Dynamics of \([\text{Ca}^{2+}]\) in the Endoplasmic Reticulum and Cytoplasm of Intact HeLa Cells

A COMPARATIVE STUDY*

Maria José Barrerø, Mayte Montero§, and Javier Alvarez¶

From the Departamento de Bioquímica y Biología Molecular y Fisiología, Instituto de Biología y Genética Molecular, Facultad de Medicina, Universidad de Valladolid and Consejo Superior de Investigaciones Científicas, E-47005 Valladolid, Spain.

We have measured the \([\text{Ca}^{2+}]\) in the endoplasmic reticulum (ER) of intact HeLa cells at both 22 °C and 37 °C using endoplasmic reticulum-targeted, low \([\text{Ca}^{2+}]\) affinity aequorin reconstituted with coelenterazine \(n\). Aequorin consumption was much slower at 22 °C, and this allowed performing a much longer study of the dynamics of \([\text{Ca}^{2+}]\) in the ER. The steady-state \([\text{Ca}^{2+}]\) (500–600 μM) was not modified by the temperature, although the rates of pumping and leak were decreased at 22 °C. The behavior of both \([\text{Ca}^{2+}]\) and cytoplasmic \([\text{Ca}^{2+}]\) (([Ca\text{\textsuperscript{2+}}] \text{\textsubscript{Cyt}})) after the addition of increasing concentrations of agonists and/or \([\text{Ca}^{2+}]\) ATPase inhibitors, or following incubation in \([\text{Ca}^{2+}]\)-free medium were compared. We show that agonists induce a fast but relatively small decrease in \([\text{Ca}^{2+}]\) in the ER, which is enough to produce a sharp increase in \([\text{Ca}^{2+}]\). Termination of \([\text{Ca}^{2+}]\) release is controlled by feedback inhibition of the inositol 1,4,5-trisphosphate receptors by \([\text{Ca}^{2+}]\), a mechanism that appears to be used to release the minimum amount of \([\text{Ca}^{2+}]\) necessary to produce the required \([\text{Ca}^{2+}]\) signal. We also show that \([\text{Ca}^{2+}]\) release is inhibited progressively when \([\text{Ca}^{2+}]\) decreases below a threshold of about 150 μM, even in the absence of \([\text{Ca}^{2+}]\) pumping or \([\text{Ca}^{2+}]\) increase. This effect is consistent with a regulation of the inositol 1,4,5-trisphosphate-gated channels by \([\text{Ca}^{2+}]\).

Monitoring directly the \([\text{Ca}^{2+}]\) inside the main \([\text{Ca}^{2+}]\) store of the cell, the endoplasmic reticulum, has been difficult to achieve in intact cells. We have reported recently (1) that using HeLa cells expressing ER\(^3\)-targeted, low \([\text{Ca}^{2+}]\) affinity aequorin reconstituted with coelenterazine \(n\) allows measuring reliably \([\text{Ca}^{2+}]\) in intact cells. In that study, however, the high \([\text{Ca}^{2+}]\) levels reached at steady state in the ER (500–600 μM) led to a fast consumption of aequorin, allowing measurement of \([\text{Ca}^{2+}]\) for only a few minutes. We have now observed that aequorin consumption is 1 order of magnitude slower at 22 °C, and this allows performing long term comparative studies of the dynamics of \([\text{Ca}^{2+}]\) and \([\text{Ca}^{2+}]\), under different conditions, e.g. in the presence of extracellular InsP\(_3\)-producing agonists, \([\text{Ca}^{2+}]\)-ATPase inhibitors, or during incubation in \([\text{Ca}^{2+}]\)-free medium. Our results reveal several unexpected characteristics of the \([\text{Ca}^{2+}]\) release phenomenon, e.g. that the magnitude of the changes of \([\text{Ca}^{2+}]\) after agonist action does not always correlate with the magnitude of the corresponding changes in \([\text{Ca}^{2+}]\). A sharp agonist-induced cytosolic \([\text{Ca}^{2+}]\) peak can be obtained with only little \([\text{Ca}^{2+}]\) release from the stores, and further release is feedback inhibited by the increase in \([\text{Ca}^{2+}]\), probably through the generation of microdomains of high \([\text{Ca}^{2+}]\).

**EXPERIMENTAL PROCEDURES**

**Calibration**—Cell lysates of the EM26 cell clone (2) stably expressing the recombinant photoprotein were reconstituted overnight at 4 °C with 5 μM coelenterazine \(n\), and incubated with solutions containing 110 mM KCl, 10 mM NaCl, 1 mM free Mg\(^2+\), 40 mM HEPES, pH 7.0, at 22 °C, and known concentrations of \([\text{Ca}^{2+}]\) prepared using buffers with 5 mM HEDTA when necessary. The fractional rate of aequorin consumption (luminescence/total luminescence remaining, \(L/L\_\text{max}\)) at steady state was calculated in every case after consuming all aequorin luminescence with 10 mM \([\text{Ca}^{2+}]\). Procedures for fitting the curve to the experimental data and other details have been described previously (3).

**[Ca\text{\textsuperscript{2+}}]** Measurements—The HeLa cell clone EM26 producing ER-targeted low \([\text{Ca}^{2+}]\) affinity mutated aequorin (2) was grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum and 0.2 mg/ml G418. Cell clones were plated onto 13-mm round coverslips. Before reconstituting aequorin, \([\text{Ca}^{2+}]\) was reduced by incubating the cells for 5 min at 37 °C with the SERCA inhibitor BHQ (10 μM) in KRB (Kreb's-Ringer modified buffer: 125 mM NaCl, 5 mM KCl, 1 mM MgCl\(_2\), 1 mM NaPO\(_4\), 5.5 mM glucose, 20 mM HEPES, pH 7.4), supplemented with 3 mM EGTA. Cells were then incubated for 1 h at room temperature in KRB containing 0.5 mM EGTA, 10 μM BHQ, and either \(500 \mu\text{M} \text{Ca}^{2+}\), which is enough to produce a sharp increase in \([\text{Ca}^{2+}]\), or \(4 \mu\text{M} \text{fura-2-AM}\) for measuring \([\text{Ca}^{2+}]\). The coverslip was then washed for 5 min in KRB containing 0.5 mM EGTA, 10 μM BHQ, and \(0.5 \mu\text{M} \text{coelenterazine} (n)\) for measuring \([\text{Ca}^{2+}]\) measurements were always depleted of \([\text{Ca}^{2+}]\) in the same way as those used for \([\text{Ca}^{2+}]\), measurements, to allow comparison of both types of data in the same conditions. Coelenterazine \(n\) and BHQ-2-AM, and \(4 \mu\text{M} \text{BAPTA-AM}\) were obtained from Molecular Probes. Other reagents were from Sigma (Madrid, Spain) or Merck (Darmstadt, Germany).

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§ To whom correspondence should be addressed: Departamento de Bioquímica y Biología Molecular y Fisiología, Facultad de Medicina, Universidad de Valladolid, E-47005 Valladolid, Spain. Tel.: 34-83-423085; Fax: 34-83-423588; E-mail: jalvarez@cpd.uv.es.

¶ The abbreviations used are: ER, endoplasmic reticulum; \([\text{Ca}^{2+}]\)\text{\textsubscript{ER}}; \([\text{Ca}^{2+}]\); \([\text{Ca}^{2+}]\); \([\text{Ca}^{2+}]\); cytoplasmic \([\text{Ca}^{2+}]\); InsP\(_3\), inositol 1,4,5-trisphosphate; KRB, Kreb's-Ringer modified buffer; BHQ, 2,4-dinitro-butyryl-benzohydroquinone; HEDTA, hydroxyethylendiaminetetraacetic acid; BAPTA, 1,2-bis(2-aminophenoxy)ethane-N,N',N''-N'-tetraacetic acid; SERCA, sarcoplasmic and endoplasmic reticulum \([\text{Ca}^{2+}]\)-ATPase; SOCC, store-operated calcium channel(s).
Fig. 1. A, calibration of the low Ca2+ affinity mutated aequorin reconstituted with coelenterazine n. The calibration curve at 22 °C was determined in vitro as described under “Experimental Procedures.” Each point is the average of four trials. The standard deviation is shown when significant. The calibration curve at 37 °C, taken from Ref. 1, is also shown for comparison. B, HeLa cells producing ER-targeted, low Ca2+ affinity mutated aequorin (2) were depleted of Ca2+ and reconstituted with coelenterazine n. At the time indicated, 1 mM Ca2+ was perfused. The upper panel shows the luminescence record. The lower panel shows the calibrated [Ca2+]c levels. This experiment is representative of 13 similar ones.

RESULTS

Fig. 1A shows the calibration curve obtained at 22 °C of low Ca2+ affinity mutated aequorin reconstituted with coelenterazine n, a prosthetic group conferring still lower Ca2+ affinity (5). The curve obtained at 37 °C, reported previously (1), is also shown for comparison. We can observe that decreasing the temperature reduces the rate of aequorin consumption by nearly 1 order of magnitude at every [Ca2+]c and the ER may have a similar temperature dependence, because the decrease in the rate of increase of [Ca2+]c was 1.8 ± 0.25%/°C. The temperature could be a major factor determining the magnitude of [Ca2+]c.

In Fig. 2, we compare the effect of adding increasing concentrations of histamine on both [Ca2+]c and [Ca2+]er. In the upper panel, we show the effect of the addition of 2.5 μM, 10 μM and 100 μM histamine on the [Ca2+]er of HeLa cells, measured using fura-2. In every case, the agonist induces a sharp initial [Ca2+]c peak, which is followed by a decreasing plateau lasting for several minutes. In the middle panel, the effect of the same histamine concentrations on [Ca2+]er is shown. The higher concentration, 100 μM histamine, produced a fast [Ca2+]er decrease of about 100 μM, coincident with the sharp cytosolic peak. Then, surprisingly, [Ca2+]er stabilized at that level for several minutes still in the presence of histamine, turning up again when histamine was washed. Therefore, the decreasing plateau of [Ca2+]c, coincides with a steady-state [Ca2+]er in which the stores keep at 80–90% of maximum loading. When the histamine concentration was only 10 μM, the initial phase of [Ca2+]er decrease was smaller (around 50 μM), and [Ca2+]er remained afterward at approximately the same levels as before, even though the [Ca2+]c peak was not much different from that obtained with 100 μM histamine. With 2.5 μM histamine, the effect on [Ca2+]er was negligible, and in many experiments it could not even be detected over the background noise. Instead, the [Ca2+]c peak was smaller, but its amplitude was still near half the maximum obtained with 100 μM histamine. The effect of histamine on [Ca2+]er was always potentiated when the increase in [Ca2+]c was blocked by loading the cells with the Ca2+-chelator BAPTA. The lower panel shows the effect of the same three histamine concentrations on [Ca2+]er in BAPTA-loaded cells. Histamine induced a much prolonged decrease in [Ca2+]er in the three cases, and the rate was proportional to the histamine concentration.

The potentiating effect of BAPTA loading could be attributed either to the inhibition of Ca2+ pumping or to a direct effect activating InsP3-gated Ca2+ release. In our previous paper (1), we showed that simultaneous addition of 100 μM histamine and the SERCA inhibitor BHQ produced only additive effects, suggesting that the main effect of BAPTA was protecting the InsP3...
Careful analysis of the decay curves of $[\mathrm{Ca}^{2+}]_{\text{er}}$ obtained after simultaneous addition of histamine and BHQ to cells loaded with BAPTA allowed us to study the possible regulation of the InsP$_3$-gated channels by $[\mathrm{Ca}^{2+}]_{\text{er}}$. Theoretically, if the ER would behave as a single compartment emptying at constant rate through the InsP$_3$ receptors, the decay of $[\mathrm{Ca}^{2+}]_{\text{er}}$ in the absence of pumping should follow a single exponential. Fig. 5 compares the rate of emptying obtained experimentally after addition of BHQ and either 2.5 $\mu$M or 100 $\mu$M histamine, at 22 °C, with a monoexponential decay calculated to fit the initial
part of the experimental curve. We can see that the experimental data follow the exponential most of the time, but clearly deviate from it when the [Ca\(^{2+}\)]\(_{\text{er}}\) decreases below 150 \(\mu\)M, no matter what is the histamine concentration used. This finding, which has been reported previously using the same technique but with Sr\(^{2+}\) as a Ca\(^{2+}\) surrogate (6), provides evidence in favor that InsP\(_3\) receptors require a certain level of [Ca\(^{2+}\)]\(_{\text{er}}\) for maximal activity.

We have studied finally the effect of histamine on [Ca\(^{2+}\)]\(_{\text{er}}\) in the absence of extracellular Ca\(^{2+}\), as well as the behavior of [Ca\(^{2+}\)]\(_{\text{er}}\) during incubation of the cells in Ca\(^{2+}\)-free medium. Fig. 6 (upper panel) shows that when histamine is added to HeLa cells incubated in Ca\(^{2+}\)-free medium, the initial [Ca\(^{2+}\)]\(_{\text{c peak}}\) peak is similar to that obtained in Ca\(^{2+}\)-containing medium, but the plateau phase is largely (though not completely) removed. If the cells are incubated for 10 or 20 min in Ca\(^{2+}\)-free medium, the height of the peak was only reduced to 73 \pm 5\% (n = 4; 78\% in the figure) and 69 \pm 12\% (n = 4; 81\% in the figure) of the control value, respectively. In all the cases, it is apparent that the return of [Ca\(^{2+}\)]\(_{\text{c peak}}\) to the base line is biphasic, with a final slow phase lasting for 1–2 min, which cannot be attributed to Ca\(^{2+}\) entry and must be due to Ca\(^{2+}\) release. The counterpart in the ER is shown in the lower panel. Addition of histamine in EGTA-containing medium produces a decrease in [Ca\(^{2+}\)]\(_{\text{er}}\), which has three consecutive phases from a kinetic point of view: a first rapid drop of about 60 \(\mu\)M, which must be responsible for the peak of [Ca\(^{2+}\)]\(_{\text{c peak}}\); a second phase of slower decrease, lasting for 1–2 min and getting 80–90 \(\mu\)M lower (coincident in time with the last part of the [Ca\(^{2+}\)]\(_{\text{c peak}}\), peak); and a third phase, in which the rate of decrease in [Ca\(^{2+}\)]\(_{\text{er}}\) approaches that obtained just by incubation in Ca\(^{2+}\)-free medium. The same kinetics was observed when histamine was added after 10 or 20 min of incubation in Ca\(^{2+}\)-free medium. It was surprising, however, to find that there was not a strict correlation between the height of the [Ca\(^{2+}\)]\(_{\text{c peak}}\), peak and the [Ca\(^{2+}\)]\(_{\text{er}}\) at the moment of histamine addition. The [Ca\(^{2+}\)]\(_{\text{er}}\) level decreased to 59 \pm 6\% (n = 4; 60\% in the figure) of the initial value after 10 min (when the [Ca\(^{2+}\)]\(_{\text{c peak}}\) peak was 73 \pm 5\% of maximum), and to 29 \pm 8\% (n = 5; 30\% in the figure) after 20 min (when the [Ca\(^{2+}\)]\(_{\text{c peak}}\) peak was still 69 \pm 12\% of maximum). In these experiments, the half-time for emptying of the stores in Ca\(^{2+}\)-free medium was 12 \pm 3 min (n = 10).

Similar effects were observed when the experiments were carried out at 37 °C, but histamine was in this case more potent, inducing Ca\(^{2+}\) release. Fig. 7 shows that addition of histamine in EGTA-containing medium produced a sharp [Ca\(^{2+}\)]\(_{\text{c peak}}\), followed by a short (30–40 s) phase in which the decrease in [Ca\(^{2+}\)]\(_{\text{c}}\) was slower. If cells with full Ca\(^{2+}\) stores were incubated in Ca\(^{2+}\)-free medium for 3 or 5 min, the [Ca\(^{2+}\)]\(_{\text{c peak}}\) peak suffered only a minor decrease to 92 \pm 6\% (n = 4; 99\% in the figure) and 82 \pm 17\% (n = 4; 91\% in the figure) of the control, respectively. The lower panel shows the effect of histamine on [Ca\(^{2+}\)]\(_{\text{er}}\). Due to the fast aequorin consumption at 37 °C, histamine had to be added during the rising phase of [Ca\(^{2+}\)]\(_{\text{er}}\) after addition of extracellular Ca\(^{2+}\). We can observe that histamine induces a decrease of [Ca\(^{2+}\)]\(_{\text{er}}\) in two phases: a first rapid decrease from 500 \(\mu\)M to about 350 \(\mu\)M, followed by a slower decrease, which continues down to about 100 \(\mu\)M [Ca\(^{2+}\)]\(_{\text{er}}\). This second phase is still much faster than the rate of decrease in [Ca\(^{2+}\)] induced only by EGTA (see the traces on the
right). The same kinetics were observed if histamine was added after 3 or 5 min of incubation in Ca\(^{2+}\)-free medium. As in the experiments at 22 °C, the initial drop was responsible for the sharp cytosolic peak and the second slower phase was coincident with the last part of the [Ca\(^{2+}\)]\(_{esr}\) peak. Again, the height of the [Ca\(^{2+}\)]\(_{esr}\) peak did not correlate with the [Ca\(^{2+}\)]\(_{er}\) level, which decreased to 46 ± 10% (n = 4; 48% in the figure) after 3 min in Ca\(^{2+}\)-free medium (the [Ca\(^{2+}\)]\(_{esr}\) peak decreased only to 92 ± 6%) and to 35 ± 10% (n = 5; 34% in the figure) after 5 min (the [Ca\(^{2+}\)]\(_{esr}\) peak decreased only to 82 ± 17%). The half-time for emptying of the stores in Ca\(^{2+}\)-free medium in these experiments was about 3 min (170 ± 50 s, n = 8).

**DISCUSSION**

We have measured directly the dynamics of [Ca\(^{2+}\)] in the endoplasmic reticulum of intact HeLa cells. Using ER-targeted, low Ca\(^{2+}\) affinity mutated aequorin reconstituted with coelenterazine \(n\), and because of the decrease in the rate of aequorin consumption at room temperature, we have been able to perform long-lasting experiments comparing the effects both in [Ca\(^{2+}\)]\(_{esr}\) and [Ca\(^{2+}\)]\(_{er}\) of the addition of extracellular agonists and/or SERCA inhibitors, both in Ca\(^{2+}\)-containing or Ca\(^{2+}\)-free medium. The results obtained, some of them quite unexpected, provide a new view on the role of the intracellular Ca\(^{2+}\) stores in Ca\(^{2+}\) homeostasis.

When using aequorin, the possibility of artifacts due to heterogeneity in [Ca\(^{2+}\)] or behavior of the pools containing the indicator should be always considered. Native aequorin had an extremely nonlinear sensitivity to Ca\(^{2+}\), and the signal could be therefore much affected by the presence of small compartments of high [Ca\(^{2+}\)]. In the mutated aequorin we have used in this paper, the near abolition of one of the Ca\(^{2+}\)-binding sites reduced considerably the slope of the calibration curve, which became near linear with [Ca\(^{2+}\)] (see Fig. 1 and Ref. 2). Using mutated aequorin, therefore, the possibility of having artifacts due to small compartments with high [Ca\(^{2+}\)] is much lower. On the other hand, because of consumption, aequorin is very well suited to detect heterogeneity in [Ca\(^{2+}\)]. We have shown recently using theoretical models (6) that, in experiments such as that of Fig. 1B, we should obtain a peak after Ca\(^{2+}\) addition in the calibrated [Ca\(^{2+}\)] signal if there were a compartment refilling to a much higher [Ca\(^{2+}\)] than the rest. The reason for this artifact is that the signal would be dominated initially by the high [Ca\(^{2+}\)] compartment until aequorin consumption in that compartment was near complete. Afterward, the [Ca\(^{2+}\)] signal would return down to end monitoring the low [Ca\(^{2+}\)] compartment isolated. Our experimental results show instead a smooth increase in [Ca\(^{2+}\)], leading to a steady state that keeps flat until near 90% of aequorin has been consumed (see Fig. 1B). Therefore, we can conclude that there are no gross differences in [Ca\(^{2+}\)] throughout the bulk of the ER.

Increasing the temperature from 22 °C to 37 °C accelerated nearly 4-fold the rate of increase in [Ca\(^{2+}\)]\(_{esr}\) after addition of extracellular Ca\(^{2+}\). On the other hand, the rate of Ca\(^{2+}\) leak from the stores, measured from the rate of decrease in [Ca\(^{2+}\)]\(_{er}\) after addition of BHQ in 1 mM Ca\(^{2+}\)-containing medium, also increased with the temperature from 2 ± 0.1 (n = 5) μM/s at 22 °C to 4.4 ± 0.8 (n = 4) μM/s at 37 °C. Similarly, emptying of the stores after incubation in Ca\(^{2+}\)-free medium was 4-fold slower at 22 °C (half-time 12 min) than at 37 °C (half-time 3 min). Given that the steady-state [Ca\(^{2+}\)]\(_{esr}\) depends on the balance between these Ca\(^{2+}\)-pumping and leak, it is not surprising to find that the steady-state [Ca\(^{2+}\)]\(_{esr}\) does not change with the temperature (590 ± 90 μM at 22 °C, compare with 550 ± 70 μM (Ref. 1), at 37 °C).

The experiments shown in Fig. 2 demonstrate that a relatively small and fast decrease in [Ca\(^{2+}\)]\(_{esr}\) is enough to produce a maximal [Ca\(^{2+}\)]\(_{esr}\) peak. A similar conclusion was reached recently observing the decrease in [Sr\(^{2+}\)] and the [Sr\(^{2+}\)] peak induced by caffeine in skeletal muscle myotubes (7). Therefore, the height of the [Ca\(^{2+}\)]\(_{esr}\) peak appears to be related much more with the rate of Ca\(^{2+}\) release than with the actual amount of Ca\(^{2+}\) released. In fact, 2.5 μM histamine produced an almost undetectable decrease in [Ca\(^{2+}\)]\(_{er}\) and a near half-maximum peak in [Ca\(^{2+}\)]\(_{esr}\). Additionally, it is also surprising to see from these experiments that the plateau of elevated [Ca\(^{2+}\)]\(_{esr}\) following the main peak coincides with a constant [Ca\(^{2+}\)]\(_{esr}\) level, still in the presence of histamine. This phenomenon has two main implications that deserve further discussion. First, the constant [Ca\(^{2+}\)]\(_{esr}\) level must be a consequence of the balance between increased Ca\(^{2+}\)-pumping (following the increase in [Ca\(^{2+}\)]\(_{esr}\)) and reduced InsP\(_3\)-activated Ca\(^{2+}\) release. Second, the plateau of [Ca\(^{2+}\)]\(_{esr}\) must be due to an increased Ca\(^{2+}\) entry from the extracellular medium due to activation of the store-operated Ca\(^{2+}\) channels under these conditions.

Regarding the first conclusion, clearly activation of Ca\(^{2+}\) release through the InsP\(_3\)-gated channels at maximal rate could never be balanced by the activity of the SERCA, given that ion flux through channels is several orders of magnitude faster than the maximal pumping activity of Ca\(^{2+}\) ATPases. Partial emptying is also not due to heterogeneous distribution of the InsP\(_3\) receptors, because in BAPTA-loaded cells histamine induces an almost complete emptying (see also Ref. 1). The only consistent explanation of these results is therefore that the InsP\(_3\)-gated channels become strongly inhibited after the initial histamine-induced Ca\(^{2+}\) release. The experiments shown in Figs. 3 and 4, where histamine-induced Ca\(^{2+}\) release is observed in the absence of Ca\(^{2+}\) pumping, indicate that the inhibition of the InsP\(_3\) receptors is quite strong at 37 °C and near 100% at 22 °C. The stronger inhibition at low temperature is probably due to the reduced production of InsP\(_3\) (8). As has been discussed already in Ref. 1, the mechanism for the inhibition probably depends on the generation of microdomains of relatively high [Ca\(^{2+}\)] in the vicinity of the InsP\(_3\) receptors, which would inhibit Ca\(^{2+}\) release according to the known bell-shaped dependence of these channels with [Ca\(^{2+}\)] (9–12). This mechanism seems to be perfectly designed from a physiological point of view to produce maximal [Ca\(^{2+}\)]\(_{esr}\) peaks with minimum Ca\(^{2+}\) release from the stores. In this way, the energetic cost is considerably reduced and, even more important, the cell is able to respond consecutively to several different stimuli because the Ca\(^{2+}\) store is maintained nearly full most of the time.

Regarding the second point, stimulation of Ca\(^{2+}\) entry by histamine in HeLa cells is believed to occur through the capacitative pathway (SOCC, store-operated calcium channels; see Refs. 13 and 14), a Ca\(^{2+}\) pathway that is activated through an unknown mechanism following the emptying of the intracellular Ca\(^{2+}\) stores. The activation of this pathway has been shown in several cell types to be proportional to the degree of emptying of the stores (15, 16). However, if the activation of Ca\(^{2+}\) entry by histamine was just a consequence of the emptying of the stores we observe, it is difficult to imagine a mechanism with such an exquisite sensitivity to detect the minute changes in the bulk [Ca\(^{2+}\)]\(_{esr}\) (<10%) induced by the different histamine concentrations. Alternative explanations could be: (i) selective emptying by the agonist of a store with specific functions in the activation of SOCC, although confocal microscopy studies have shown no specific sites for histamine-induced Ca\(^{2+}\) release in HeLa cells (17); (ii) direct activation of SOCC by the agonist through a mechanism either independent or cooperative with the emptying of the stores (18–20). Further study will be nec-
ecessary to determine the correlation between the activation of SOCC and the actual level of \([Ca^{2+}]_{\text{cyt}}\).

The results shown in Fig. 5 suggest also that \(Ca^{2+}\) release through the InsP₃-gated channels may be regulated by \([Ca^{2+}]_{\text{er}}\). \(Ca^{2+}\) release follows a single exponential while \([Ca^{2+}]_{\text{er}}\) is above 150 \(\mu M\), but slows down progressively when \([Ca^{2+}]_{\text{er}}\) gets below that. An alternative interpretation for this phenomenon could only be found in terms of heterogeneity of the rate of \(Ca^{2+}\) release through the InsP₃ receptors between different pools of the ER, assuming that there was a small portion of the ER having a rate of \(Ca^{2+}\) release several times slower than the rest. We have previously shown the same phenomenon using \(Sr^{2+}\) as a \(Ca^{2+}\) surrogate (6), although in that case the \([Sr^{2+}]_{\text{er}}\) required to activate maximally \(Sr^{2+}\) release was higher, around 500 \(\mu M\). Regulation by \([Ca^{2+}]_{\text{er}}\) of the sensitivity of the InsP₃ receptors has been reported previously (21, 22), but some other groups failed to detect this effect (23–26) or found the regulation to be significant only at very low luminal \([Ca^{2+}]\) (<10 \(\mu M\), Ref. 27). Our data suggest that regulation of \(Ca^{2+}\) release by \([Ca^{2+}]_{\text{er}}\) takes place in intact cells, slowing \(Ca^{2+}\) release when \([Ca^{2+}]_{\text{er}}\) gets below 150 \(\mu M\). This threshold was independent of the histamine concentration and therefore of the rate of \(Ca^{2+}\) release, so excluding alternative explanations such as the build-up of a diffusion membrane potential across the ER membrane. Our results cannot be interpreted either in terms of an effect of luminal \(Ca^{2+}\) acting at the cytosolic inhibitory site (28), because the same effect was observed (6) using cells loaded with \(Sr^{2+}\), which does not bind to the inhibitory site (1, 29, 30). Regarding the physiological significance of this mechanism, it is important to note that the stimulatory effect of \(Ca^{2+}\) saturates at \([Ca^{2+}]_{\text{er}}\) values around 150 \(\mu M\), that is 30% of its steady-state value. This means that inhibition of \(Ca^{2+}\) release by this mechanism will probably take place only when cells are strongly stimulated. In those conditions, this mechanism may be designed as a negative feedback able to stop cell activation, avoiding complete depletion of \(Ca^{2+}\) of the ER.

Finally, another initially unexpected result from this study was the relative lack of correlation between the height of the histamine-induced-\([Ca^{2+}]\), peaks and the \([Ca^{2+}]_{\text{er}}\) level during incubations in \(Ca^{2+}\)-free medium. The height and the shape of the \([Ca^{2+}]_{\text{er}}\) peak were only slightly modified even when the \([Ca^{2+}]_{\text{er}}\) had been reduced to about 30% of the initial value (see Figs. 6 and 7). This result, however, could be expected from the data shown in Fig. 2. Given that a decrease in \([Ca^{2+}]_{\text{er}}\) of 50–100 \(\mu M\) is enough to produce a maximum \([Ca^{2+}]\) peak and that the affinity for \(Ca^{2+}\) of the most abundant \(Ca^{2+}\)-binding proteins in the ER is in the millimolar range (31), we can predict that a similar amount of \(Ca^{2+}\) should be released after a fast \([Ca^{2+}]_{\text{er}}\) decrease from 500 \(\mu M\) to 450 \(\mu M\) or from 200 \(\mu M\) to 150 \(\mu M\). In fact, the inhibition of the InsP₃ receptors by \([Ca^{2+}]\) limits the total amount of \(Ca^{2+}\) released after histamine action, and therefore the effect of the agonist on \([Ca^{2+}]\) becomes nearly independent of the \([Ca^{2+}]_{\text{er}}\), within a certain range of levels (below 150 \(\mu M\), \(Ca^{2+}\) release starts to be inhibited, see above). This may also be important from a physiological point of view, because this mechanism allows cell responses to InsP₃-producing extracellular agonists to be nearly independent of the \(Ca^{2+}\) content of the stores. On the other hand, these results suggest that the content of the \(Ca^{2+}\) stores cannot be adequately estimated from the height of the peaks of \([Ca^{2+}]_{\text{er}}\) induced by an agonist. Direct measurement of \([Ca^{2+}]_{\text{er}}\) appears now essential and will surely throw new light in the next few years on many long-known phenomena related to \(Ca^{2+}\)-homeostasis.

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