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Uptake of Ca^{2+} and refilling of intracellular Ca^{2+} stores in Ehrlich-ascites-tumour cells and in rat thymocytes

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We have studied the uptake of Ca^{2+} and its redistribution between the cytoplasm and the intracellular stores in Ehrlich-ascites-tumour cells and rat thymocytes previously depleted of Ca^{2+} by incubation in Ca^{2+}-free medium. Measurements included changes of the cytoplasmic Ca^{2+} concentration ($[Ca^{2+}]_i$), uptake of $^{45}Ca^{2+}$ and uptake of Mn^{2+}, a Ca^{2+} surrogate for Ca^{2+} channels. Refilling of the Ca^{2+} stores in thymocytes was very fast (half-filling time: 4 s at 37 °C) and very sensitive to temperature (10 times slower at 20 °C). It was always preceded by an increase of $[Ca^{2+}]_i$. In the Ehrlich cell, both refilling and increase of $[Ca^{2+}]_i$ were about one order of magnitude slower. The increase of $[Ca^{2+}]_i$ and the refilling of the intracellular stores were both almost completely blocked by Ni^{2+} in thymocytes, but only partially in the Ehrlich cell. The rates of $^{45}Ca^{2+}$ and Mn^{2+} uptake varied consistently with temperature and the kind of cell. These results suggest that the intracellular stores are refilled by Ca^{2+} taken up from the cytoplasm. We also find that filling of the Ca^{2+} stores decreases by about 90% the rate of Mn^{2+} uptake in thymocytes. This is direct evidence of modulation of the plasma-membrane Ca^{2+} entry by the degree of filling of the intracellular stores. This modulation occurs in the absence of agonists, suggesting some kind of signalling between the intracellular stores and the Ca^{2+} entry pathways of the plasma membrane.

INTRODUCTION

Many cell agonists, including hormones and neurotransmitters, produce transient increases of the cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$) by releasing Ca^{2+} from intracellular stores [1–3]. Whereas the role of the inositol phosphates in Ca^{2+} release is widely acknowledged [4,5], the mechanism for refilling of these stores after stimulation is scarcely known. Extracellular Ca^{2+} seems to be essential for refilling. The conventional view is that Ca^{2+} is taken up first to the cytoplasm and then from there to the intracellular stores. However, it has been proposed recently that Ca^{2+} could be taken up directly from the extracellular medium to the endoplasmic reticulum [6–8]. Activation of Ca^{2+} influx by agonists has been proposed on the basis of comparisons of the changes of $[Ca^{2+}]_i$ or of agonist actions in the presence and in the absence of extracellular Ca^{2+} [6,9], of measurements of ^{45}Ca uptake [10,11] or of measurements of the uptake of Mn^{2+}, a Ca^{2+} surrogate for Ca^{2+} channels [12–14]. Refilling should occur either by uptake of Ca^{2+} from the cytoplasm, the uptake through the cell membrane remaining at basal rate [11,15], or through a Ca^{2+} pathway that remains activated for some time after the releasing effect of the agonist has vanished [6,10]. If such a pathway were regulated by intracellular messengers, like inositol phosphates, then the levels of these compounds should remain increased for some time after release has finished. It has also been proposed recently that emptying of the Ca^{2+} stores could activate Ca^{2+} entry, either directly to the Ca^{2+} stores through a pathway analogous to a gap junction [6] or into the cytoplasm. Mechanisms proposed for the last include either an unknown mediator [16,17] or a decrease of $[Ca^{2+}]_i$ at the inner surface of the plasma membrane, resulting in activation of a plasma-membrane Ca^{2+}-influx mechanism [18]. The experiments referred to above were carried out by emptying the intracellular Ca^{2+} pools with agonists. Consequently a direct or indirect role of the agonists on the pool-dependent Ca^{2+} entry cannot be excluded.

Here we have compared the kinetics of the uptake of Ca^{2+} through the plasma membrane, the changes of $[Ca^{2+}]_i$ and the filling of the intracellular stores. The intracellular Ca^{2+} pools were depleted by incubation in Ca^{2+}-free medium before the measurements. This condition simulates the state of the cell after stimulation, but in the absence of agonist-induced second messengers. Our results indicate that Ca^{2+} stores are refilled by Ca^{2+} taken up from the cytoplasm and that the plasma-membrane Ca^{2+} entry is regulated by the degree of filling of the intracellular stores. This regulation occurs in the absence of agonists, suggesting direct signalling between the intracellular stores and the plasma membrane.

METHODS

Ehrlich-ascites-tumour cells [19] and rat thymocytes [20] were prepared as described previously. The cells, either Ehrlich cells or thymocytes, were suspended at 5% cytocrit in standard incubation medium containing (in mM): NaCl, 150; $MgCl_2$, 1; sodium pyruvate, 10; potassium Hepes, 10, pH 7.4. For measurements of $^{45}Ca^{2+}$ uptake, intracellular Ca^{2+} stores were first depleted by incubation at room temperature for 1 h in standard medium containing 0.2 mM-EGTA. Then the cells were washed twice and resuspended at 5% cytocrit in standard incubation medium (containing no EGTA). The experiment was started by adding 1 mM-$CaCl_2$ containing $^{45}Ca^{2+}$ as the tracer (sp. radioactivity 10^{10} c.p.m./mol.). Samples (0.1 ml) were taken after different incubation times and washed twice with 1 ml of standard medium containing 0.5 mM-EGTA. Cell pellets were extracted with 0.6 ml of 6% trichloroacetic acid, and 0.5 ml of the supernatant was used for liquid-scintillation counting of radioactivity. In some experiments the cells were first loaded with quin2 by adding 0.2 mM-quin2 acetoxymethyl ester (quin2/AM) during the initial 1 h Ca^{2+}-depleting incubation. For measurement of quin2 concentration in the cells, 0.2 ml of the final cell suspension was mixed with 0.8 ml of water, heated for 10 min at 100 °C and centrifuged for 5 min at 12000 *g*. Fluorescence (340 nm excitation; 500 nm emission) of the supernatant in the

Abbreviations used: $[Ca^{2+}]_i$, cytoplasmic free Ca^{2+} concentration; /AM, acetoxymethyl ester; $R_{340/380}$, ratio of fluorescences at 340 and 380 nm.

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presence of either 1 mM-Ca^{2+} (maximum fluorescence) or 1 mM-Mn^{2+} (quenched fluorescence) was then compared with a standard of quin2 free acid.

For measurements of $[Ca^{2+}]_i$, both cell types were first loaded with fura-2 by incubation at 1% cytocrit in standard medium containing 2 μM-fura-2/AM for 30 min at room temperature. Then the cells were washed twice and resuspended at 1% cytocrit in standard medium. Incubation in EGTA-containing medium was omitted in this case, since it was found that Ca^{2+} depletion took place during this preparation procedure. $[Ca^{2+}]_i$ measurements were performed at constant temperature (17–37 °C) in 0.5 ml aliquots of the cell suspension under magnetic stirring using a fluorescence spectrophotometer constructed by Cairn Research (Newnham, Sittingbourne, Kent, U.K.). The system allows quick (30–300 Hz) alternation of up to six excitation wavelengths and separate reading of the corresponding fluorescence emission. Fluorescence emission was set at 530 nm. Readings were integrated at 1 s intervals. $[Ca^{2+}]_i$ was calculated from the ratio of fluorescences excited at 340 and 380 nm ($R_{340/380}$; [21]). The uptake of Mn^{2+} was monitored by the quenching of fura-2 fluorescence excited at 360 nm. At this excitation wavelength, changes of $[Ca^{2+}]_i$ do not modify fluorescence emission. On the other hand, quenching by Mn^{2+} does not modify $R_{340/380}$. This allows simultaneous and independent measurement of $[Ca^{2+}]_i$ and Mn^{2+} uptake. This procedure has been described in detail elsewhere [12,13].

$^{45}Ca^{2+}$ was obtained from Amersham International. Fura-2/AM was obtained from Molecular Probes, Eugene, OR, U.S.A. Quin2/AM and quin2 free acid were obtained from Sigma. Ionomycin was purchased from Calbiochem. Other chemicals were obtained either from Sigma or Merck.

RESULTS

Experimental approach

We measured four parameters in order to monitor the uptake and pool (cytoplasm/Ca^{2+} stores) redistribution of Ca^{2+} into the cells. The uptake of ^{45}Ca reflects the total incorporation into the cell, but does not give information on pool distribution. When cells heavily loaded with quin2 are used, the size of the cytoplasmic pool is increased. The data obtained under this condition then reflect mainly the uptake into the cytoplasmic pool. Mn^{2+} is a Ca^{2+} surrogate for several Ca^{2+}-transport mechanisms [12–14; 22]. Mn^{2+} uptake measures, then, influx through the plasma membrane into the cytosol. In the experimental design in which Ca^{2+} is added to Ca^{2+}-depleted cells, the changes of $[Ca^{2+}]_i$ mainly reflect uptake from the medium to the cytoplasm minus extrusion by the Ca^{2+} pump minus uptake to the cell stores. Finally, the degree of filling of the intracellular stores was estimated here from the increase of $[Ca^{2+}]_i$ after addition of the Ca^{2+} ionophore ionomycin to cells incubated in EGTA-containing medium.

$[Ca^{2+}]_i$ increase after addition of Ca^{2+} to Ca^{2+}-depleted cells

Ca^{2+}-depleted cells incubated in nominally Ca^{2+}-free medium (about 10 μM-Ca^{2+}) had low $[Ca^{2+}]_i$, usually between 30 and 60 nM. Fig. 1 shows that addition of 1 mM-Ca^{2+} to either Ehrlich cells (Fig. 1*a*) or thymocytes (Fig. 1*b*) induced an increase of $[Ca^{2+}]_i$ that reached a new steady state at about 100–120 nM-Ca^{2+}. The time course of the $[Ca^{2+}]_i$ increase was, however, very different between the two cells. In the Ehrlich cell the half-time was (mean ± S.D.; $n = 3$) 94 ± 9 s at room temperature (about 20 °C). The increase of $[Ca^{2+}]_i$ was much faster in thymocytes, where the half-time was only 10 ± 1 s ($n = 4$) at room temperature. At 37 °C the increase of $[Ca^{2+}]_i$ in thymocytes was even faster (half-time 1.6 ± 0.3 s; $n = 7$). At this temperature $[Ca^{2+}]_i$ reached a maximum at about 6 s and then decreased slowly to the steady state. This suggests hysteresis of either the membrane Ca^{2+} permeability or the Ca^{2+} pumping. Experiments with fura-2-loaded Ehrlich cells could not be carried out at 37 °C because of rapid leakage of fura-2 at this temperature. Fig. 1 shows also that 1 mM-Ni^{2+} produced a partial inhibition of the $[Ca^{2+}]_i$ increase in Ehrlich cells and a complete inhibition in thymocytes.

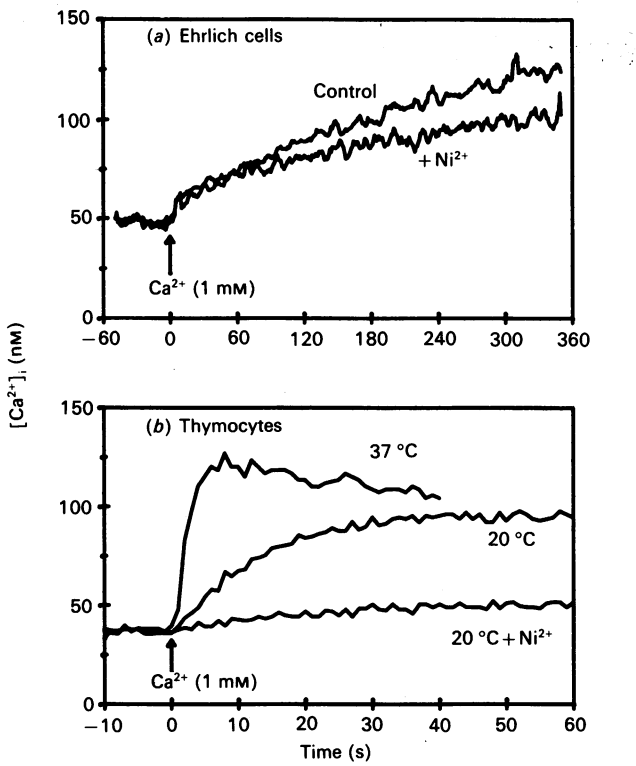

Fig. 1. Effects of the addition of Ca^{2+} (1 mM) on the intracellular Ca^{2+} concentration in fura-2-loaded Ca^{2+}-depleted Ehrlich cells (*a*) or in rat thymocytes (*b*)

The experiment with the Ehrlich cells was performed at 20 °C. For thymocytes, results obtained at 20 °C and 37 °C are shown. $NiCl_2$ (1 mM) was added immediately before Ca^{2+}. Note the differences in the time scales. The decrease of $[Ca^{2+}]_i$ in thymocytes after the first 5–10 s incubation with Ca^{2+} was observed in all the experiments.

Refilling of the intracellular Ca^{2+} stores

In a series of experiments, fura-2-loaded cells were allowed to refill their stores by incubation with 1 mM-Ca^{2+} for several periods of time. Then excess EGTA and ionomycin were added. The amount of Ca^{2+} present in the intracellular stores was estimated in each case from the peak of $[Ca^{2+}]_i$ obtained after addition of ionomycin. Fig. 2 shows the results of typical experiments performed at room temperature in Ehrlich cells (Fig. 2*a*) and in rat thymocytes (Fig. 2*b*). Ionomycin produced a $[Ca^{2+}]_i$ peak 5–10 s after its addition. The magnitude of this peak increased with the time of preincubation with Ca^{2+}. If no external Ca^{2+} was added (curves A and a) basal $[Ca^{2+}]_i$ was lower and ionomycin had little or no effect, indicating that the intracellular Ca^{2+} stores were empty. In the Ehrlich cell, full refilling of the intracellular stores required about 60 min, whereas only about 2 min was sufficient in thymocytes. Fig. 3 represents data from similar experiments, but in this case the magnitude of the $[Ca^{2+}]_i$ peak was plotted against the time of preincubation with 1 mM-Ca^{2+}. In the Ehrlich cell (Fig. 3*a*) the half-time for the refilling calculated from three similar experiments was (mean ± S.D.)

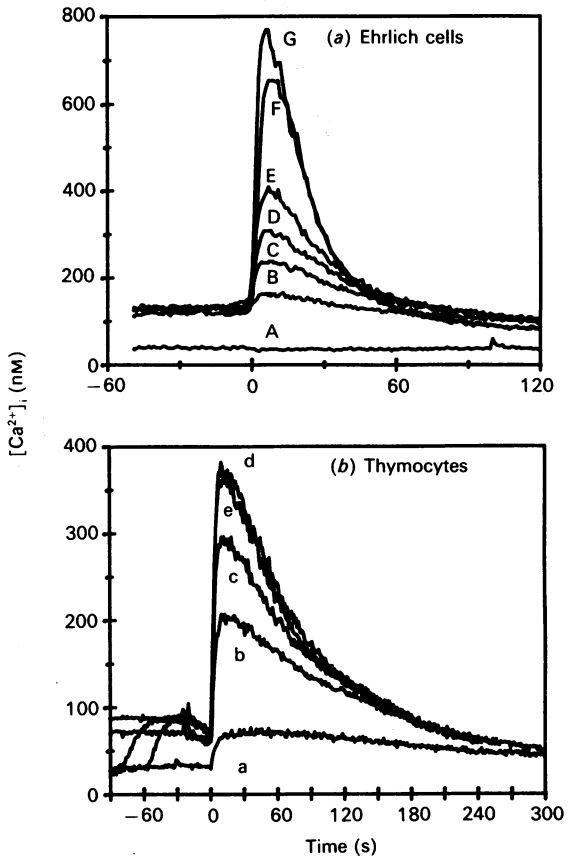

Fig. 2. Refilling of intracellular Ca^{2+} stores after different periods of incubation with 1 mM-Ca^{2+} at 20 °C in fura-2-loaded Ca^{2+}-depleted Ehrlich cells (*a*) or in rat thymocytes (*b*)

The cells were incubated with 1 mM-Ca^{2+} for different time periods and then 5 mM-EGTA and 200 nM-(Ehrlich cells) or 30 nM-(thymocytes) ionomycin was added. The addition of ionomycin corresponds to zero time in the Figure. The incubation periods with Ca^{2+} before the addition of ionomycin were (in min): Ehrlich cells: A, none; B, 5; C, 10; D, 15; E, 20; F, 50; G, 65. Thymocytes: a, none; b, 0.5; c, 1; d, 2; e, 5. Note the differences in both time and $[Ca^{2+}]_i$ scales. In curves b and c the increase of $[Ca^{2+}]_i$ after Ca^{2+} addition can be seen. In all the others most of the $[Ca^{2+}]_i$ increase had taken place before the earliest times shown in the Figure.

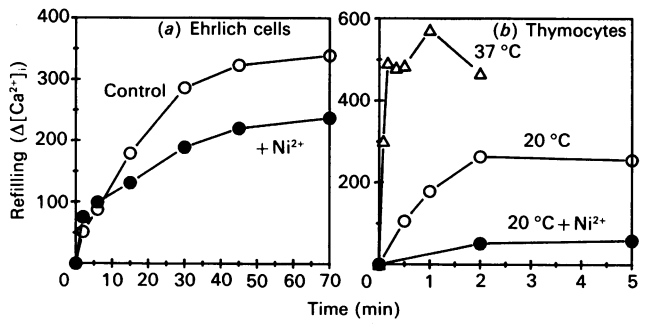

Fig. 3. Time course of intracellular Ca^{2+} stores refilling in the Ehrlich cells (*a*) and in rat thymocytes (*b*)

Experiments were performed as described in the legend to Fig. 2. Refilling is expressed as the increase of $[Ca^{2+}]_i$ (in nM) over the basal level at the peak after ionomycin addition. Incubation time with 1 mM-Ca^{2+} before the addition of ionomycin is plotted on the abscissa. The experiments with the Ehrlich cells were performed at 20 °C. For thymocytes, two incubation temperatures are shown, as labelled in the Figure. Note the differences in both scales for each kind of cells.

14 ± 8 min. The presence of 1 mM-Ni^{2+} produced only a small inhibition of the refilling. Fig. 3(*b*) shows the time course of refilling of the intracellular Ca^{2+} stores in rat thymocytes at room temperature and 37 °C, and the effect of 1 mM-Ni^{2+}. The half-times obtained were 40 s at room temperature, more than one order of magnitude faster than in the Ehrlich cell, and 4 s at 37 °C. In contrast with the Ehrlich cells, 1 mM-Ni^{2+} almost completely prevented the refilling of intracellular stores in thymocytes.

$^{45}Ca^{2+}$ uptake into Ca^{2+}-depleted cells

Fig. 4(*a*) shows a representative experiment with either Ca^{2+}-depleted or Ca^{2+}-depleted and quin2-loaded Ehrlich cells. Loading with millimolar concentrations of quin2 was performed in order to estimate the rate of $^{45}Ca^{2+}$ uptake without interference by back Ca^{2+} pumping. The incubation temperature was 37 °C. Cells loaded with quin2 showed linear uptake during the first 20 min. By contrast, $^{45}Ca^{2+}$ uptake did not progress after the 2 min in cells not loaded with quin2. Control experiments showed that, after the addition of ionomycin and excess EGTA, the background $^{45}Ca^{2+}$ remaining in the cells amounted to about 20 μmol/litre of cells (results not shown). This suggests that a large proportion (about 50%) of $^{45}Ca^{2+}$ uptake in cells not loaded with quin2 is 'artefactual' and that the uptake is probably linear from the origin in the quin2-loaded cells. On the basis of these premises, the rates estimated in quin2-loaded cells in several similar experiments were (in μmol/min per litre of cells; mean $\pm$ S.D..; $n = 3$) 2.3 ± 0.4 at room temperature and 6 ± 2 at 37 °C. Addition of ionomycin produced a decrease in the $^{45}Ca^{2+}$ content in cells not loaded with quin2 (Fig. 4). This paradoxical effect has been described previously, and it is due to the release of Ca^{2+} from the intracellular stores and subsequent pumping to the external medium [23]. In quin2-loaded cells, ionomycin produced an increase of $^{45}Ca^{2+}$ uptake. In this case most of the $^{45}Ca^{2+}$ taken up is bound by quin2 and hence 'not seen' by the Ca^{2+} pump.

Fig. 4(*b*) shows a similar experiment performed in rat thymocytes at room temperature. The uptake of $^{45}Ca^{2+}$ was linear for the first 2 min in the quin2-loaded cells. The estimated value for the rate of uptake was (mean $\pm$ S.D.; $n = 3$) 32 ± 2 μmol/min per litre of cells. The uptake in cells not loaded with quin2 did not progress after the first 1 min. Ionomycin here also increased the uptake of $^{45}Ca^{2+}$ by quin2-loaded cells.

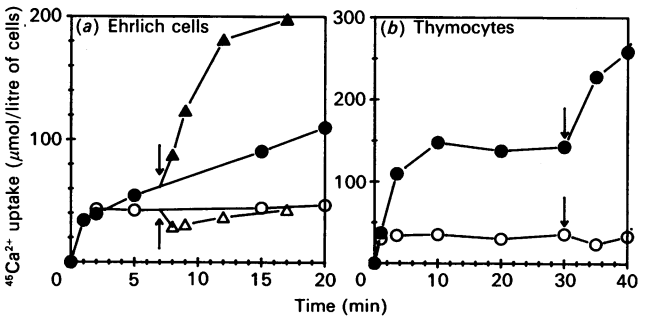

Fig. 4. $^{45}Ca^{2+}$ uptake by Ca^{2+}-depleted Ehrlich cells (*a*) and rat thymocytes (*b*)

The experiments with Ehrlich cells were performed at 37 °C, and those with thymocytes at 20 °C. In all the cases the concentration of Ca^{2+} was 1 mM. Ionomycin was added at the times shown by the arrows to give final concentrations of either 200 nM (Ehrlich cells) or 10 nM (thymocytes). Experiments were performed with either control cells (open symbols) or cells first loaded with quin2 (closed symbols). The contents of quin2 in the cells were (in mmol/litre of cells) 1.22 in Ehrlich cells and 1.43 in thymocytes. The values of uptake for Ehrlich cells treated with ionomycin are shown by triangles.

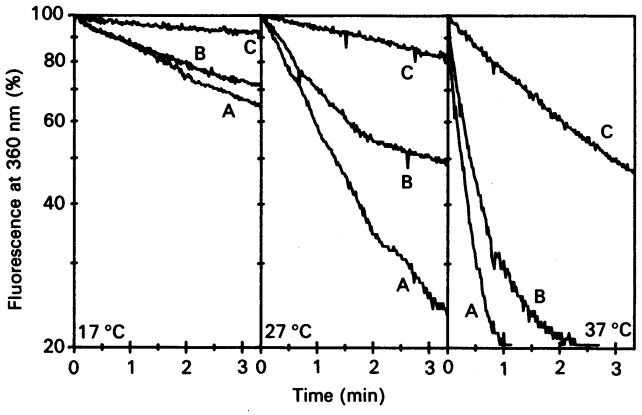

Fig. 5. Uptake of Mn^{2+} by fura-2-loaded thymocytes

Fluorescence emission excited at 360 nm is shown on the ordinate (note the logarithmic scale). The values were normalized to 100% just before Mn^{2+} addition. Quenching of fluorescence is proportional to Mn^{2+} uptake. Experiments performed at three different temperatures (17, 27 and 37 °C) with the same batch of cells are shown from left to right. In all the cases the cells had previously been depleted of Ca^{2+} by incubation in nominally Ca^{2+}-free medium (about 10 μM-Ca^{2+}). In curves labelled A, 1 mM-$MnCl_2$ was added at zero time. In curves labelled B, $CaCl_2$ and $MnCl_2$, each at 1 mM, were added simultaneously at zero time. In curves labelled C, Ca^{2+} was added either 5 min (experiments at 17 °C and 27 °C) or 1 min (experiments at 37 °C) before Mn^{2+}; zero time corresponds to Mn^{2+} addition. Half-times for Mn^{2+} uptake in these experiments are shown in Table 1.

Table 1. Half-times of the initial rates of the Mn^{2+} uptake by rat thymocytes

Data were calculated from the experiments shown in Fig. 5.

Condition	Temperature (°C)...	Half-time (s) 17	27	37
Mn^{2+} alone		300	81	14
$Mn^{2+}+Ca^{2+}$ simultaneously		300	119	20
First Ca^{2+} and then Mn^{2+} (% inhibition)		1817 (83)	723 (89)	176 (92)

Mn^{2+} uptake

As commented on above, Mn^{2+} has been used here as a Ca^{2+} surrogate for entry through the plasma membrane. Moreover, we have taken advantage of the fact that the Mn^{2+}-uptake procedure can be used to estimate Ca^{2+} fluxes in both Ca^{2+}-depleted and Ca^{2+}-depleted and then refilled cells. The aim of the experiments described below was to test whether or not the refill of the Ca^{2+} stores produces a decrease in the plasma-membrane permeability to Ca^{2+} (Mn^{2+}). The Ehrlich cell was not a good model for this study because Ca^{2+}-depleted cells had a very slow rate of Mn^{2+} uptake at room temperature (half-time 7.7 ± 0.8 min; mean $\pm$ S.D.; $n = 4$; results not shown). On the other hand, leakage of fura-2 made the experiments at 37 °C difficult. In thymocytes, Mn^{2+} uptake was much faster and highly dependent on temperature. Fig. 5 shows the entry of Mn^{2+} into thymocytes at 17, 27 and 37 °C. The rate of Mn^{2+} uptake (curve A) increased 21-fold from 17 to 37 °C (see also Table 1). Preincubation of the cells with 1 mM-Ca^{2+} for 5 min at 17 and 27 °C or for 1 min at 37 °C inhibited Mn^{2+} uptake by about 90% at every temperature

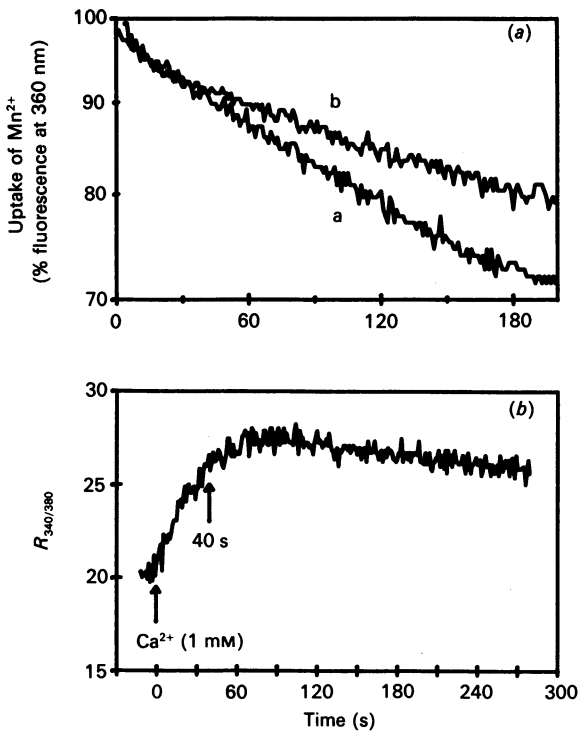

Fig. 6. Effect of a 40 s preincubation with Ca^{2+} on the uptake of Mn^{2+} by Ca^{2+}-depleted thymocytes loaded with fura-2

(*a*) Compares the uptake of Mn^{2+} when $CaCl_2$ and $MnCl_2$ (1 mM each) were added simultaneously (curve a) and when Ca^{2+} was added 40 s before Mn^{2+} (curve b); zero time corresponds to the addition of Mn^{2+}. Other details were as in Fig. 5. (*b*) Shows the time course of $R_{340/380}$ changes after addition of 1 mM-Ca^{2+} (at the first arrow) in the same batch of cells to illustrate that $[Ca^{2+}]_i$ was already at or near its maximum concentration 40 s after the Ca^{2+} addition (second arrow). Experiments were performed at 17 °C.

(curve C, Fig. 5; Table 1). This inhibtion was not due to the external Ca^{2+}, as the simultaneous addition of Ca^{2+} and Mn^{2+} (curve B, Fig. 5) did not produce significant variations in the initial rate of Mn^{2+} uptake (Table 1). The last condition, however, produced a delayed inhibition of Mn^{2+} uptake and the extent of this inhibition increased with time.

Fig. 6(*a*) compares the effects of adding Ca^{2+} at the same time (curve a) or 40 s before Mn^{2+} (curve b) on the uptake of Mn^{2+}. The experiment was performed at 17 °C. The initial rate of Mn^{2+} uptake was the same in both cases, although inhibition developed earlier when Ca^{2+} preceded Mn^{2+}. Fig. 6(*b*) shows the evolution of $[Ca^{2+}]_i$ after the addition of external Ca^{2+}. It can be seen that $[Ca^{2+}]_i$ had already risen to a maximum after 40 s, the time at which Mn^{2+} was added. These results indicate that the inhibition of Mn^{2+} uptake is not directly caused by the increase of $[Ca^{2+}]_i$.

DISCUSSION

We have made a detailed study of the Ca^{2+} uptake and redistribution in Ehrlich cells and rat thymocytes. Both cells possess ionomycin-sensitive intracellular Ca^{2+} stores. In the Ehrlich cells this pool is identical with the ATP-sensitive pool [23]. In thymocytes it is not known whether the ionomycin-sensitive pool is also agonist-sensitive. We have purposely avoided the use of agonists to release Ca^{2+} from the intracellular stores. Instead the stores were depleted by incubation in Ca^{2+}-free medium. This condition resembles the state of the cell after

stimulation, but in the absence of second messengers induced by agonists. This enables us to show the direct effects of the stores on Ca^{2+} redistribution.

Ca^{2+}-depleted cells appeared to be well preserved in every aspect that was tested: (i) they were able to accumulate fura-2 and retain it for long time; (ii) after addition of external Ca^{2+} they kept $[Ca^{2+}]_i$ low and accumulated Ca^{2+} in the intracellular stores; (iii) they responded to ionomycin by pumping out the Ca^{2+} released from the stores; (iv) after calcium refilling, Ehrlich cells were able to respond to extracellular ATP with release of Ca^{2+} from the intracellular stores, giving a $[Ca^{2+}]_i$ peak identical with that given by non-depleted cells (experiments not shown; see also [9,23]).

The uptake of Ca^{2+} ($^{45}Ca^{2+}$ and Mn^{2+}), the increase in $[Ca^{2+}]_i$ and the refilling of the Ca^{2+} stores were all very fast in thymocytes at 37 °C, and all of them were decreased by about one order of magnitude by lowering the temperature to 20 °C. In the Ehrlich cell, every one of these parameters was about an order of magnitude smaller. The time course of the increase of $[Ca^{2+}]_i$ was always faster than the refilling of the intracellular stores. In every case (temperature, different kinds of cell) both rates changed in close correlation. Ni^{2+} inhibited the refilling of the intracellular stores in every kind of cell to the same extent as it inhibited the entry of Ca^{2+} into the cytoplasm (compare Figs. 1 and 3). All these results suggest that Ca^{2+} entry to the Ca^{2+} stores takes place in these cells through the cytoplasm.

The increase in Ca^{2+} uptake by the intracellular stores at the higher temperatures in thymocytes may be partially explained by a temperature-dependent increase in the activity of the endoplasmic-reticulum Ca^{2+} pump. However, we find that the temperature-dependence of Mn^{2+} uptake was very similar. This suggests that most of the increase in the uptake of Ca^{2+} by the intracellular stores does ultimately depend on the increase in the rate of entry of Ca^{2+} into the cytoplasm through Mn^{2+}-permeable Ca^{2+} channels. Channels are usually scarcely sensitive to temperature. Therefore the high thermic coefficient found here is probably related to the mechanism of activation of the channels.

Addition of Ca^{2+} to the incubation medium produced a delayed inhibition of Ca^{2+} (Mn^{2+}) entry into Ca^{2+}-depleted thymocytes (Fig. 5). This delayed inhibition could also explain the decrease in $[Ca^{2+}]_i$ observed after the first 6 s of incubation with Ca^{2+} at 37 °C (Fig. 1*b*). Similar kinetics have recently been observed in parotid acinar cells [17]. It has been suggested that Ca^{2+} channels could be inhibited by an increase in the Ca^{2+} concentrations on the inner surface of the plasma membrane [18]. However, in our case, the inhibition took place some time after cytoplasmic Ca^{2+} had reached its maximum (Fig. 6). The time scale of delayed inhibition correlates well with the rate of refilling of the intracellular Ca^{2+} stores. The half-times for $[Ca^{2+}]_i$ increase and refilling were 10 and 40 s respectively at the temperature used in this experiment. Therefore, when $[Ca^{2+}]_i$ had reached its maximum, 40 s after the Ca^{2+} addition, the Ca^{2+} stores were still about half-filled and the inhibition of Mn^{2+} uptake did not begin to appear until about 30 s later.

We conclude that refilling of the intracellular Ca^{2+} stores in thymocytes is dependent on Ca^{2+} uptake through a plasma-membrane Ca^{2+} pathway whose activity is somehow modulated by the degree of filling of the Ca^{2+} stores. Evidence for this modulation in other cells is controversial at the moment. Studies in parietal gastric cells [15] and adrenal-glomerulosa cells [11] have found no increase in the Ca^{2+} influx during refilling. Original studies in parotid acinar cells also failed to note any additional Ca^{2+} influx during refilling [6,24], and they concluded that Ca^{2+} did not pass through the cytosol in its way to the intracellular stores. However, recent studies in these cells [16,17] have shown that emptying of the Ca^{2+} stores induces an increase in Ca^{2+} influx across the plasma membrane, and that this increase is independent of the presence of second messengers linked to receptor activation. An increase in Ca^{2+} influx during refilling has also been reported in pancreatic acinar cells, even when $CaCl_2$ was added 10 min after the termination of agonist action [10].

The mechanism of the pool-dependent Ca^{2+} influx is unknown, but our results show that it occurs in the absence of external agonists. The temperature-dependence of the activity of the Ca^{2+}-entry pathway reported here suggests that it is already activated in the Ca^{2+} depleted unstimulated cell, perhaps by an enzymically produced mediator or by a channel modification such as phosphorylation. Filling of the Ca^{2+} stores could inhibit one of these processes.

The role of a pool-dependent Ca^{2+}-influx mechanism during agonist action is unclear. The relative timings of stimulation of internal release and influx of Ca^{2+} vary according to the cell type and the agonist. In ADP-stimulated platelets, influx of Mn^{2+} precedes release of Ca^{2+} from intracellular stores [25]; in thrombin-stimulated endothelial cells internal release of Ca^{2+} precedes Mn^{2+} influx [26]; in neutrophils they both occur simultaneously [14]; and in parotid acinar cells, agonists do not stimulate Mn^{2+} influx [27], although there is evidence for stimulated Ca^{2+} influx [28]. The provisional conclusion is that a diversity of mechanisms could be responsible for receptor-mediated Ca^{2+} entry. Our results show that, at least in the kinds of cells studied here, the emptying of the intracellular Ca^{2+} stores is able to stimulate plasma-membrane Ca^{2+} entry directly. This opens the question of whether this mechanism could co-operate in the increase in Ca^{2+} influx induced by agonists known to release Ca^{2+} from the intracellular stores without the need for additional agonist-induced second messengers.

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