Characterization of receptor-mediated and store-regulated Ca^{2+} influx in human neutrophils

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1. It is not known to what extent the emptying of intracellular Ca^{2+} stores participates in the mediation of chemoattractant-induced Ca^{2+} influx in human neutrophils. To study this question, we compared the properties of bivalent-cation influx in response to the chemoattractant *N*-formyl-L-methionyl-L-leucyl-L-phenylalanine (f-MLP) and to the microsomal Ca^{2+} -ATPase inhibitor thapsigargin. 2. The influx pathway activated by f-MLP and thapsigargin had identical properties of permeation. Mn^{2+} influx became saturated at around 1 mM extracellular Mn^{2+} , whereas Ca^{2+} influx did not become saturated up to concentrations of 10 mM. 3. The influx of the two bivalent cations, Mn^{2+} and Ca^{2+} , was activated to a similar extent and with identical kinetics of

activation. 4. The Mn^{2+} influx activated by f-MLP and thapsigargin was blocked, with identical dose-inhibition curves, by four imidazole analogues. 5. The same relationship between the emptying of Ca^{2+} stores and bivalent-cation influx was observed for f-MLP and thapsigargin, with a half-maximal activation of the influx at 40% emptying of intracellular stores. 6. In conclusion, neutrophils possess a single type of Ca^{2+} -influx pathway that is activated by receptor agonists and by store depletion. Receptor agonists activate this influx pathway to a large extent, if not completely, through the depletion of intracellular Ca^{2+} stores.

INTRODUCTION

Chemoattractants elevate the cytosolic free Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$) in human neutrophils. This elevation of $[\text{Ca}^{2+}]_i$ consists of an initial Ca^{2+} release from intracellular stores and a more delayed Ca^{2+} influx across the plasma membrane (Pozzan et al., 1983; Merritt et al., 1989; Sage et al., 1990). The chemoattractant-induced Ca^{2+} release is mediated by $\text{Ins}(1,4,5)\text{P}_3$ (Prentki et al., 1984). There is, however, considerable uncertainty concerning the mediation of Ca^{2+} influx. Neutrophils do not possess voltage-operated Ca^{2+} channels (Demaurex et al., 1992b), and their Ca^{2+} -influx pathway falls into the category of so-called receptor-mediated Ca^{2+} influx. The latter term designates a very heterogeneous group of Ca^{2+} -influx pathways. Receptor-mediated Ca^{2+} influx may be operated (i) directly by the receptor, (ii) by second messengers [i.e. $[\text{Ca}^{2+}]_i$ or $\text{Ins}(1,3,4,5)\text{P}_4$], (iii) by the filling state of intracellular Ca^{2+} stores, or (iv) by a combination of all three (for reviews see Rink, 1990; Taylor, 1990; Putney, 1990; Irvine, 1990; Meldolesi et al., 1991). Studies in neutrophils have shown that the depletion of intracellular Ca^{2+} stores, either by inhibitors of the Ca^{2+} pump of intracellular Ca^{2+} stores (Foder et al., 1989; Demaurex et al., 1992a,b) or by prolonged incubation of cells in Ca^{2+} -free medium (Montero et al., 1991), leads to activation of Ca^{2+} influx. Thus neutrophils clearly possess a store-regulated Ca^{2+} -influx pathway. However, it is not clear whether this pathway participates in the mediation of chemoattractant-induced Ca^{2+} influx, and, if so, to what extent. Indeed, the store-regulated Ca^{2+} influx might be a safety device that is operative only under circumstances of extreme Ca^{2+} depletion (Irvine, 1990; Clementi et al., 1992), rather than representing a mechanism of receptor-mediated Ca^{2+} influx.

Observations in other cell types add to the level of complexity. $\text{Ins}(1,4,5)\text{P}_3$ releases Ca^{2+} from intracellular stores and might thereby indirectly activate the store-regulated Ca^{2+} influx. How-

ever, some cell types appear to have $\text{Ins}(1,4,5)\text{P}_3$ -sensitive Ca^{2+} channels in the plasma membrane (Kuno and Gardner, 1987; Fujimoto et al., 1992; Khan et al., 1992; Sharp et al., 1992) and $\text{Ins}(1,4,5)\text{P}_3$ may therefore also directly activate Ca^{2+} influx. Some cell types possess several types of receptor-operated Ca^{2+} influx pathways, one of which shows similarities to store-regulated Ca^{2+} influx, whereas the others show different properties with respect to regulation, kinetics of activation, sensitivity to blockers and permeation of bivalent cations (Sage et al., 1992; Clementi et al., 1992).

In this study we have analysed neutrophil Ca^{2+} and Mn^{2+} influx in response to the Ca^{2+} -ATPase inhibitor thapsigargin as a model for store-regulated influx, and Ca^{2+} and Mn^{2+} influx in response to the chemotactic peptide *N*-formyl-L-methionyl-L-leucyl-L-phenylalanine (f-MLP) as a model for receptor-mediated influx. Our results favour a crucial role for store-regulated Ca^{2+} influx in the mediation of Ca^{2+} influx by the receptor agonist.

MATERIALS AND METHODS

Materials

f-MLP, thapsigargin, econazole, dimethyl sulphoxide (DMSO), Hepes and *NN*-bis-[2-[bis(carboxymethyl)amino]ethyl]glycine-penta-acetic acid (DTPA) were purchased from Sigma (St. Louis, MO, U.S.A.), and fura-2 (acetoxymethyl ester) was from Molecular Probes (Eugene, OR, U.S.A.). Isoconazole was supplied by Schering Aktiengesellschaft (Berlin, Germany), SK&F 96365 was provided by Dr. J. Merritt, SmithKline Beecham (Welwyn, Herts., U.K.), and SC 38249 was kindly given by Dr. U. T. Ruegg, Sandoz Chemicals (Basel, Switzerland) (for systematic names, see title-page footnote). Other chemicals were of analytical grade and were obtained from Sigma. The medium referred to as ' Ca^{2+} -free medium' contained (in mM): NaCl 138; KCl 6; MgCl_2 1; glucose 20; Hepes 20; pH 7.4. The medium referred to

Abbreviations used: $[\text{Ca}^{2+}]_i$, cytosolic free Ca^{2+} concentration; f-MLP, *N*-formyl-L-methionyl-L-leucyl-L-phenylalanine; DMSO, dimethyl sulphoxide; DTPA, *NN*-bis-[2-[bis(carboxymethyl)amino]ethyl]glycine-penta-acetic acid; SK&F 96365, 1- β -[3-(4-methoxyphenyl)propoxy]-4-methoxyphenethyl-1-*H*-imidazole hydrochloride; SC 38249, $(\pm)$ -1,2,3-bis[4-methoxyphenyl]methoxy]propyl-1-*H*-imidazole.

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Figure 1 Determination of Mn^{2+} influx in human neutrophils

Human neutrophils were loaded with the fluorescent Ca^{2+} indicator fura-2, and the fluorescence was measured at the Ca^{2+} -independent wavelength of fura-2 ($\lambda_{\text{ex}} = 360 \text{ nm}$). The trace is an example of determination of Mn^{2+} influx in cells exposed to $1 \mu\text{M}$ f-MLP. After the achievement of a stable baseline (F_0), 0.5 mM Mn^{2+} was added to cells at zero time in the graph. Mn^{2+} addition caused a rapid initial fall in fluorescence (ΔF_e), due to quenching of extracellular fura-2 by Mn^{2+} . The increase in fluorescence was then approximately linear during 40 s (from F_0 to F_{40}) and decayed exponentially thereafter. At the end of the experiment, DTPA (at a concentration corresponding to twice the added Mn^{2+}) was added to determine ΔF_e . Mn^{2+} influx was defined as $F_0 - F_{40} - \Delta F_e$.

as 'Ca $^{2+}$ medium' contained in addition 1.0 mM CaCl_2 . When drugs were added as DMSO solutions, the final concentration of DMSO in the recording medium did not exceed 0.1% .

Purification of neutrophils

Human neutrophils were obtained from the blood of healthy volunteers, and purified by dextran sedimentation and Ficoll density-gradient centrifugation, as previously described (Krause et al., 1985). Contaminating red cells were disrupted by hypotonic lysis, and the purified neutrophils were suspended at a concentration of 2×10^7 cells/ml in Ca^{2+} medium. Preparations routinely contained $> 95 \%$ viable neutrophils, as assessed by Trypan Blue exclusion.

Fluorescence measurements

Neutrophils were loaded with fura-2 by incubation with $2 \mu\text{M}$ fura-2/AM at 37°C for 45 min, then centrifuged and resuspended at 10^7 cells/ml in Ca^{2+} medium and kept at room temperature. Just before use, a 0.5 ml portion of the cell suspension was centrifuged and resuspended in 2.5 ml of the indicated medium. Experiments were performed on a Perkin-Elmer fluorimeter (LS3), thermostatically maintained at 37°C . Fluorescence emission was set at 505 nm , and fluorescence excitation (λ_{ex}) at 360 or 340 nm (see below).

Determination of Mn^{2+} influx

Mn^{2+} influx was measured by using the fura-2 quenching technique, as previously described (Demaurex et al., 1992a). Mn^{2+} was added to cells, and the Mn^{2+} -dependent decrease in intracellular fura-2 fluorescence (= quenching) was measured at the Ca^{2+} -independent wavelength of fura-2 ($\lambda_{\text{ex}} = 360 \text{ nm}$). Upon

Mn^{2+} addition, the fura-2 fluorescence decayed with an exponential time course, as shown in Figure 1(a) for the Mn^{2+} influx in response to $1 \mu\text{M}$ f-MLP. This exponential decay was also observed when the fluorescence changes were of small amplitude, and thus cannot be entirely accounted for by the non-linearity of the fura-2/ Mn^{2+} interaction. As preincubation of cells with Mn^{2+} greatly diminished, in a time-dependent manner, the f-MLP-induced Mn^{2+} influx (results not shown), this time course might be due to a time-dependent block of the f-MLP-stimulated Mn^{2+} influx by Mn^{2+} . Therefore, in our opinion, only the initial rate of Mn^{2+} entry is a parameter that truly reflects the opening state of the Ca^{2+} -influx pathway. However, the initial slope of fura-2 quenching is difficult to determine, because it is masked by a concomitant fluorescence decrease due to quenching of extracellular fura-2 fluorescence by Mn^{2+} . To standardize our measurement procedure and to avoid the use of an arbitrary selected 'initial slope', we quantified fura-2 quenching by measuring two parameters: (i) the fluorescence decrease within 40 s after Mn^{2+} addition ($= F_0 - F_{40}$), reflecting both Mn^{2+} entry into the cytosol and quenching of extracellular fura-2, and (ii) the fluorescence increase after addition of the heavy-metal chelator DTPA (ΔF_e), reflecting the 'unquenching' of the extracellular fura-2 fluorescence. Mn^{2+} influx was thus defined as: $F_0 - F_{40} - \Delta F_e$, as shown in Figure 1a, and expressed as decrease in fluorescence during 40 s (U/40 s).

Determination of Ca^{2+} influx

Ca^{2+} influx was measured by using conventional fura-2 measurements. Ca^{2+} was added to stimulated or non-stimulated cells in a nominally Ca^{2+} -free medium, and the Ca^{2+} -dependent increase in intracellular fura-2 fluorescence was measured at the Ca^{2+} -dependent wavelength of fura-2 ($\lambda_{\text{ex}} = 340 \text{ nm}$). Upon Ca^{2+} addition, the fluorescence increased with a sigmoidal time course (results not shown). This non-linear increase might be due to the non-linear behaviour of fura-2 fluorescence, to a stimulation of Ca^{2+} efflux (via activation of the plasma-membrane Ca^{2+} -ATPase), or to a feed-back inhibition of Ca^{2+} influx (through refilling of intracellular Ca^{2+} stores or through feed-back inhibition of the Ca^{2+} -influx pathway by a high $[\text{Ca}^{2+}]_i$). Therefore, the initial slope of the fluorescence increase reflects best the opening state of the Ca^{2+} -influx pathway at a given time point. As Ca^{2+} influx was very fast compared with Mn^{2+} influx, we measured the fluorescence increase during the first 10 s after Ca^{2+} addition. During this period, the fluorescence changes were linear (between 45% and 65% fura-2 saturation). At these fura-2 saturations, the fluorescence response of fura-2 upon Ca^{2+} binding is nearly linear (Grynkiewicz et al., 1985). We therefore preferred to express Ca^{2+} influx as changes in fluorescence units/time rather than as changes in $[\text{Ca}^{2+}]_i$ /time to allow comparison with the Mn^{2+} -influx data. A correction for the extracellular fura-2 was not necessary, as the latter was already saturated at the Ca^{2+} concentration ($\sim 5 \mu\text{M}$) of our nominally Ca^{2+} -free medium. Ca^{2+} influx was thus defined as $F_{10} - F_0$, and expressed as increase in fluorescence during 10 s (Fluo U/10 s).

Assessment of the filling state of intracellular stores

The Ca^{2+} content of intracellular stores was determined by adding the ionophore ionomycin ($1 \mu\text{M}$) to cells suspended in a Ca^{2+} -free medium. Under these circumstances, ionomycin induced a transient increase in Ca^{2+} -dependent fura-2 fluorescence, which was exclusively due to Ca^{2+} release from intracellular stores. We therefore estimated the amount of intracellularly stored Ca^{2+} by measuring the maximal amplitude of the ionomycin-induced fluorescence transient.

Data analysis and statistics

The fluorimetric data were presented on a strip-chart recorder and, when needed, were digitized on-line at a rate of 50 Hz by using a 12-bits A/D interface (Acqui, SICMU, Geneva, Switzerland), and stored on an AT computer for subsequent analysis. From these fluorescence traces, Mn²⁺ influx, Ca²⁺ influx and the filling state of intracellular stores were calculated as described above. Data shown are means $\pm$ S.E.M. of 3–5 different experiments. Statistical analysis was performed with Origin software (Northampton, MA, U.S.A.). The paired *t* test was used to compare the different conditions. When the experiments resulted in data not paired on *x* or *y* values (Figure 6), a computer fit was performed on one set of data. From this fit, values corresponding to the *x* values of the other set of data were extracted, and the paired *t* test was performed.

RESULTS

Permeation of bivalent cations

To analyse the permeation of bivalent cations in human neutrophils, we measured the influx of Mn²⁺ and Ca²⁺ ions at different extracellular Mn²⁺ or Ca²⁺ concentrations. Figure 2(a) shows the dependence of Mn²⁺ influx on the extracellular Mn²⁺ concentration, in cells stimulated with f-MLP (1 μ M), thapsigargin (30 nM) or with the solvent vehicle DMSO (0.1%). Figure 2(b) shows the dependence of Ca²⁺ influx on the extracellular Ca²⁺ concentration. Significant stimulation of influx by f-MLP and thapsigargin could be detected at extracellular concentrations as low as 10 μ M (Mn²⁺) or 50 μ M (Ca²⁺). Over the whole range of

Figure 2 Permeation of Mn²⁺ and Ca²⁺ in neutrophils stimulated by f-MLP or by thapsigargin

Cells were exposed to 1 μ M f-MLP (○), 30 nM thapsigargin (●) or 0.1% of the solvent vehicle DMSO (+) in a Ca²⁺-free medium. At 2 min after drug application, various concentrations of Mn²⁺ or Ca²⁺ were added, and the influx of bivalent cations was measured. (a) Dependence of Mn²⁺ influx on the extracellular Mn²⁺ concentration. (b) Dependence of Ca²⁺ influx on the extracellular Ca²⁺ concentration. Values are means $\pm$ S.E.M. of 3 different experiments; when not shown, the error bar is smaller than the symbol.

ion concentrations tested, f-MLP and thapsigargin induced an approx. 3.5-fold increase in the rate of entry of both Mn²⁺ and Ca²⁺ (Figures 2c and 2d). f-MLP and thapsigargin were equally potent in stimulating Mn²⁺ and Ca²⁺ influx (*P* = 0.93 and 0.96 respectively; paired *t* test). However, permeation of Mn²⁺ and Ca²⁺ showed distinct properties that did not depend on the stimulus: Mn²⁺ influx was half-maximally activated around 100 μ M extracellular Mn²⁺, and became saturated at around 1 mM (Figure 2a). In contrast, Ca²⁺ influx did not become saturated up to concentrations of 10 mM (Figure 2b).

Activation kinetics of bivalent-cation influx

The identical permeation properties of the f-MLP- and thapsigargin-activated bivalent-cation influx did not suggest heterogeneity of influx pathways. However, these studies were performed at one given time point after addition of the stimulus, and different influx pathways might be activated at early or late time points after agonist stimulation, as previously demonstrated in platelets (see the Discussion section). We therefore investigated the activation kinetics of the Mn²⁺ and Ca²⁺ influx (Figure 3). f-MLP caused a rapid (< 20 s) increase in the rate of entry of Mn²⁺, but the subsequent kinetics were different at low or at high external [Ca²⁺]. At low external [Ca²⁺], the f-MLP-induced Mn²⁺ influx continued to increase to a new steady-state value with a half-time of 120 s (Figure 3a), whereas at high external Ca²⁺ the rate of Mn²⁺ entry decreased, with similar kinetics, to control values (Figure 3b). In contrast with f-MLP, the kinetics of thapsigargin-induced increase in Mn²⁺ influx were slow and increased monotonically at high and at low external Ca²⁺.

Measurements of Ca²⁺ influx were performed by re-adding Ca²⁺ to cells stimulated in a Ca²⁺-free medium. Both f-MLP and thapsigargin caused a sustained increase in the rate of entry of Ca²⁺ (Figure 3c). The activation kinetics of Ca²⁺ influx closely paralleled the activation kinetics of Mn²⁺ influx (Figure 3d).

Apparent affinity of block by imidazole derivatives

In a further attempt to isolate components of receptor-mediated Ca²⁺ influx that differ from store-regulated influx, we have compared the inhibition of f-MLP and thapsigargin-induced Mn²⁺ influx by imidazole derivatives (Figure 4). Cells were incubated for 1 min with four different imidazole derivatives (isoconazole, econazole, SK&F 96365 and SC 38249), stimulated with f-MLP, thapsigargin or DMSO, and Mn²⁺ influx was assessed.

All imidazole derivatives inhibited, in a dose-dependent manner, the Mn²⁺ influx activated by f-MLP and thapsigargin, with an order of potency: isoconazole > econazole > SK&F 96365 > SC 38249. For each imidazole derivative, the concentration producing half-maximal inhibition of Mn²⁺ influx (IC₅₀) was derived from a Boltzman fit of the dose-inhibition curves. Values for IC₅₀ were, for f-MLP and thapsigargin respectively: isoconazole 0.17 $\pm$ 0.03 μ M and 0.16 $\pm$ 0.07 μ M; econazole 0.48 $\pm$ 0.19 μ M and 0.57 $\pm$ 0.23 μ M; SK&F 96365 3.1 $\pm$ 0.4 μ M and 2.8 $\pm$ 0.5 μ M; SC 38249 13.0 $\pm$ 2.6 μ M and 25 $\pm$ 17.8 μ M. No statistically significant differences between f-MLP and thapsigargin were observed for the inhibition by the imidazole derivatives (*P* > 0.5 for all conditions, paired *t* test).

Correlation between the filling state of intracellular Ca²⁺ stores and bivalent-cation influx

The results shown so far strongly suggest that f-MLP and thapsigargin activate the same Ca²⁺-influx pathway. However,

Figure 3 Time course of f-MLP- and thapsigargin-induced Mn^{2+} and Ca^{2+} influx

Cells were exposed to either 1 μ M f-MLP (○) or 30 nM thapsigargin (●) at zero time in the graphs, and Mn^{2+} (0.5 mM) or Ca^{2+} (1 mM) was added at different times after drug application to measure bivalent-cation influx. (a) Time course of Mn^{2+} influx, measured in a Ca^{2+} -free medium. (b) Time course of Mn^{2+} influx, measured in a Ca^{2+} -containing medium. (c) Time course of Ca^{2+} influx, measured in a Ca^{2+} -free medium. Values are means $\pm$ S.E.M. of 3 different experiments; lines were fitted to the data by eye. (d) Time course of the relative Mn^{2+}/Ca^{2+} influx, derived from the averaged respective rate of entry measured in a Ca^{2+} -free medium.

Figure 4 Block by imidazole derivatives of the Mn^{2+} influx activated by f-MLP or by thapsigargin

Cells were incubated for 1 min with various concentrations of the different imidazole derivatives in a Ca^{2+} -containing medium, and subsequently exposed to 1 μ M f-MLP (○), 30 nM thapsigargin (●), or 0.1% DMSO (x). Then 2 min later, 0.5 mM Mn^{2+} was added and Mn^{2+} influx was measured. (a) Dose-response for block by isoconazole. (b) Dose-response for block by econazole. (c) Dose-response for block by SKF 96365. (d) Dose-response for block by SC 38249. Values are means $\pm$ S.E.M. of 3 different experiments; when not shown, the error bar is smaller than the symbol. Lines were fitted to the data by eye.

Figure 5 Parallel dose-responses of the f-MLP- and thapsigargin-induced store depletion and Mn²⁺ influx

Cells were incubated for 8 min in a Ca²⁺-free medium with various concentrations of either f-MLP (white symbols) or thapsigargin (black symbols), and, in parallel experiments, store depletion and Mn²⁺ influx were simultaneously measured. (a) and (b): Parallel fura-2 fluorescence measurements at $\lambda_{ex} = 340$ nm (upper trace) and at $\lambda_{ex} = 360$ nm (lower traces) of cells preincubated with thapsigargin at the indicated concentrations. At zero time in the graphs (arrow), cells were exposed either to 1 μ M ionomycin to release Ca²⁺ from stores (upper traces, store Ca²⁺), or to 0.5 mM Mn²⁺ to determine Mn²⁺ influx (lower traces, Mn²⁺ influx). (c) Dose-responses of f-MLP for store depletion (○) and Mn²⁺ influx (□). (d) Dose-responses of thapsigargin for store depletion (●) and Mn²⁺ influx (■). In (c) and (d), left axis shows peak value of the ionomycin-induced fluorescence increase, taken as an estimate of the amount of store Ca²⁺, and the right axis shows Mn²⁺ influx. Values are means $\pm$ S.E.M. of 3 different experiments; when not shown, the error bar is smaller than the symbol. Lines were fitted to the data by eye.

this does not exclude the possibility that the physiological activation of this pathway by receptor agonists is mediated by a mechanism other than depletion of intracellular stores. To determine the relative contribution of store depletion in the mediation of f-MLP-induced influx, we have measured in parallel the filling state of intracellular stores and the rate of entry of Mn²⁺, at different f-MLP and thapsigargin concentrations.

Figures 5(a) and 5(b) show original fluorescence traces of parallel measurements of the Ca²⁺ content of intracellular stores (= ionomycin-releasable Ca²⁺, upper traces; $\lambda_{ex} = 340$ nm) and activation of the Ca²⁺-influx pathway (= Mn²⁺ quenching of fura-2 fluorescence, lower traces; $\lambda_{ex} = 360$ nm), at two different thapsigargin concentrations. Figures 5(c) and 5(d) show the dose-response of f-MLP and thapsigargin for store depletion (left axis) and Mn²⁺ influx (right axis). Increasing the concentration of f-MLP or thapsigargin increased Mn²⁺ influx and decreased, with a similar dose-response, the amount of ionomycin-releasable Ca²⁺. To correlate Mn²⁺ influx with store depletion, we have plotted the rate of Mn²⁺ entry against the filling state of stores. Data from Figure 5 were normalized in every experiment to the Mn²⁺ influx induced by 300 nM thapsigargin (taken as 100% Mn²⁺ influx) and to the fluorescence released by ionomycin in control cells (taken as 100% filled stores).

Figure 6 shows the correlation of store depletion with Mn²⁺ influx in cells stimulated with f-MLP or thapsigargin. No difference was observed between f-MLP and thapsigargin in the correlation of store depletion with Mn²⁺ influx ($P > 0.5$, paired t test). In both cases, half-maximal activation of Mn²⁺ influx was observed at 48% emptying of intracellular stores. A maximally

Figure 6 Correlation between filling state of intracellular Ca²⁺ stores and Mn²⁺ influx

Data are taken from Figure 5. For every experiment, the Mn²⁺ influx was normalized to the influx induced by 300 nM thapsigargin (taken as 100% influx), and the filling state of stores was normalized to control cells (taken as 100% filled stores). For each concentration of f-MLP (○) or thapsigargin (■), the Mn²⁺ influx is plotted against the percentage of store depletion. Values are means $\pm$ S.E.M. of 3 different experiments. The line is a computer fit of the form $y = 4.38 \pm 5.15e^{(x/22.54)}$, which fitted equally well the f-MLP data ($r = 0.98$) and the thapsigargin data ($r = 0.99$).

effective concentration of thapsigargin caused a 66% emptying of stores, whereas a maximal f-MLP concentration caused a

63 % emptying of stores. In accordance, a maximal thapsigargin concentration induced slightly more Mn^{2+} influx (100 %) than a maximal f-MLP concentration (90 %). Thus the f-MLP-induced Mn^{2+} influx, similar to the thapsigargin-induced Mn^{2+} influx, is strictly correlated with the depletion of intracellular Ca^{2+} stores.

DISCUSSION

In this study we characterize receptor-mediated Ca^{2+} influx in neutrophils and study its relationship with store-regulated Ca^{2+} influx. Our results suggest that (i) neutrophils have a Ca^{2+} -influx pathway which is poorly permeable to Mn^{2+} , (ii) receptor-mediated Ca^{2+} influx involves a single type of bivalent-cation influx pathway, (iii) receptor-mediated and store-regulated influx occur through the same pathway, and (iv) activation of receptor-mediated Ca^{2+} influx occurs predominantly through the depletion of intracellular Ca^{2+} stores.

Technical aspects of this study

As an assay for Mn^{2+} and Ca^{2+} influx, we measured the changes in intracellular fura-2 fluorescence upon addition of the ions to the extracellular medium. The observed fluorescence changes reflect changes in the cytosolic concentration of the respective ions and do not directly reflect the entry of ions through the plasma membrane. This is particularly important with respect to Ca^{2+} influx, because Ca^{2+} is rapidly removed from the cytosol by various mechanisms, and because a $[Ca^{2+}]_i$ increase might interfere with Ca^{2+} influx through refilling of Ca^{2+} stores or through blockage of the influx pathway. Similarly, prolonged exposure (> 1 min) of cells to Mn^{2+} also appears to interfere with bivalent-cation influx by an unknown mechanism (see the Materials and methods section). To overcome these limitations, we analysed only the early changes in fluorescence upon Mn^{2+} or Ca^{2+} addition (see the Materials and methods section). Measuring influx over short time periods minimized the contribution of the mechanisms discussed above, and measuring over a restricted range of fluorescence values minimized the errors due to the non-linear response of fura-2. By this technique, influx could be reliably measured over a broad range of values: Figure 2(a) shows that, although the basal and stimulated Mn^{2+} influx differ by a factor of 3.5, the observed dependence of Mn^{2+} influx on the extracellular Mn^{2+} concentration is identical in both conditions.

Thus Mn^{2+} and Ca^{2+} influx could be compared semi-quantitatively. However, the limitations of fluorescence measurements do not provide an estimate of the permeability values for Mn^{2+} and Ca^{2+} .

The receptor-mediated and store-regulated influx pathway is poorly permeable to Mn^{2+}

The influx pathway activated by f-MLP or by thapsigargin had identical properties of permeation, suggesting the activation of a common influx pathway. However, Mn^{2+} influx through this pathway saturated around concentrations of approx. 1 mM extracellular Mn^{2+} , whereas Ca^{2+} influx did not saturate up to a concentration of 10 mM extracellular Ca^{2+} . The saturation of the Mn^{2+} influx was not due to a saturation of our assay system, as higher rates of fura-2 quenching could be observed after addition of the ionophore ionomycin (results not shown), and was not due to a decreased activation of the influx pathway, as the increase over basal influx was virtually identical over the whole range of Mn^{2+} concentrations tested (Figure 2d). Thus the observed saturation is most likely due to a saturation of the Mn^{2+} fluxes through the influx pathway. Saturation of ion fluxes occurs when

the permeation through the ion-transporting pore is slower than the supply of ions from the surrounding medium, which is a function of the concentration of the ion, or more precisely of its ionic activity (for references, see Hille, 1992). The observed saturation is most likely due to a saturation of the Mn^{2+} fluxes through the influx pathway, suggesting, given the respective saturations of ~ 1 mM and > 10 mM, that Mn^{2+} permeates at least 10-fold slower than Ca^{2+} through the influx pathway. Consistent with a slow permeation of Mn^{2+} , the observed fluorescence changes upon Mn^{2+} addition are of small amplitude and slower than the changes observed upon Ca^{2+} addition, despite the very high affinity of fura-2 for Mn^{2+} (approx. 5 nM, compared with 250 nM for Ca^{2+} ; Grynkiewicz et al., 1985).

Other types of receptor-mediated Ca^{2+} -influx pathways have different characteristics of Mn^{2+} and Ca^{2+} permeation. Some pathways appear not to conduct Mn^{2+} at all (Sage et al., 1992; Llopis et al., 1992), whereas others have an equal permeability to Ca^{2+} and Mn^{2+} , with no saturation of Mn^{2+} influx up to Mn^{2+} concentrations of 140 mM (Lückhoff and Clapham, 1992).

Evidence for a store-regulated Ca^{2+} channel with low Mn^{2+} permeability exists in mast cells: electrophysiological studies (which have equal sensitivity for Ca^{2+} and Mn^{2+}) detect only Ca^{2+} but not Mn^{2+} currents in this cell type (Hoth and Penner, 1992), whereas fluorescence measurements (which show an exquisite sensitivity for Mn^{2+} because of the high affinity of the probe for this ion) detect the permeation of both Mn^{2+} and Ca^{2+} (Fasolato et al., 1993). Our results suggest that, similarly to mast cells, neutrophils have a Ca^{2+} -influx pathway which is poorly permeable to Mn^{2+} .

Mn^{2+} and Ca^{2+} influx have identical kinetics of activation

In some cell types, both Mn^{2+} -impermeable and Mn^{2+} -permeable receptor-mediated Ca^{2+} -influx pathways coexist, which may be distinguished on the basis of their different kinetics (Sage et al., 1989; Llopis et al., 1992; Clementi et al., 1992). In neutrophils, we did not find any discrepancy between the time courses of f-MLP-stimulated Ca^{2+} and Mn^{2+} influx, although the relatively low time resolution of our measurements would not allow the detection of a very rapidly activating and inactivating component. However, consistent with our results, previous studies in human neutrophils using stopped-flow fluorimetry have demonstrated that the kinetics of activation of Ca^{2+} and Mn^{2+} influx were comparable (Sage et al., 1990), arguing against the transient activation of an Mn^{2+} -impermeant pathway at early times after f-MLP stimulation. Our observation that, for both f-MLP and thapsigargin, the ratio of Mn^{2+} influx to Ca^{2+} influx remains constant over the whole time period tested, strongly suggests the activation of a single pathway over the whole time course of Ca^{2+} influx.

Receptor-mediated and store-regulated influx are equally sensitive to block by imidazole derivatives

In addition to having identical properties of permeation, f-MLP- and thapsigargin-activated influx pathways are equally sensitive to inhibition by four different imidazole derivatives. These compounds are relatively non-selective inhibitors: in addition to some types of receptor-mediated Ca^{2+} influx (Merritt et al., 1990; Ciardo and Meldolesi, 1990; Garcia-Sancho et al., 1992), they also block voltage-operated Ca^{2+} channels (Merritt et al., 1990; Ciardo and Meldolesi, 1990; Villalobos et al., 1992), Ca^{2+} -activated K^+ channels (Alvarez et al., 1992) and cytochrome P-450 enzymes (Rodrigues et al., 1987; Yoshida and Aoyama, 1987). However, despite this relative lack of selectivity, the

sensitivity to imidazole derivatives is a useful tool to distinguish different types of receptor-mediated Ca²⁺ influx. Some types of receptor-mediated Ca²⁺ channels, such as the *N*-methyl-D-aspartate-receptor Ca²⁺ channel in cerebellar neurons and the ATP-receptor-operated Ca²⁺ channels in PC12 cells, are insensitive to imidazoles (Ciardo and Meldolesi, 1990; Fasolato et al., 1990).

In neutrophils, both the receptor-mediated and the store-regulated Ca²⁺ influx are equally sensitive to block by four different imidazole analogues. This contrasts with results obtained in other cellular systems. Carbachol-stimulated PC12 cells and ADP- and thrombin-stimulated platelets show Ca²⁺-influx components that are resistant to imidazole derivatives, whereas the thapsigargin-induced Ca²⁺ influx in the same cells can be efficiently inhibited by these blockers (Sargeant et al., 1992; Sage et al., 1992; Clementi et al., 1992). This suggests that in certain cell types, such as PC12 cells and platelets, several types of receptor-mediated Ca²⁺-influx pathways exist, whereas neutrophils appear to have a single type of receptor-mediated Ca²⁺-influx pathway.

Activation of the influx pathway occurs through the depletion of intracellular stores

The different kinetics of activation of receptor-mediated and store-regulated bivalent-cation influx suggest that the refilling of intracellular Ca²⁺ stores is the signal that terminates Ca²⁺ influx. Refilling of intracellular Ca²⁺ stores does not occur in cells exposed to thapsigargin and in cells exposed to f-MLP in a Ca²⁺-free medium, and Mn²⁺ influx is sustained in these conditions. In contrast, refilling of intracellular Ca²⁺ stores occurs in cells exposed to f-MLP in a Ca²⁺ medium (Montero et al., 1991), and Mn²⁺ influx is transient in this condition. Interestingly, in Ca²⁺-free medium Ca²⁺-influx activation by f-MLP appeared, after a rapid phase completed within less than 1 min, to increase continuously for another 3–4 min. As in these conditions f-MLP-induced PLC activation, inositol phosphate generation and Ca²⁺ release are terminated within 1–2 min (Pittet et al., 1989), this time course might be indicative of the generation of a transient inhibitory, rather than stimulatory, signal of Ca²⁺ influx activation by f-MLP. Indeed, a transient inhibitory signal generated by f-MLP has been described (Montero et al., 1993) and is presumably due to protein kinase C activation (McCarthy et al., 1989; Foder et al., 1989).

Most importantly, the parallel measurements of Ca²⁺-store depletion and Mn²⁺ influx demonstrated that, for both f-MLP and thapsigargin, Mn²⁺ influx strictly correlated with store depletion. To our knowledge, this is the first quantitative study that compares the relationship between store depletion and Ca²⁺ influx in response to an agonist and to a Ca²⁺-ATPase inhibitor. Studies in other cell types have shown that receptor occupancy activates Ca²⁺ influx partially, or even predominantly, by mechanisms other than store depletion (see above). In contrast, in neutrophils store depletion is clearly the predominant mechanism of receptor-mediated Ca²⁺ influx. How can these diverging observations in different cell types be reconciled? Given its widespread tissue and species distribution, store-regulated Ca²⁺ influx has probably developed early in evolution, possibly in parallel with intracellular Ca²⁺ stores to provide an efficient mechanism of refilling. Some cell types have kept the function of store-regulated Ca²⁺ influx restricted to this refilling of Ca²⁺

stores and have developed other mechanisms (such as second-messenger-operated, receptor-operated and voltage-operated Ca²⁺ channels) to achieve stimulated Ca²⁺ influx. In contrast, neutrophils, and probably some other cell types, have exploited store-regulated Ca²⁺ influx in a way that makes it the predominant, if not the only, source of stimulated Ca²⁺ influx.

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