Control of Ca^{2+} entry into HL60 and U937 human leukaemia cells by the filling state of the intracellular Ca^{2+} stores

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Differentiation of HL60 cells by treatment with dimethyl sulphoxide induces the expression of membrane receptors for *N*-formylmethionyl-leucyl-phenylalanine (fMLP) and for platelet-activating factor (PAF). In these cells both agonists produced an increase in the cytosolic Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$) by release of Ca^{2+} from the intracellular stores, followed shortly by an acceleration of the entry of Ca^{2+} or Mn^{2+} , used here as a Ca^{2+} surrogate for Ca^{2+} channels. Cytochrome *P*-450 inhibitors blocked the agonist-induced entry of Ca^{2+} or Mn^{2+} with no modification of Ca^{2+} release from the stores. Emptying the intracellular Ca^{2+} stores either by treatments inducing no inositol phosphate production, such as prolonged incubation in Ca^{2+} -

free medium or treatment with the Ca^{2+} ionophore ionomycin, increased the plasma-membrane permeability to Ca^{2+} and Mn^{2+} . This Ca^{2+} -store-regulated Mn^{2+} entry was inhibited by Ni^{2+} and by cytochrome *P*-450 inhibitors. Refilling of the Ca^{2+} stores by incubation in Ca^{2+} -containing medium restored low Mn^{2+} permeability. The same mechanism is present and functional in non-differentiated cells, before expression of membrane receptors for fMLP and PAF. These results suggest that agonist-induced Ca^{2+} (Mn^{2+}) entry is secondary to the emptying of the intracellular Ca^{2+} stores, which in turn activates plasma-membrane channels by a mechanism involving cytochrome *P*-450.

INTRODUCTION

In many cell types, receptor activation results in an increase in the intracellular Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$), which is due to both Ca^{2+} release from the intracellular Ca^{2+} stores and Ca^{2+} entry through the plasma membrane [1]. It is generally accepted that $\text{Ins}(1,4,5)\text{P}_3$, produced by activation of phospholipase C on occupation of the membrane receptors, is the mediator for Ca^{2+} release from the stores [2]. The nature of the mechanism controlling 'receptor-operated' Ca^{2+} entry has proved harder to elucidate [3,4]. Proposed mechanisms include direct coupling to the membrane receptors by G-proteins [5,6] or indirect coupling via a second messenger such as $\text{Ins}(1,3,4,5)\text{P}_4$, InsP_3 or the concerted action of both [4,7,8]. An alternative view, first proposed by Putney [9] and confirmed later in several cell types [10–23], postulates that the emptying of the intracellular Ca^{2+} stores may increase the plasma-membrane permeability to Ca^{2+} . In this scheme, activation of 'receptor-operated' Ca^{2+} channels by agonists may take place by a rather indirect mechanism, involving no direct interactions with the channels, nor even direct generation of a channel-activating second messenger.

We have shown recently that such a mechanism can account for the entry of Ca^{2+} induced by the chemotactic peptide *N*-formylmethionyl-leucyl-phenylalanine (fMLP) or the platelet-activating factor (PAF) in human neutrophils [18]. As in other cell types, the agonist-induced Ca^{2+} / Mn^{2+} entry, but not the release of Ca^{2+} from the stores, was prevented by cytochrome *P*-450 inhibitors, suggesting that *P*-450 may be the link between the stores and the plasma-membrane Ca^{2+} channels. The existence of an entry pathway for bivalent cations which becomes activated on depletion of the intracellular stores by treatment with inhibitors of Ca^{2+} -ATPase has been documented recently in HL60 cells differentiated towards granulocytes by treatment with dimethyl sulphoxide [24].

In the present paper we show that the entry of Ca^{2+} / Mn^{2+} induced by fMLP or PAF in differentiated HL60 cells is prevented by cytochrome *P*-450 inhibitors. Emptying the Ca^{2+} stores by other treatments not producing InsP_3 , such as prolonged incubation in Ca^{2+} -free medium, or treatment with ionomycin produced a similar entry of Mn^{2+} , which was also prevented by cytochrome *P*-450 inhibitors. This store-controlled Ca^{2+} -entry pathway was also present in non-differentiated HL60 cells, before expression of membrane receptors for fMLP and PAF. A similar Ca^{2+} -entry pathway was found in both non-differentiated and monocyte-macrophage-differentiated U937 leukaemia cells.

METHODS

Cell culture

HL60 and U937 human leukaemia cells were propagated and cultured as described elsewhere [25]. HL60 cells were differentiated towards granulocytes by culture in medium containing 1.3 % dimethyl sulphoxide during 4 days [26]. U937 cells were differentiated towards monocytes-macrophages by culture in medium containing 0.1 μM phorbol 12-myristate 13-acetate [27].

Measurements of $[\text{Ca}^{2+}]_i$ and Mn^{2+} entry

$[\text{Ca}^{2+}]_i$ and the uptake of Mn^{2+} were measured simultaneously and independently in fura-2-loaded cells. For loading with fura-2, the cells were first washed twice with a nominally Ca^{2+} -free medium of the following composition (mM): NaCl, 145; KCl, 5; MgCl_2 , 1; glucose, 10; Na-Hepes, 10; pH 7.4. Then they were incubated at 2 % cytocrit with 4 μM fura-2/AM. After 30–45 min at room temperature, the cells were washed twice, resuspended at 1 % cytocrit in the same medium and stored at room temperature until used. All the above steps were performed in nominally

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

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Ca^{2+} -free medium in order to deplete the intracellular Ca^{2+} stores of Ca^{2+} [15,17,18]. Measurements of the stores' Ca^{2+} content at this stage [15] revealed 65–90 % depletion. Incubation of the cells with 1 mM Ca^{2+} for 5–10 min allowed full refilling of the stores.

Fluorescence measurements were performed in 0.5 ml samples of the cell suspension, kept under magnetic stirring and at 37 °C, in a fluorescence spectrophotometer constructed by Cairn Research (Newnham, Sittingbourne, Kent, U.K.), which allows quasi-simultaneous (30–300 Hz) measurements of fluorescence excited at up to six different wavelengths. The emission wavelength was set at 530 ± 10 nm and readings were integrated at 1 s intervals. $[\text{Ca}^{2+}]_i$ was estimated from the ratio of the fluorescences excited at 340 and 380 nm ($R_{340/380}$; [28]). The uptake of Mn^{2+} was monitored by the quenching of fluorescence excited at 360 nm. At this excitation wavelength, changes of $[\text{Ca}^{2+}]_i$ do not modify fluorescence emission [12,28]. On the other hand, quenching by Mn^{2+} does not modify $R_{340/380}$ as long as the total fura-2 content of the cells is larger than the total content of Mn [29,30]. Mn^{2+} was used here as a tracer for Ca^{2+} entry (see the Results section).

Single-cell fluorescence measurements

For these experiments HL60 cells were first allowed to attach to glass coverslips coated with polylysine for about 30 min. Then the coverslips were transferred to wells containing 1 ml of Ca^{2+} -free medium and 5 μM fura-2/AM and incubated with gentle shaking for 2 h at room temperature. The coverslips were then mounted under the microscope in a chamber thermostatically maintained at 36 °C and epi-illuminated alternately at 340 and 380 nm. Light emitted at wavelengths above 520 nm was recorded by an extended ISIS-M camera (Photonic Science, Robertbridge, East Sussex, U.K.) and analysed with a Joyce-Loebl (Newcastle, U.K.) Magical image processor with 32 Mbyte video RAM. Four video frames of each wavelength were averaged by hardware, with an overall time resolution of about 3 s for each pair of images at alternate wavelengths. $[\text{Ca}^{2+}]_i$ was monitored from F_{340}/F_{380} [28]. For monitoring of quenching by Mn (F_{total} in Figure 6), each pair of images, taken at 340 and 380 nm, were added in such a way that the result was insensitive to changes of $[\text{Ca}^{2+}]_i$. For these purposes the images taken at 380 nm were multiplied by a constant factor before addition. This factor was calculated as (increase in F_{340} /decrease in F_{380}) when $[\text{Ca}^{2+}]_i$ was increased with ionomycin [30].

Chemicals

Fura-2/AM was obtained from Molecular Probes, Eugene, OR, U.S.A. PAF and ionomycin were purchased from Calbiochem. Econazole, miconazole, clotrimazole, ketoconazole and fMLP were obtained from Sigma. Other chemicals were obtained from either Sigma or Merck.

RESULTS

On differentiation, HL60 cells express receptors for agonists of mature neutrophils, such as those for the chemotactic peptide fMLP or for PAF [31,32]. Figure 1(a) compares the effects of the addition of either 2 mM Ca^{2+} , 0.1 μM fMLP or both together on $[\text{Ca}^{2+}]_i$ of differentiated HL60 cells suspended in low (0.1 mM)- Ca^{2+} medium. The effects of fMLP together with Ca^{2+} were more than additive, suggesting that fMLP accelerates Ca^{2+} entry. The

effects of Ca^{2+} + fMLP were largely prevented by the cytochrome P-450 inhibitor econazole (Figure 1b), whereas the effects of fMLP alone were little affected (Figure 1c). These results suggest that, as shown previously in neutrophils [18], fMLP-induced Ca^{2+} entry, but not Ca^{2+} release from the stores, is prevented by cytochrome P-450 inhibitors. However, precise quantification of the differences of Ca^{2+} permeability from these experiments is not possible, since the size of the $[\text{Ca}^{2+}]_i$ peak is decreased by active pumping of Ca^{2+} , which increases with $[\text{Ca}^{2+}]_i$. Mn^{2+} has been shown previously to be a Ca^{2+} surrogate for many plasma-membrane Ca^{2+} pathways, including the receptor-operated Ca^{2+} channels of human neutrophils [18]. Mn^{2+} 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.

Figure 2 shows the effects of fMLP on $[\text{Ca}^{2+}]_i$ and on the entry of Mn^{2+} in non-differentiated and in differentiated HL60 cells with filled Ca^{2+} stores. The extracellular medium contained no Ca^{2+} , so that any increase in $[\text{Ca}^{2+}]_i$ must arise from Ca^{2+} release from the stores. The chemotactic peptide had no effect in non-differentiated cells, but produced an immediate increase of $[\text{Ca}^{2+}]_i$ (upper panels) in differentiated cells, followed 10–15 s later by an acceleration of Mn^{2+} entry (lower panels). This lag corresponded roughly to the time required to empty the intracellular Ca^{2+} stores, as estimated from the time course of the $[\text{Ca}^{2+}]_i$ increase. Econazole blocked the entry of Mn^{2+} , with little effect on the release of Ca^{2+} from the stores (Figure 2). Similar results were obtained with PAF as the agonist. PAF had no effect on non-differentiated cells, but produced an immediate increase in $[\text{Ca}^{2+}]_i$, followed by an acceleration of Mn^{2+} entry in differentiated HL60 cells. Econazole blocked the PAF-induced entry of Mn^{2+} , with little or no effect on the release of Ca^{2+} from the stores (results not shown).

The intracellular Ca^{2+} stores can be depleted of Ca^{2+} by treatment with Ca^{2+} ionophores. We have shown previously that low concentrations of ionophores may effectively permeabilize endomembranes and deplete the intracellular stores of Ca^{2+} , with little effect on the plasma-membrane permeability [15,17,18, 21,33]. Figure 3 (lower panels) shows the effects of the addition of 1 nM ionomycin on the entry of Mn^{2+} in non-differentiated and differentiated HL60 cells with filled Ca^{2+} stores. During the

Figure 1 Changes of $[\text{Ca}^{2+}]_i$ in differentiated HL60 cells stimulated with fMLP: effects of extracellular Ca^{2+} and econazole

Cells were loaded with fura-2 (see the Methods section) and stored in medium containing 0.1 mM Ca^{2+} . At the time shown by arrows, either 2 mM Ca^{2+} , 0.1 μM fMLP or both together were added (panel a). In panels (b) and (c) the cell suspension was incubated with 10 μM econazole for 2 min before these additions. $[\text{Ca}^{2+}]_i$ was estimated from the ratio F_{340}/F_{380} . Each trace is the average of 2–4 similar experiments.

Figure 2 Effects of fMLP on $[Ca^{2+}]_i$ (upper panels) and on the entry of Mn^{2+} (lower panels) in non-differentiated (left) and in differentiated (right) HL60 cells with filled intracellular Ca^{2+} stores

Ca²⁺-depleted fura-2-loaded cells (see the Methods section) were first incubated with 1 mM Ca²⁺ for 10 min in order to refill the intracellular Ca²⁺ stores. Then 1 mM MnCl₂ was added, followed 2 min later by fMLP (0.1 μ M; $t = 0$ in the Figure). The $[Ca^{2+}]_i$ changes, measured from the ratio F_{340}/F_{380} (see the Methods section), are shown in the upper panels. The influx of Mn^{2+} is shown in the lower panels. Fluorescence was excited at 360 nm (under which conditions it is not sensitive to Ca²⁺, but is quenched by Mn²⁺; see the Methods section) and normalized to 100% at the time of Mn²⁺ addition. In some experiments with differentiated (d) HL60 cells, econazole (concentration indicated by the values at the right of the traces) was added 2 min before fMLP. This experiment is representative of two similar ones. The same results were obtained with PAF (20 ng/ml) as the agonist.

Figure 3 Effects of ionomycin (1 nM) on $[Ca^{2+}]_i$ (upper panels) and on the entry of Mn^{2+} (lower panels) in non-differentiated (left) and differentiated (d; right) HL60 cells with filled intracellular Ca^{2+} stores

Ca²⁺-depleted fura-2-loaded cells (see the Methods section) were first incubated with 1 mM Ca²⁺ for 10 min in order to refill the intracellular Ca²⁺ stores. Then 1 mM MnCl₂ was added, followed 2 min later by ionomycin (1 nM; $t = 0$ in the Figure). In the traces labelled '+Ni' and '+Eco', 5 mM NiCl₂ or 5 μ M econazole respectively was added 2 min before Mn²⁺. Other details are as in Figure 2.

first few seconds after ionomycin addition there was no effect on the rate of entry, but then it suddenly increased to 3–6-fold the value before ionophore addition. Simultaneous measurements of

$[Ca^{2+}]_i$ showed an immediate increase after the addition of ionomycin, which levelled off within a few seconds (Figure 3, upper panels). These results indicate that the intracellular Ca²⁺

Figure 4 Comparison of the entry of Mn^{2+} in cells with either empty or refilled Ca^{2+} stores: effects of econazole in cells with empty stores

Ca^{2+} -depleted fura-2-loaded cells (d, differentiated) suspended in Ca^{2+} -free medium (see the Methods section) were placed in the spectrofluorimeter cuvette and 1 mM $MnCl_2$ was added at $t = 0$ (traces labelled 'E'). To some of these, econazole (concentration shown at right of each trace) was added 2 min before Mn^{2+} . Other samples were incubated with 1 mM $CaCl_2$ for 10 min before $MnCl_2$ addition ($t = 0$ in the Figure) to allow refilling of the intracellular Ca^{2+} stores (traces labelled 'F'). The experiments shown are representative of three similar ones performed with different cell batches. In some experiments (results not shown), cells with filled Ca^{2+} stores were sedimented and resuspended in Ca^{2+} -free medium before Mn^{2+} addition; the entry of Mn^{2+} in these cells was as in 'F' in the Figure. In other experiments (results not shown) 1 mM each of Ca^{2+} and Mn^{2+} was added together to cells with empty Ca^{2+} stores; the entry of Mn^{2+} in these cells was 80–90% as fast as in 'E' in the Figure. Other details are as in Fig. 2.

Table 1 IC_{50} values for inhibition by several imidazole antimycotics of Mn^{2+} entry in cells with empty Ca^{2+} stores

Values were estimated from experiments similar to those shown in Figure 4, and are expressed as means $\pm$ S.E.M. for the numbers of individual measurements given in parentheses.

Inhibitor	Cell type ...	IC_{50} (μM)				
		HL60	Differentiated HL60	U937	Differentiated U937	Others*
Econazole		1.0 ± 0.2 (7)	1.1 ± 0.2 (3)	1.9 ± 0.2 (6)	1.7 ± 0.2 (8)	0.2–3
Miconazole		2.3 ± 0.7 (4)	1.0 ± 0.2 (3)	3.8 ± 0.7 (8)	3.0 ± 0.4 (5)	0.5–3
Clotrimazole		10 ± 3 (4)	2.1 ± 0.2 (4)	19 ± 6 (4)	11 ± 1 (6)	2–6
Ketoconazole		22 ± 6 (3)	9 ± 1 (3)	80 ± 30 (4)	23 ± 4 (5)	15–25

* Range of values obtained previously in thymocytes, neutrophils and platelets [17,18,21].

stores had been instantly permeabilized by ionomycin, resulting in the discharge of their contents to the cytoplasm and an increase in $[Ca^{2+}]_i$ within a few seconds. The delay in the onset of Mn^{2+} entry corresponded roughly to the time required for the increase in $[Ca^{2+}]_i$, suggesting that it is secondary to the emptying of the intracellular Ca^{2+} stores effected by the ionophore. The acceleration of Mn^{2+} uptake induced by 1 nM ionomycin (Figure 3), but not the increase in $[Ca^{2+}]_i$ (results not shown), was blocked by Ni^{2+} and by econazole. These results reinforce the view that the acceleration of Mn^{2+} entry does not take place directly through the ionophore, since neither Ni^{2+} nor econazole interferes with ionomycin-mediated transport, but that it is secondary to the emptying of the intracellular Ca^{2+} stores. The similarities between the effects of the agonists (Figure 2) and ionomycin (Figure 3) in differentiated HL60 cells suggest that the trigger for Mn^{2+} entry may be the same in both cases, the emptying of the intracellular stores. Non-differentiated HL60 cells seem to possess the same plasma-membrane Mn^{2+} -entry pathway which is activated on emptying the stores with ionomycin (Figure 3). However, the agonists are not able to release Ca^{2+} from the stores in non-differentiated cells and,

consistently, they are not able to activate Mn^{2+} entry, either (Figure 2).

The intracellular Ca^{2+} stores can be depleted of Ca^{2+} by prolonged incubation in Ca^{2+} -free medium and then refilled by incubation with Ca^{2+} at 37 °C (see the Methods section). Figure 4 compares the entry of Mn^{2+} in cells with either empty or filled stores. In all the cases the entry of Mn^{2+} , as measured from the rate of quenching of fura-2, was faster (4–7-fold) in the cells whose Ca^{2+} stores were empty (labelled 'E' in Figure 4, compared with 'F' for filled). The inhibition of the uptake of Mn^{2+} in cells with empty Ca^{2+} stores by different concentrations of econazole (as shown at the right of the traces) is also documented in Figure 4. The inhibitory effect increased with the concentration of econazole, and the maximal inhibition was about the same as that achieved by refilling the Ca^{2+} stores. Other imidazole antimycotics were also efficient inhibitors. The values estimated for IC_{50} in experiments similar to those of Figure 4 are shown in Table 1. Econazole and miconazole were the most potent inhibitors, followed by clotrimazole and ketoconazole. α -Naphthoflavone was also a potent inhibitor (IC_{50} 5–9 μM ; not shown in Table 1). The addition of 5 mM $NiCl_2$ to the incubation

Figure 5 Comparison of the entry of Ca²⁺ in single HL60 cells with either empty or refilled Ca²⁺ stores: effects of Ni²⁺

Cells were attached to polylysine-coated coverslips and loaded by incubation with 10 μ M fura-2/AM in Ca²⁺-free medium for about 1 h at room temperature. The coverslips were then placed under the microscope in a chamber thermostatically regulated to 36 °C and perfused at 3 ml/min. Solutions were changed as indicated in the Figure. Extracellular Ca²⁺ ([Ca²⁺]_o) was changed between 1 and 0 mM. The Ca²⁺-free solution contained 200 μ M EGTA. Ionomycin (iono) was dissolved in Ca²⁺-free medium at 100 nM. NiCl₂ was dissolved in either Ca²⁺-free or Ca²⁺-containing medium at 5 mM. The stores were fully depleted of Ca²⁺ by incubation with 100 nM ionomycin in Ca²⁺-free medium for 2 min, followed by 2 min more in Ca²⁺-free medium before the first perfusion with Ca²⁺ shown in the Figure. The trace labelled 'AV' is the average of 44 cells present in the same microscope field.

medium also blocked completely the entry of Mn²⁺ in cells with empty Ca²⁺ stores. In cells with filled Ca²⁺ stores, either Ni²⁺ or imidazole antimycotics had little or no effect on the uptake of Mn²⁺ (results not shown).

The effects of emptying and refilling the intracellular Ca²⁺ stores on the entry of extracellular Ca²⁺ and Mn²⁺ were also studied at the single-cell level. Figure 5 illustrates the [Ca²⁺]_i overshoot observed on addition of Ca²⁺ to cells with empty Ca²⁺ stores. The upper trace represents the average behaviour of 44 cells present in the same microscope field. Addition of Ca²⁺ to cells with empty Ca²⁺ stores produced a quick increase of [Ca²⁺]_i from 20 to 360 nM. [Ca²⁺]_i then decreased to about 100 nM within the next 4 min. After this period, in which the stores had been refilled, removal followed by re-addition of extracellular Ca²⁺ produced a much smaller [Ca²⁺]_i overshoot (from 50 to 85 nM), indicating that the plasma-membrane permeability to Ca²⁺ had decreased. The [Ca²⁺]_i peak induced by ionomycin addition in Ca²⁺-free medium (Figure 5a) demonstrates that the stores had been refilled with Ca²⁺. The average response described above arises from the addition of the individual behaviours of about 75% 'typical cells' (such as cell 9 in the Figure) and 25% 'atypical cells' (such as cell 12). These atypical cells showing no [Ca²⁺]_i overshoot on re-addition of Ca²⁺ after store depletion with ionomycin had no functional Ca²⁺ stores, either, as illustrated by the failure of ionomycin to increase [Ca²⁺]_i in Ca²⁺-free medium for cell 12 in the Figure. Figure 5 shows also that the

Figure 6 Effects of refilling the intracellular Ca²⁺ stores on the entry of Mn²⁺ into single HL60 cells

Solutions were changed as indicated in the Figure. The Ca²⁺ concentration ([Ca²⁺]_o) was either 1 mM or 0 (50 μ M EGTA). MnCl₂ was dissolved in nominally Ca²⁺-free solution at 0.5 mM. The perfusion with 1 mM Ca²⁺ in the right-hand panels was maintained for 5 min. F_{TOTAL} , which is insensitive to Ca²⁺ but quenched by Mn²⁺, was calculated as described in the Methods section.

presence of Ni²⁺ prevented the [Ca²⁺]_i overshoot. Removal of Ni²⁺ revealed that the increase in membrane permeability to Ca²⁺ was still present, as shown by the [Ca²⁺]_i overshoot, very similar to that obtained previously.

The entry of Mn²⁺ was always faster in cells with empty Ca²⁺ stores, although the rate varied considerably among different cells. For example, in 21 Ca²⁺-depleted cells visible in the same microscope field and exposed to 0.5 mM Mn²⁺ the rates of fluorescence quenching ranged between 0.6 and 3.5%/s (mean $\pm$ S.D. = 1.4 $\pm$ 0.7). The decrease in fluorescence with time was a first-order linear function (see, e.g., cell 'a' in Figure 6), suggesting that Mn²⁺ was entering the cells at a constant rate and that essentially all the Mn²⁺ was quickly bound by fura-2. The apparent decrease in the rate of fluorescence quenching with time often seen in the cuvette experiments (Figures 2–4) must then arise from the heterogeneity among cells, the 'fast' ones becoming fully quenched soon so that the subsequent 'mean' rate of quenching decreases.

Figure 6 shows records of quenching of fura-2 fluorescence by entering Mn²⁺ in a few representative single cells. In the left-hand panels the protocol included two 1 min pulses with medium containing 0.5 mM Mn²⁺, separated by a 1 min period with Ca²⁺- and Mn²⁺-free medium. In all the cases the intracellular stores had been first depleted of Ca²⁺ by a 60 min incubation in Ca²⁺-free medium. Cell 'a' was a 'fast' one which was completely quenched during the first Mn²⁺ pulse. In cells 'b' and 'c', in which the first Mn²⁺ pulse did not produce complete quenching, the rate of quenching during the second Mn²⁺ pulse was essentially the same as during the first one. In the right-hand panels (cells 'd', 'e' and 'f') the two Mn²⁺ pulses were separated by a 5 min perfusion with 1 mM Ca²⁺ in order to refill the intracellular Ca²⁺ stores. The rate of quenching decreased 5–10-fold after perfusion with 1 mM Ca²⁺. These results confirm, at

the single-cell level, that refilling of the intracellular Ca^{2+} stores decreases the rate of Mn^{2+} entry in HL60 cells.

The existence of a Ca^{2+} -store-regulated plasma-membrane Mn^{2+} permeability was also studied in U937 cells, both non-differentiated and differentiated towards monocytes-macrophages with phorbol 12-myristate 13-acetate. The results (not shown) were similar to those described for HL60 cells. The entry of Mn^{2+} was increased on emptying the stores with 1 nM ionomycin (as in Figure 3) or by prolonged incubation in Ca^{2+} -free medium (as in Figure 4). The acceleration of Mn^{2+} uptake was prevented by imidazole antimycotics (Table 1).

DISCUSSION

We show here that, as documented previously in human neutrophils [18], the capacitative mechanism proposed by Putney's group [11] may explain the agonist-induced Ca^{2+} entry in HL60 cells differentiated towards granulocytes by treatment with dimethyl sulphoxide. The $\text{Ca}^{2+}/\text{Mn}^{2+}$ pathway opened by fMLP and PAF seems to be the same as that opened by emptying the intracellular Ca^{2+} stores by prolonged incubation in Ca^{2+} -free medium or treatment with low concentrations of ionomycin, conditions which do not induce inositol phosphate production [34,35]. It has also been shown recently that inhibition of Ca^{2+} pumping to the stores by three different Ca^{2+} -ATPase inhibitors does also increase the plasma-membrane $\text{Ca}^{2+}/\text{Mn}^{2+}$ permeability with no modifications of cell InsP_3 [24]. In all the cases the bivalent-cation entry stimulated by either fMLP or the other store-emptying treatments had a similar sensitivity to inhibitors (this study, and ref. [24]).

The store-regulated plasma-membrane $\text{Ca}^{2+}/\text{Mn}^{2+}$ pathway was blocked by imidazole antimycotics, which are considered to be selective inhibitors of cytochrome *P*-450 [36,37]. The $\text{Ca}^{2+}/\text{Mn}^{2+}$ entry induced by Ca^{2+} -ATPase inhibitors has been reported to be prevented by SK&F 96365 [24], which we have reported to be also a cytochrome *P*-450 inhibitor [38]. The IC_{50} values found here (Table 1) were similar to those reported previously for inhibition of the store-regulated permeability in thymocytes [17], neutrophils [18] and platelets [21]. We have proposed in these cell types that the inhibitors act on a microsomal cytochrome *P*-450, which is activated by emptying the intracellular stores of Ca^{2+} , this leading to activation of the plasma-membrane Ca^{2+} channels (see details of the model in ref. [39]).

It should be noted that the mechanism for store-regulated permeability is present and functional in non-differentiated HL60 cells, even though membrane receptors for the agonists are not yet expressed. Similar results were found in the promyelocytic line U937. This suggests that its significance may be more general than a mere 'receptor-controlled' pathway, in the sense of a mechanism designed to allow Ca^{2+} entry when the Ca^{2+} stores are empty, whatever the cause is. This would sustain in time, if required, an increase in $[\text{Ca}^{2+}]_i$ initiated by discharge of the intracellular stores and would facilitate its refilling once the signal causing Ca^{2+} release has ceased.

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