incubated with 1 mM-Ca^{2+} for different time periods: A, no Ca^{2+}; B, 10 s; C, 20 s; D, 30 s; E, 3 min. At the end of every refilling period ($t = 0$ in the Figure), either 5 mM-EGTA and 100 nM-ionomycin (Iono) (upper panels) or 5 mM-Mn^{2+} (lower panels) was added. This high Mn^{2+} concentration was used in order to inhibit any further Ca^{2+} uptake.

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 [28]. 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 incubation medium. Cells were then washed twice and resuspended at 2% cytocrit in nominally Ca^{2+}-free standard medium. The intracellular Ca^{2+} stores were then depleted by treatment with low concentrations of ionomycin (see below) and the cells were resuspended at 1% cytocrit in nominally Ca^{2+}-free medium. $[Ca^{2+}]_i$ was measured in 0.5 ml samples of this cell suspension, kept at either 37 °C or at 25 °C and under magnetic stirring, by using a fluorescence spectrophotometer constructed by Cairn Research (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 $[Ca^{2+}]_i$ was calculated from the ratio of the fluorescences excited at 340 and 380 nm [29]. Mn^{2+} uptake was monitored simultaneously by the quenching of the fura-2 fluorescence excited at 360 nm, which is insensitive to variations in $[Ca^{2+}]_i$ [21–24, 27,30,31]. Thus this procedure allows simultaneous and independent monitoring of plasma-membrane permeability (Mn^{2+} entry) and release of Ca^{2+} from the intracellular stores ($[Ca^{2+}]_i$) when the cells are incubated in Ca^{2+}-free Mn^{2+}-containing medium [31].

The starting material for most of the experiments described in this paper was cells whose intracellular stores had been depleted of Ca^{2+}. In a previous study, depletion of Ca^{2+} from the intracellular stores was achieved by prolonged incubation of the cells in Ca^{2+}-free medium [23]. With this procedure there was a residual Ca^{2+} pool, amounting to about 20–30% of the original content, which was not removed from the stores within reasonable incubation periods (2–3 h). In the present work, a shorter as well as a more efficient procedure was designed. The cells were incubated with a low concentration of ionomycin (2 nM) for 10 min in Ca^{2+}-free medium (containing 0.2 mM-EGTA), and the ionophore was removed thereafter by washing the cells once with Ca^{2+}-free medium containing 2 mg of albumin/ml. The treatment with this low concentration of ionomycin produces only a very moderate increase in $[Ca^{2+}]_i$ (to less than 200 nM [23]), which does not alter the subsequent responses to cell agonists, and allows complete Ca^{2+} depletion of the intracellular stores (see below).

The calcium content of the intracellular Ca^{2+} stores was

measured as described previously. Briefly, excess EGTA was added to a portion of the cell suspension, followed immediately by 100 nM-ionomycin, and the increase in $[Ca^{2+}]_i$ was recorded. Under these conditions, the magnitude of the ionomycin-induced $[Ca^{2+}]_i$ peak is proportional to the degree of filling of the intracellular Ca^{2+} stores [21–23]. The use of this procedure, as well as the efficiency of the procedure described above for depletion of the intracellular Ca^{2+} stores, are illustrated in the upper panels of Fig. 1. Trace A shows that ionomycin does not produce any increase in $[Ca^{2+}]_i$ in Ca^{2+}-depleted cells, indicating that the Ca^{2+} stores were completely empty. This compares with trace E, in which the Ca^{2+} stores were allowed to refill to completion before EGTA and ionomycin addition.

Fura-2/AM was obtained from Molecular Probes, Eugene, OR, U.S.A. Platelet-activating factor (PAF) and ionomycin were purchased from Calbiochem. Econazole and formylmethionyl-leucyl-phenylalanine (fMLP) were obtained from Sigma (London). Other chemicals were obtained either from Sigma (London) or E. Merck, Darmstadt, Germany.

RESULTS

We have shown previously that refilling of the Ca^{2+} stores of human neutrophils on incubation of Ca^{2+}-depleted cells in medium containing 1 mM-Ca^{2+} is very fast ($t_{\frac{1}{2}}$ about 30 s at 37 °C; [23]). Fig. 1 compares the time course of the refilling of the Ca^{2+} stores and their effects on the entry of Mn^{2+}, used here as a tracer for Ca^{2+} entry through the plasma membrane (see the Methods section), by using two different refilling protocols. In the left panels, the Ca^{2+} stores were refilled by a 3 min incubation with different extracellular Ca^{2+} concentrations (0.05–1 mM). This procedure is termed throughout the paper the 'steady-state' method of refilling, since the Ca^{2+} content of the stores had reached a stable level after 3 min at every extracellular $[Ca^{2+}]$ tested (results not shown). In the right panels, the Ca^{2+} stores were refilled by incubation with 1 mM-Ca^{2+} for different periods of time. This procedure is termed the 'fast' method of refilling. At the end of the refilling period, the Ca^{2+} contents of the stores (upper panels) and the plasma-membrane Mn^{2+} permeabilities (lower panels) were measured.

In all the cases refilling of the Ca^{2+} stores decreased Mn^{2+} entry, but there were important quantitative differences between the results obtained with each of the refilling procedures. The Ca^{2+} content of the stores needed for full inhibition of Mn^{2+} entry was smaller when refilling was performed under the 'steady-state' condition of the left panels. For example, the refilling attained after a 3 min incubation with 100 μM-Ca^{2+} (Fig. 1, trace C in upper left panel) was similar to that obtained after a 10 s incubation with 1 mM-Ca^{2+} (trace B in upper right panel). However, whereas inhibition of Mn^{2+} uptake was almost complete in the first case (trace C, lower left panel), no significant inhibition could yet be observed in the second (trace B, lower right panel).

The upper panel of Fig. 2 shows the correlation between the Ca^{2+} content of the stores and the Mn^{2+} permeability found in several experiments similar to those of Fig. 1 by using either the 'steady-state' (white symbols) or the 'fast' procedure (black symbols) to refill the stores. It is apparent again that inhibition of Mn^{2+} permeability was obtained with smaller refilling of the stores when the 'steady-state' procedure was used. Under this condition a 40 % refilling was enough to inhibit almost completely the entry of Mn^{2+}. In contrast, with the 'fast' refilling method a roughly linear decrease of Mn^{2+} entry was observed within the full refilling range (0–100 %). The lower panel of Fig. 2 shows the results of similar experiments, but performed at 25 °C. When the 'steady-state' refilling procedure was used, a higher extracellular Ca^{2+} concentration was required at 25 °C in order to refill the stores to an extent enough to inhibit Mn^{2+} entry fully (200 μM instead of 100 μM at 37 °C; results not shown). This may be due to a decrease in the rate of Ca^{2+} pumping to the stores at 25 °C, which should lead to a smaller degree of refilling at a given extracellular Ca^{2+} concentration. In any case, full inhibition of Mn^{2+} uptake was obtained at the same degree of store refilling (about 40 %) as at 37 °C (compare upper and lower panels in Fig. 2). The lower panel of Fig. 2 shows that, when the 'fast' refilling procedure was used at 25 °C, a clearly different pattern of inhibition of the Mn^{2+} uptake was observed again. The inhibition was smaller for a given level of store-refilling, and the relationship between refilling and Mn^{2+} entry was roughly linear.

The differences observed with both refilling procedures could arise from a non-homogeneous distribution of Ca^{2+} among different intracellular stores. For example, let us suppose that Ca^{2+} entering the cytosol refills first a specific intracellular store not related to the control of plasma-membrane Ca^{2+} permeability. Then the 'fast' procedure would favour selective refilling of the quickly refilling store with little change of plasma-membrane Ca^{2+} permeability, whereas the steady-state method would allow Ca^{2+} redistribution and homogeneous refilling of all the stores. This interpretation would fit the results of Figs. 1 and 2. In order to test it, we compared the time course of the refilling of the agonist-sensitive and the whole Ca^{2+} pool. For these purposes we measured the release of Ca^{2+} from the Ca^{2+} stores, refilled to different extents by using the 'fast' procedure, induced either by

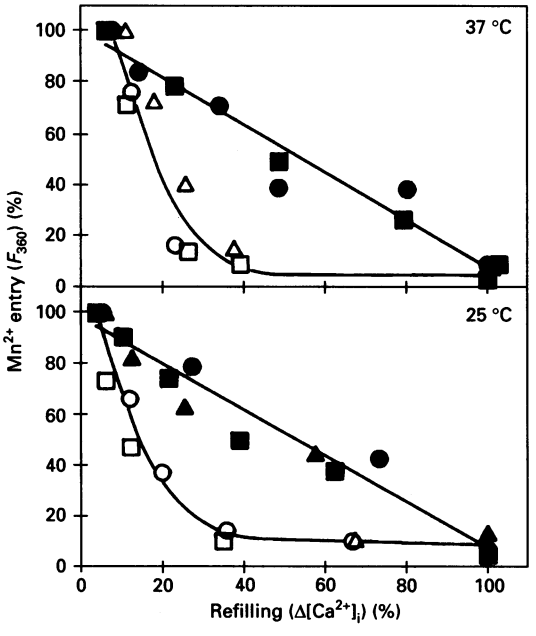

Fig. 2. Relationship between the Mn^{2+} entry and the filling state of the intracellular Ca^{2+} stores

Data were obtained in experiments similar to those of Fig. 1, performed at both 37 °C (upper panel) and 25 °C (lower panel). The refilling (Δ) of the intracellular Ca^{2+} stores was quantified as the peak $[Ca^{2+}]_i$ minus the basal $(Ca^{2+})_i$ of ionomycin-induced peaks. They were normalized in every experiment by taking the maximum level as 100 %. Mn^{2+} entry was calculated from the initial rate of quenching of F_{360} and normalized by taking the maximum rate as 100 %. Black symbols represent experiments performed by the 'fast' method of refilling (as in right panels of Fig. 1) and white symbols represent experiments performed by the 'steady-state' method of refilling (as in left panels of Fig. 1). Experiments performed with different cell batches are shown with different symbols.

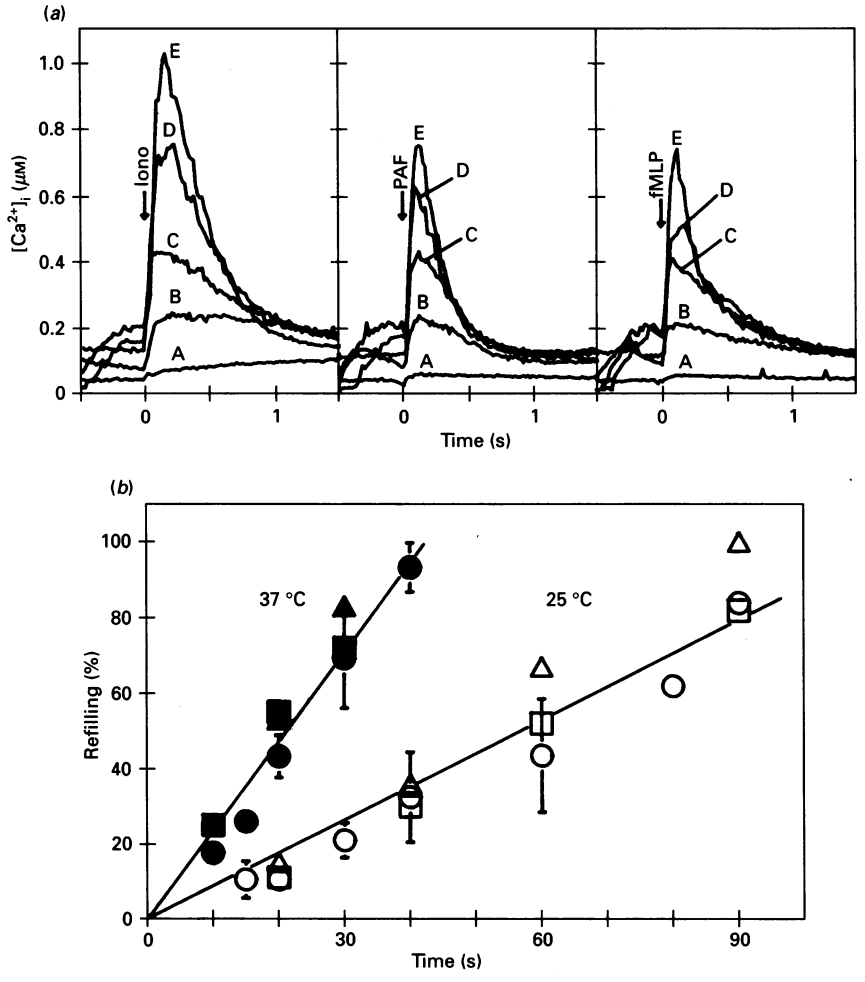

Fig. 3. Comparison of the time course of the refilling of the agonist-sensitive intracellular Ca^{2+} stores and the whole (agonist-sensitive + insensitive) Ca^{2+} pool

The refilling of the intracellular stores was estimated from the release of Ca^{2+} to the cytosol induced either by ionomycin (Iono) (whole pool) or by the agonists fMLP or PAF (agonist-sensitive pool). (*a*) Fura-2-loaded Ca^{2+}-depleted human neutrophils were incubated at 37 °C with 1 mM-Ca^{2+} for different periods of time: A, no Ca^{2+}; B, 10 s; C, 20 s; D, 30 s; E, 3 min. At the end of every incubation ($t = 0$ in the figure), 5 mM-EGTA and 100 mM-ionomycin (left panel), 20 ng of PAF/ml (centre panel) or 100 nM-fMLP (right panel) were added. (*b*) Summary of the results of several experiments similar to those in (*a*). Refilling was performed either at 37 °C (black symbols) or 25 °C (white symbols). It was quantified from the height of the $[Ca^{2+}]_i$ peak induced by either ionomycin (○, ●) or the agonists (△, ▲, fMLP; □, ■, PAF), and expressed as a percentage of the maximal release (stores filled by incubation with 1 mM-Ca^{2+} for 3 min at 37 °C or for 5 min at 25 °C). Vertical bars represent S.D. of mean values obtained in 3–6 similar experiments. Half-refilling times are 20 s at 37 °C and 60 s at 25 °C.

ionomycin or by the Ins(1,4,5)P_3-producing agonists fMLP or PAF. We have shown previously that releasing Ca^{2+} from the stores with agonists does increase Mn^{2+} influx [23], so that the quickly refilled store mentioned above, which would not control Mn^{2+} entry, should be the agonist-insensitive Ca^{2+} pool. Fig. 3(*a*) shows that the amount of Ca^{2+} released with ionomycin was larger than with the agonists. However, the relative rates of refilling of the ionomycin-releasable and the agonist-sensitive pools seemed to be similar. Thus refilling with 1 mM-Ca^{2+} for 20 s (traces C in Fig. 3) produced about half-maximal refilling in all the cases, as estimated from the relative height of the $[Ca^{2+}]_i$ peaks. Fig. 3(*b*) illustrates the correlation between refilling and time obtained in several similar experiments, performed at both 37 °C and 25 °C. In all the cases the points obtained either with ionomycin or with agonists overlapped within the same correlation lines, indicating that the rates of refilling of the agonist-sensitive and the agonist-insensitive Ca^{2+} pools are similar. Therefore, the delay in the inhibition of the Mn^{2+} uptake observed when the 'fast' method of refilling was used cannot be explained by a non-homogeneous Ca^{2+} distribution among different types of Ca^{2+} stores.

An alternative explanation would be the existence of a second messenger whose levels take some time to decay after sudden arrest of its synthesis once the stores are refilled. According to our results under the 'steady-state' refilling condition (upper panel in Fig. 2), the generation of the mediator would cease once the stores are 40% refilled. However, when the stores are refilled by the 'fast' procedure, the inhibition of Mn^{2+} entry would be delayed, since some time would be required for clearance of the mediator from the cytoplasm before closing of the Ca^{2+} channels. We have studied the time course of the decrease of Mn^{2+} entry after fast refilling of the intracellular Ca^{2+} stores to about 40% of its full capacity by incubation of Ca^{2+}-depleted cells with 1 mM-Ca^{2+} for 20 s at 37 °C. Then excess EGTA was added to avoid further refilling, and the Mn^{2+} permeability was measured after different times. The Ca^{2+} content of the stores changed little after addition of EGTA. The inset to Fig. 4 shows that refilling, measured by the ionomycin procedure, was about 40% by the

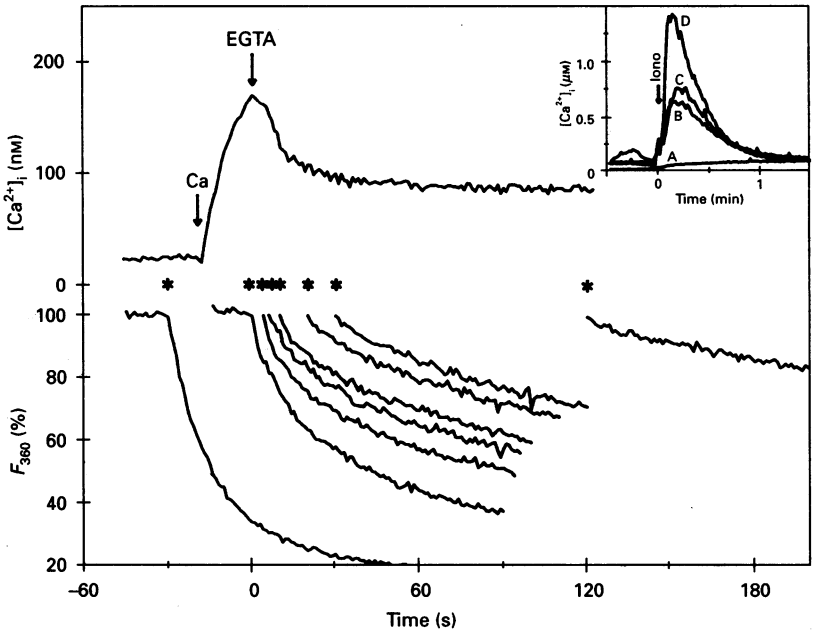

Fig. 4. Time-dependent inactivation of Mn^{2+} entry after partial refilling of the intracellular Ca^{2+} stores

The intracellular Ca^{2+} stores of fura-2 loaded Ca^{2+}-depleted human neutrophils were quickly refilled by addition of 1 mM Ca^{2+} (arrow labelled 'Ca'). After 20 s at 37 °C, 2 mM-EGTA and 2 mM-Tris were added (arrow labelled 'EGTA') in order to stop refilling. The upper panel shows the $[Ca^{2+}]_i$ trace. The inset illustrates measurements of the degree of filling of stores before refilling (A), immediately after partial refilling by a 20 s period in 1 mM-Ca^{2+} (B), after 20 s refilling in 1 mM-Ca^{2+}, followed by 2 min with EGTA as described above (C), and after complete refilling by a 3 min incubation with 1 mM-Ca^{2+} (D) (Iono = Ionomycin). In four similar experiments, the $[Ca^{2+}]_i$ peak obtained in (C) was (mean ± S.E.M.) 0.95 ± 0.05 times the $[Ca^{2+}]_i$ peak obtained in (B). The lower panel illustrates the entry of Mn^{2+} before and after partial refilling of the intracellular Ca^{2+} stores. For these experiments, 5 mM-Mn^{2+} was added either before refilling (first asterisk from the left) or immediately after the 20 s period of refilling (instead of EGTA, second asterisk from the left). The third to eighth asterisks from left to right show the effects observed on addition of 7 mM-Mn^{2+} at 4 s, 6 s, 10 s, 20 s, 30 s and 120 s after addition of EGTA.

time of EGTA addition (trace B) and remained at the same level 2 min later (trace C). In contrast, the entry of Mn^{2+}, shown in the main body of Fig. 4, decreased with time and was fully inhibited 2 min after addition of EGTA. This inhibition therefore cannot be attributed to an increase in the Ca^{2+} content of the stores, but is consistent with the existence of a mediator produced by the stores and cleared with time once its production is inhibited by refilling. Fig. 5 shows the decay with time of the initial rate of Mn^{2+} uptake obtained in experiments similar to those of Fig. 4, performed either at 37 °C (black symbols) or at 25 °C (white symbols). Half-maximal decay after 40% refilling of the stores took place within approx. 7 s at 37 °C and 20 s at 25 °C.

If, in accordance with our model [22–24,27], this second messenger were a product of the activity of a cytochrome *P*-450, then sudden inhibition of the cytochrome should lead to a time-dependent decrease of Mn^{2+} entry with kinetics similar to that seen in the experiments of Figs. 4 and 5. The experiments of Fig. 6 were designed to measure the entry of Mn^{2+} in cells with empty Ca^{2+} stores at different time periods after addition of the cytochrome *P*-450 inhibitor econazole. For these purposes, the addition of Mn^{2+}, which starts the measurement of the uptake, was delayed by different times with respect to addition of econazole. At 37 °C (upper panel), the inhibition by econazole had a lag of 8–10 s when it was added at the same time as Mn^{2+}. When econazole preceded Mn^{2+} by 5 s, the delay was decreased to 3–4 s. Finally, when econazole was added 2 min before Mn^{2+}, the inhibition of the entry was complete from the beginning. At 25 °C the inhibition of the initial rate was half-maximal when the cells had been preincubated with econazole for 10–20 s. Although half-times cannot be estimated precisely from these experiments, it is clear that the delays for inhibition of Mn^{2+} entry either by econazole (Fig. 6) or by fast refilling of the stores (Figs. 4 and 5)

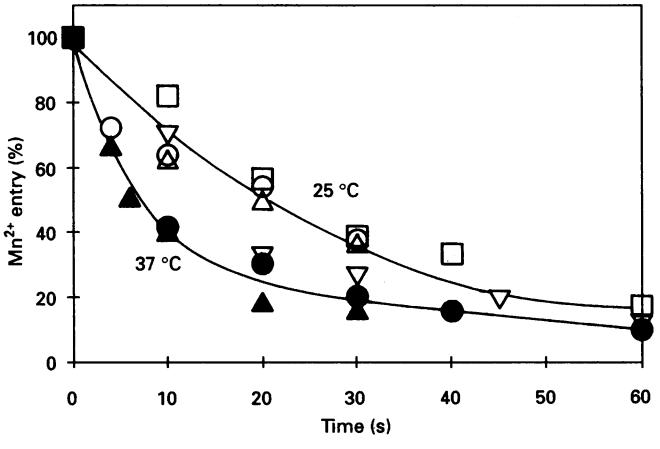

Fig. 5. Time-dependent inactivation of Mn^{2+} entry after partial refilling of the intracellular Ca^{2+} stores at either 37 °C (black symbols) or 25 °C (white symbols)

Data were obtained in experiments similar to those of the lower panel of Fig. 4. The stores of Ca^{2+}-depleted cells were refilled to about 40% of the maximum by incubation with 1 mM-Ca^{2+} for either 20 s at 37 °C or 40 s at 25 °C before addition of 2 mM-EGTA + 2 mM-Tris. The Mn^{2+} entry, estimated from the initial rate of quenching of F_{360}, is plotted against the time elapsed from the addition of EGTA (end of the refilling period). Mn^{2+}-entry values were normalized by taking the Mn^{2+}-uptake rate obtained just at the end of the refilling period as 100% (second asterisk from the left in Fig. 4), and the Mn^{2+}-uptake rate obtained long after the addition of EGTA as 0% (120 s at 37 °C or 180 s at 25 °C, last asterisk from the left in Fig. 4). Experiments made with different cell batches are shown with different symbols.

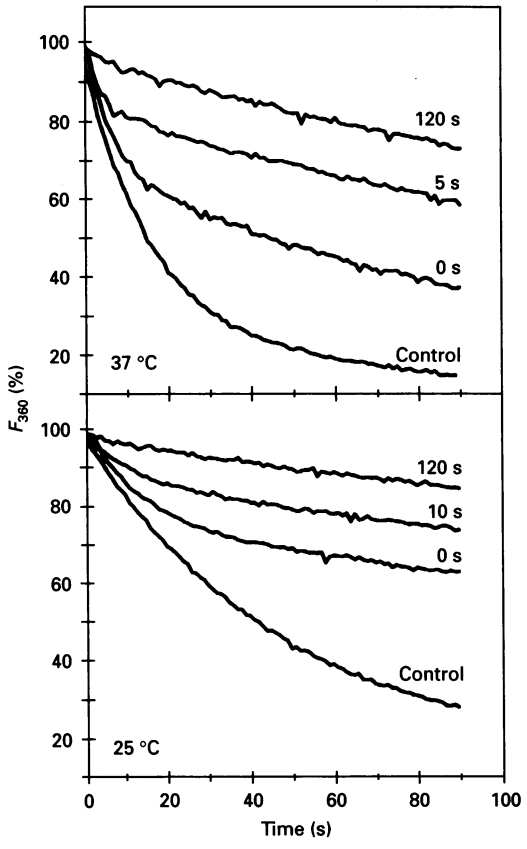

Fig. 6. Time-dependence of inhibition of Mn^{2+} entry into fura-2-loaded Ca^{2+}-depleted human neutrophils by econazole

Mn^{2+} entry was measured either in the absence of econazole (labelled 'Control') or in cells exposed to 5 μM-econazole for different time periods (as indicated on the right) before addition of Mn^{2+} ($t = 0$ in the figure). The effect of the simultaneous addition of Mn^{2+} and econazole is shown in the curve labelled '0 s'. Experiments were performed at either 37 °C (upper panel) or 25 °C (lower panel).

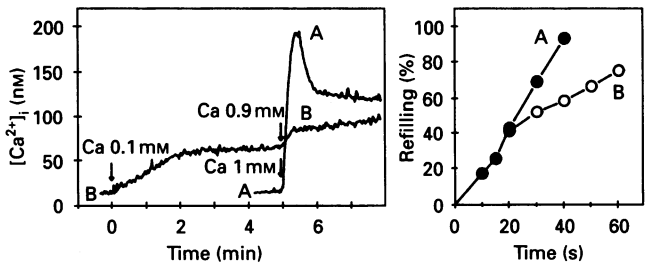

Fig. 7. Comparison of the entry of Ca^{2+} (left) and the refilling of the Ca^{2+} stores (right) in cells with Ca^{2+} stores either empty or partly refilled by the 'steady-state' procedure

(Left) Cells with empty stores were incubated either with no Ca^{2+} (trace A, shown from $t = 4$ min) or with 0.1 mM-Ca^{2+} (trace B, 0.1 mM-Ca^{2+} addition at $t = 0$, as shown by arrow). A 5 min incubation with 0.1 mM-Ca^{2+} allows a 40 % steady-state refilling of the stores (see Fig. 1 and right panel in this Figure). At $t = 5$ min Ca^{2+} was added to give a final concentration of 1 mM (shown by arrows in both traces). (Right) The refilling degree of the stores was measured in both cell kinds by the ionomycin procedure (see Figs. 1 and 2 for details). Labels A and B are as in the left panel. In all the cases refilling was expressed as a percentage of the maximum refilling observed in condition A (3 min incubation of cells with empty stores with 1 mM-Ca^{2+}). In trace A, $t = 0$ corresponds to the time of addition of 1 mM-Ca^{2+}. Trace B has been shifted 20 s to the right ($t = 20$ s in the Figure corresponds to the time of addition of 0.9 mM-Ca^{2+}) in order to facilitate comparison of the rates of refilling at comparable degrees of refilling.

are in the same range. The delay observed for the effect of econazole could also be attributed to the time required to reach its site of action, the intracellular Ca^{2+} stores. Econazole is, however, a highly lipophilic molecule that should redistribute very quickly through the plasma membrane. With ionomycin, a molecule of similar lipid-solubility, we find no delay (< 1 s) on the release of Ca^{2+} from the stores (results not shown). In addition, as previously shown for the related compound SKF96365 [32], high (more than 5 μM) concentrations of econazole produced a small immediate increase in $[Ca^{2+}]_i$ by release of Ca^{2+} from the intracellular stores (results not shown). Whatever the mechanism for this effect, it suggests that econazole gains immediate access to the Ca^{2+} stores. The above considerations therefore suggest that the time lag for inhibition of Mn^{2+} influx by econazole cannot be explained by diffusional delay in reaching the intracellular stores. They are consistent, however, with this delay being due to the time required to clear a cytochrome *P*-450 product from the cytoplasm once its production is arrested by econazole.

It could be argued that all our evidence on control of plasma-membrane Ca^{2+} channels by the intracellular Ca^{2+} stores is based on Mn^{2+}-entry data and the fact that Mn^{2+} may be inadequate as a surrogate for plasma-membrane Ca^{2+} channels. It has been reported that Mn^{2+} may be a poor substitute of Ca^{2+} for receptor-operated Ca^{2+} channels of hepatocytes [33], lacrimal acinar cells [34] or salivary acinar cells [35,36]. We have shown previously that Mn^{2+} is a good surrogate for receptor-operated Ca^{2+} channels of neutrophils [23]. However, we wanted to test this point in the present series of experiments. We have documented above that 40 % refilling of the stores under the 'steady-state' condition almost abolished Mn^{2+} entry (Fig. 2). Should Ca^{2+} use the same pathway for entry, we would expect that the increase in $[Ca^{2+}]_i$ resulting from Ca^{2+} entry should be greatly decreased in cells with partly refilled Ca^{2+} stores. This prediction was tested by comparing the increase in $[Ca^{2+}]_i$ observed in cells with empty Ca^{2+} stores and in cells with 40 %-refilled stores on incubation with 1 mM external Ca^{2+}. Fig. 7 (left panel) shows that the increase in $[Ca^{2+}]_i$ was much faster in cells with empty Ca^{2+} stores. The right panel in Fig. 7 shows the degree of filling of the stores in the same experiment. Curve A, similar to that shown in Fig. 3, shows the time course of the refilling of cells with empty Ca^{2+} stores on addition of 1 mM-Ca^{2+} (at $t = 0$ in the Figure). Curve B shows the time course of the refilling in the cells with partly refilled stores on incubation with 1 mM-Ca^{2+}. The curve has been shifted 20 s to the right to facilitate comparison of the rates of refilling at comparable degrees of filling. It is clear that the filling of the stores was slower when they had been allowed to reach a 40 % steady-state refilling previously. This is what should be expected if the store-refilling was limited by slow plasma-membrane Ca^{2+} entry under this condition. In fact, refilling became even slower after the first 1 min, reaching only about 80 % refilling after 5 min, the longer time tested (results not shown).

DISCUSSION

We have studied the control of the plasma-membrane Ca^{2+} (Mn^{2+}) permeability by the level of Ca^{2+} in the stores under two different conditions: a steady-state condition, in which the stores are refilled to a given extent and the cells are kept in that condition for several minutes, and a more dynamic condition, in which the Mn^{2+} permeability was measured while the Ca^{2+} stores were being refilled. Under the steady-state condition the cor-

relation found between the level of Ca^{2+} in the stores and the plasma-membrane Ca^{2+} (Mn^{2+}) permeability was hyperbolic, with a rapid decrease in the Mn^{2+} permeability up to near the basal level when the Ca^{2+} stores were still 40 % full. This kind of correlation is similar to that previously found in thymocytes [22] and in neutrophils [23] with Ca^{2+} stores refilled under the steady-state condition. This hyperbolic correlation would be unfavourable to allow fast and complete refilling of the stores after release of Ca^{2+} by physiological signals, since the stimulation of Ca^{2+} entry to the cells would slow down when the stores were only still 40 % full. When Mn^{2+} permeability was measured during refilling, the inhibition of the plasma-membrane Ca^{2+} (Mn^{2+}) permeability was delayed. The delay was just enough to allow a linear correlation between the level of Ca^{2+} in the stores and the plasma-membrane Ca^{2+} (Mn^{2+}) permeability. Therefore the stores-dependent control of the plasma-membrane Ca^{2+} (Mn^{2+}) permeability of human neutrophils seems designed to allow, owing to the existence of the delay, a fast and complete refilling of the stores after physiological stimulation. The fact that the same straight line correlated the refilling degree of the Ca^{2+} stores and the plasma-membrane Mn^{2+} permeability at 25 °C and at 37 °C (Fig. 2) suggests that both the refilling and the mechanism responsible for the delay have similar temperature-dependence. In other cells, these mechanisms may work differently. For example, in endothelial cells a linear correlation between the filling state of the stores and Mn^{2+} entry was found in the steady-state condition [19].

We have proposed recently that the link between the emptying of the Ca^{2+} stores and the plasma-membrane Ca^{2+} (Mn^{2+}) channels could be a cytochrome *P*-450 sited at the stores, which is activated when the Ca^{2+} content of the stores decreases [22–24,27]. This proposal was based on the inhibition of the Mn^{2+} permeability by several N^1-substituted imidazole compounds, which are strong and selective inhibitors of cytochrome *P*-450. If this model were correct, then the same delay in deactivation of Mn^{2+} permeability found after quick refilling of the stores (Figs. 4 and 5) should appear in cells with empty stores after direct inhibition of cytochrome *P*-450 with an imidazole antimycotic. This prediction was confirmed in the experiments of Fig. 6.

The interaction between the Ca^{2+} stores and the plasma-membrane Ca^{2+} channels may be indirect, perhaps through a soluble mediator travelling between the Ca^{2+} stores and the plasma membrane [14,26,37]. This mediator would be produced when the Ca^{2+} stores are empty, and its production would cease when the Ca^{2+} stores are about half-full. The mediator would then be inactivated with a half-time of about 7 s at 37 °C and 20 s at 25 °C. Direct interaction of the store-filling sensor and the plasma-membrane channels cannot be excluded by the present results, but the slow relaxation rates required to fit the half-times found here seem unlikely for a protein–protein interaction.

This work was supported by the Spanish Dirección General de Investigación Científica y Técnica (DGICYT), grant no. PB89-0359. We thank Mr. Jesús Fernandez for excellent technical assistance and The University Hospital Blood Bank for blood supply.

Received 13 April 1992/22 May 1992; accepted 2 June 1992

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