Capacitative Ca^{2+} Entry Is Closely Linked to the Filling State of Internal Ca^{2+} Stores: A Study Using Simultaneous Measurements of I_{CRAC} and Intraluminal $[Ca^{2+}]$

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Abstract. I_{CRAC} (the best characterized Ca^{2+} current activated by store depletion) was monitored concurrently for the first time with $[Ca^{2+}]$ changes in internal stores. To establish the quantitative and kinetic relationship between these two parameters, we have developed a novel means to clamp $[Ca^{2+}]$ within stores of intact cells at any level. The advantage of this approach, which is based on the membrane-permeant low-affinity Ca^{2+} chelator N,N,N′,N′-tetrakis (2-pyridylmethyl)ethylene diamine (TPEN), is that $[Ca^{2+}]$ within the ER can be lowered and restored to its original level within 10–15 s without modifications of Ca^{2+} pumps or release channels. Using these new tools, we demonstrate here that Ca^{2+} release–activated Ca^{2+} current (I_{CRAC}) is activated (*a*) solely by reduction of free $[Ca^{2+}]$ within the ER and (*b*) by any measurable decrease in $[Ca^{2+}]_{ER}$. We also demonstrate that the intrinsic kinetics of inactivation are relatively slow and possibly dependent on soluble factors that are lost during the whole-cell recording.

THE Ca^{2+} influx pathway activated by depletion of intracellular stores (Putney, 1986, 1990) modulates a host of key cellular processes ranging from photoreceptor excitability to Ca^{2+} oscillations and growth factor signaling (Tsien and Tsien, 1990; Berridge, 1995). The intervening molecular steps between the depletion of internal stores and the activation of Ca^{2+} entry at the plasma membrane, however, remain obscure.

Evidence for a variety of diverse mechanisms has been presented previously to account for this phenomenon; among these, the production of a soluble messenger gating Ca^{2+} entry (Parekh et al., 1993; Randriamampita and Tsien, 1993), and direct physical coupling of inositol 1,4,5-trisphosphate ($InsP_3$)[1] receptors to plasma membrane channels (by analogy with the interactions between the ryanodine receptor/dihydropyridine receptor in skeletal muscle; Irvine, 1990; Berridge, 1995; Petersen and Berridge, 1996). In addition, roles for tyrosine kinases (Lee et al., 1993), inositol 1,3,4,5-tetrakisphosphate ($InsP_4$) (Lückhoff and Clapham, 1993), small GTP-binding proteins (Fasolato et al., 1993; Bird and Putney, 1993), cyclic GMP (Bahnson et al., 1993), heterotrimeric G-proteins (Petersen and Berridge, 1995; Berven et al., 1995), and cytochrome P-450 (Alvarez et al., 1992) have also been implicated. The possibility that combinations of some or all of these elements may participate in activating or modulating capacitative Ca^{2+} entry, or that cell-type specific mechanisms exist, cannot be excluded.

Not only is the nature of the triggering event mysterious, but the molecular identity of the ion channels as well as the relationship between the $[Ca^{2+}]$ drop within the store and the activation of the current remain unknown. As to the former, the TRP channels, first identified in *Drosophila* photoreceptors (and their mammalian homologues) are, at present, the best candidates (Hardie and Minke, 1993; Vaca et al., 1994; Petersen et al., 1995; Wes et al., 1995). As to the latter problem, the hypothesis has been recently proposed that Ca^{2+} release–activated Ca^{2+} current (I_{CRAC}) activation is an all-or-none event with respect to $InsP_3$-induced depletion of $[Ca^{2+}]_{ER}$. In particular, Parekh and co-workers have proposed that opening of CRAC channels occurs only once the $[Ca^{2+}]_{ER}$ reaches a threshold level, possibly in a specialized subcompartment of the store (Parekh et al., 1997). The mechanistic and functional implications of this hypothesis are extremely important for the understanding of the physiological role of the Ca^{2+} signaling pathway, but the evidence supporting it is still rather indirect. A key issue in particular, namely the direct quantitative correlation between current activa-

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1. *Abbreviations used in this paper*: BAPTA, 1,2-bis (2-amino-phenoxy)ethane-*N,N,N′,N*-tetraacetic acid; I_{CRAC}, Ca^{2+} release–activated Ca^{2+} current; $InsP_3$, inositol 1,4,5-trisphosphate; $InsP_4$, inositol 1,3,4,5-tetrakisphosphate; TPEN, N,N,N′,N′-tetrakis (2-pyridylmethyl)ethylene diamine.

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tion–inactivation and the $[Ca^{2+}]_{ER}$ has not yet been addressed experimentally. Another important issue concerning capacitative Ca^{2+} influx that has received virtually no consideration in the past, due in part to its experimental inaccessibility, is the kinetics of the current inactivation once stores have been refilled. Such information is important because it may help to define the mechanisms by which the putative messengers that turn on the influx are metabolized or inactivated.

In the present study, we have overcome the limitations mentioned above (*a*) by measuring simultaneously the Ca^{2+} level in the ER and the activation–inactivation of I_{CRAC} (as well as Ca^{2+} influx) and (*b*) by developing a new approach that allows the rapid reduction of $[Ca^{2+}]_{ER}$ at will without affecting $InsP_3$ receptors or the Ca^{2+} ATPases of the stores. This method also allows a very rapid refilling of stores, thus permitting the investigation of the intrinsic rate of current inactivation once the ER Ca^{2+} level is restored to normal. Our results indicate that the magnitude of Ca^{2+} entry is closely correlated with the extent of store depletion and that there is no (or undetectable) depletion threshold for activating the current. Furthermore, our data show that the Ca^{2+} entry process, once activated, has an intrinsically slow rate of turn off. The lack of a clear threshold and the low rate of inactivation are difficult to reconcile with models dependent on protein–protein interaction, and they indicate the existence of a relatively slow metabolic step in both the turning on and turning off of the capacitative Ca^{2+} entry pathway.

Materials and Methods

Simultaneous Measurement of I_{CRAC} and Internal Store $[Ca^{2+}]$ Using Compartmentalized Mag-Fura-2

RBL-1 cells, grown on glass coverslips and cultured as described previously (Fasolato et al., 1993), were loaded with 2 μM mag-fura-2-AM (Raju et al., 1989) for 20–30 min at 37°C (Hofer and Machen, 1993). Cytoplasmic indicator was dialyzed from the cells via the patch pipette (1.5–3 MΩ resistance in standard Ringer's solution, sylgard-coated) in the tight seal whole-cell configuration at room temperature (Hamill et al., 1981). The external solution consisted of standard NaCl Ringer's (in mM: 140 NaCl, 2.8 KCl, 10 glucose, 10 Hepes, 2 $MgCl_2$, pH 7.4) supplemented with 10 mM $CaCl_2$; 5 mM CsCl was routinely added to block inwardly rectifying K^+ channels (Hoth, 1995). Unless otherwise stated, pipette solution contained (in mM): 145 Cs glutamate, 8 NaCl, 1 $MgCl_2$, 10 Hepes, 1,2-bis (2-amino-phenoxy)ethane-*N,N,N′,N*-tetraacetic acid (BAPTA) (Cs salt), with CaBAPTA added to yield a free $[Ca^{2+}]$ of 100 nM (to prevent spontaneous activation of I_{CRAC}) and a total [BAPTA] of 10 mM, and 0.5 mM MgATP, pH 7.2. Current and fluorescence measurements of mag-fura-2 were carried out with an inverted microscope (model Axiovert100; Carl Zeiss, Inc., Thornwood, NY) equipped for epifluorescence and photometry (T.I.L.L. Photonics, Planegg, Germany). The light source was a xenon short arc lamp (model 75X-O; Ushio, Inc., Tokyo, Japan) and a diffraction grating mounted on a high-speed scanner, providing monochromatic light at 345 and 375 nm wavelengths. Light from the photometer was directed through a quartz optical fiber to a gray filter (Oriel, Italia, Milano) before entering the microscope. To reduce photobleaching further, the monochromatic light was pulsed for 20 ms at each wavelength, followed by 200 ms of dark. Light was deflected by a 420-nm dichroic mirror into the microscope objective (Fluar 40×/1.30 oil; Carl Zeiss, Inc.). The emitted light was directed through a 450-nm cut-off filter (Oriel) to a photomultiplier tube (model R928; Hamamatsu Photonics, Italia, Milano). To collect fluorescence from a single cell, a pin hole was placed in the image plane of the phototube. Current recordings and mag-fura-2 fluorescence were acquired by a computer-based patch clamp amplifier system (model EPC-9; HEKA, Lambrecht, Germany) controlled by the Pulse software (HEKA). Capacitative currents were canceled before each voltage ramp using the automatic capacitance compensation of the EPC-9. Uncompensated series resistances were in the range of 4-8 MΩ. All voltages were corrected for a liquid junction potential of 8 mV between external and internal solutions. High-resolution currents were acquired at a sampling rate of 10 kHz, low-pass filtered at 2.3 kHz, and digitally filtered to 1 kHz for presentation. Voltage ramps of 50-ms duration, from −100 to +100 mV, were delivered at 0.5 Hz from a holding potential of 0 mV. Baseline current ramps (taken before the onset of I_{CRAC}) were subtracted from current–voltage relationships for presentation. The holding current, the mag-fura-2 fluorescence, and other parameters were synchronously recorded at low resolution (5 Hz) by the X-Chart software (HEKA). Test solutions (1 μM ionomycin prepared in the same external solution or 5 mM N,N,N′,N′-tetrakis [2-pyridylmethyl]ethylene diamine [TPEN] in a nominally Ca^{2+}-free external solution with 3 mM $MgCl_2$) were applied by local pressure from a wide-tipped micropipette (5–10 μm). In some patch clamp experiments (where noted), cells were continuously superfused with extracellular solution so that there was rapid washing of test solution when the application was stopped.

Measurement of Cytoplasmic $[Ca^{2+}]$ with Fura-2

Cells were loaded with 5 μM fura-2-AM at 37°C for 30 min with a subsequent wash period (10–15 min) at room temperature. The 345/375 nm excitation ratio (emission 450 nm) was acquired from individual cells within the microscope field every 4 s using a commercial imaging system (Georgia Instruments, Roswell, GA) described previously (Gamberucci et al., 1994). Cells were superfused continuously with standard NaCl Ringer's (as described above) containing 1 mM $CaCl_2$ and no CsCl. Ratios were calibrated into free $[Ca^{2+}]$ according to the procedures of Grynkiewicz et al. (1985).

Measurement of Internal Store $[Ca^{2+}]$ in Digitonin-permeabilized Mag-Fura-2-AM–loaded Cells

RBL-1 cells or BHK-21 fibroblasts (cultured as described previously; Hofer et al., 1995) were loaded with 2 μM mag-fura-2-AM for 20–30 min at 37°C. Dye-loaded cells were rinsed briefly in a high K^+ solution (in mM: 125 KCl, 25 NaCl, 10 Hepes, pH 7.25, 0.1 $MgCl_2$) and then exposed for 2–3 min to an "intracellular buffer" at 37°C (the same solution supplemented with 0.5 mM MgATP, Ca/EGTA buffers, 0.1 mM total [EGTA], 200 nM free Ca^{2+}, pH 7.25) also containing 5 μg/ml digitonin. After plasma membrane permeabilization, cells were continuously superfused with intracellular buffer (without digitonin). Imaging experiments were performed as above for fura-2–loaded cells.

Results

Simultaneous Measurement of I_{CRAC} and Luminal $[Ca^{2+}]$: Release with Ionomycin

Although intracellularly trapped fluorescent Ca^{2+} indicators have often been used to measure the dynamics of $[Ca^{2+}]$ within $InsP_3$-sensitive stores, simultaneous recording of capacitative Ca^{2+} currents and $[Ca^{2+}]$ within stores has not been carried out previously. A major difficulty with these experiments has been that cell types traditionally used for measurement of I_{CRAC} (RBL, Jurkat, and mast cells) tend to accumulate dye in organelles rather poorly. However, by paying careful attention to loading conditions for the dye, we have been able to optimize compartmentalization of fluorophore into agonist sensitive stores, permitting measurement of $[Ca^{2+}]$ changes in that compartment. Fig. 1 shows a typical experiment in which whole-cell currents were measured concurrently with intracellular mag-fura-2 fluorescence in RBL-1 cells. The beginning of the record marks the point where the whole-cell configuration was established. The top traces represent the individual fluorescence intensities at 345 nm (the isoexcitation wavelength for this indicator) and 375 nm

(which is maximally Ca^{2+} sensitive). Both intensities declined after dialysis of the cytoplasmic indicator via the patch pipette, while the 345/375 ratio rose (indicating an increase in mean cellular $[Ca^{2+}]$) to a new steady-state level as the signal from Ca^{2+}-containing stores began to predominate. 290 s after establishment of the whole-cell configuration (average dialysis time 233 ± 17 s, mean ± SEM; data from 16 experiments), the cell was treated with ionomycin (1 μM) via an application pipette, resulting in a rapid drop in the mag-fura-2 ratio. On average, the maximum drop in the mag-fura-2 ratio occurred within 23 ± 3 s. An inward current (normalized to cell capacitance, shown at 0 mV in the bottom record) developed more slowly. An appreciable lag period, which averaged 5.5 ± 0.7 s, was always observed between the onset of the current and the initial decrease in $[Ca^{2+}]_{ER}$. The current (0.50 ± 0.04 pA/pF) required 77 ± 8 s from the time of the ionomycin addition to reach its peak. Shown in the inset at a higher time resolution is the normalized current (peak value) in response to the voltage ramp protocol depicted (−100 to +100 mV), which shows an inwardly rectifying current with the high positive reversal potential characteristic of I_{CRAC}. In four cells (out of 79), a clear drop in $[Ca^{2+}]$ was observed in the stores after ionomycin treatment, but no I_{CRAC} could be detected.

The experiment presented above clearly demonstrates not only that there is a substantial delay between the decrease in $[Ca^{2+}]$ within the stores and the onset of the current, but also that it takes several tens of seconds (over 30, on average) once maximal depletion of the stores has been achieved to get the maximal activation of the store-operated current. These long delays are hardly compatible with models in which I_{CRAC} activation is due to a mechanism similar to that triggering the opening of ryanodine receptors in skeletal muscle, i.e., direct modulation of channel opening by protein–protein interaction. Taking advantage of the possibility of measuring simultaneously I_{CRAC} and $[Ca^{2+}]_{ER}$, we then asked the question whether a threshold level of depletion is necessary to activate the current, as predicted by the all-or-none model of Parekh et al. (1997). Using the same approach shown in Fig. 1, we administered graded puffs of ionomycin from an application pipette to produce stepwise drops in stored Ca^{2+} (Fig. 2, *arrows*). Often this protocol of applying very small ionomycin doses to the cell produced a slow leak of Ca^{2+} out of the store, but occasionally ($n = 6$), discrete steps of Ca^{2+} release could be resolved in the fluorescence ratio recording, as seen in Fig. 2 *A*. The first small puff produced a barely detectable decrease in the mag-fura-2 ratio, but after some delay, a small inward current could be discerned, better resolved in current ramp *a*. Note that the kinetic properties of the onset of I_{CRAC} were quite different when the entire store was rapidly emptied (as in Fig. 1), compared with the case where a slow or incomplete release of internal stores was induced (Fig. 2, *A* and *B*). An apparent maximal activation of I_{CRAC} (ramp *b*) occurred when stores were partially depleted by a second puff of ionomycin, as a third, larger application of the ionophore resulted in a further drop in the mag-fura-2 ratio, but without any additional increase in the inward current. These results suggest that a modest drop in internal store $[Ca^{2+}]$ can stimulate I_{CRAC}. Therefore, if intraluminal $[Ca^{2+}]$ must fall to a threshold value to evoke Ca^{2+} influx, this drop likely constitutes a very small percentage of the whole store. In addition, these results also indicate that the entire store does not necessarily have to be released to achieve maximal activation of the current.

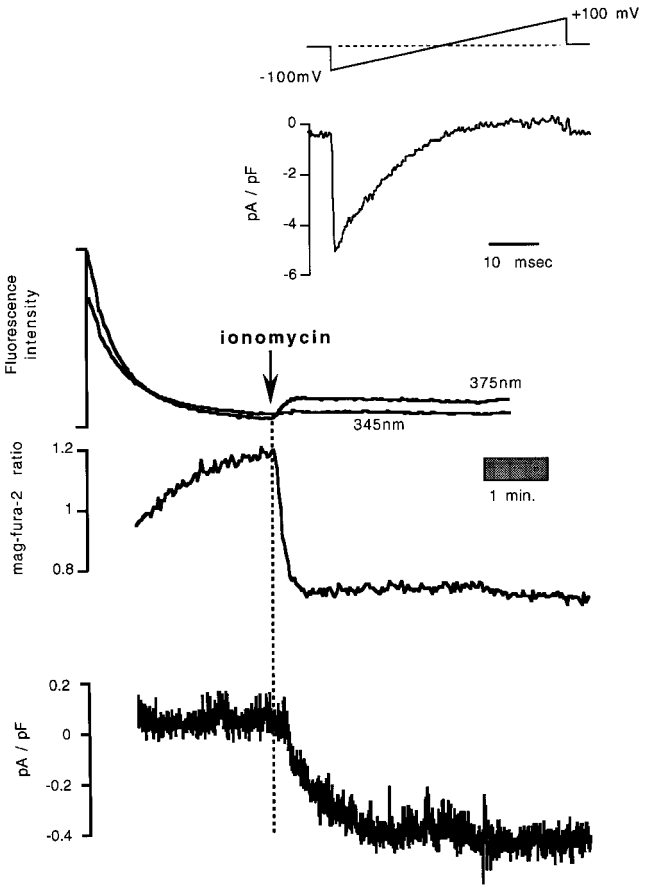

Figure 1. Simultaneous measurement of I_{CRAC} and internal store $[Ca^{2+}]$ using compartmentalized mag-fura-2. (*Top record*) Decline in individual 345- and 375-nm mag-fura-2 intensities after dialysis of the cytoplasmic indicator via the patch pipette. 290 s after establishment of the whole-cell configuration, the cell was treated with ionomycin (1 μM) via an application pipette, resulting in a rapid drop in the mag-fura-2 ratio (a measure of internal store $[Ca^{2+}]$; *middle record*). An inward current (normalized to cell capacitance, shown at 0 mV, *bottom record*; holding potential 0 mV) developed more slowly after a variable lag period that averaged 5.5 ± 0.7 s (±SEM; data from 16 cells). The current required 77 ± 8 s to reach its peak. (*Inset*) Normalized background subtracted current–voltage relationship (peak value) and voltage ramp protocol (−100 to +100 mV) shows inward rectification characteristic of I_{CRAC}.

A second type of experiment (shown in Fig. 2 *B*) further supports the conclusion that the activation of I_{CRAC} is a graded phenomenon that depends on the level of store $[Ca^{2+}]$. Internal stores were depleted passively using a Ca^{2+}-free patch pipette solution (containing also 10 mM BAPTA, i.e., free $[Ca^{2+}]$ about 10^{-9} M). Since store depletion would start as soon as the whole-cell configuration was attained (while dilution of cytosolic dye into the pipette was still occurring), we were not able to measure $[Ca^{2+}]$ in the store in this type of experiment. The spontaneous decrease of $[Ca^{2+}]_{ER}$ resulted in a slow, partial activation of I_{CRAC}. To confirm that indeed the partial activa-

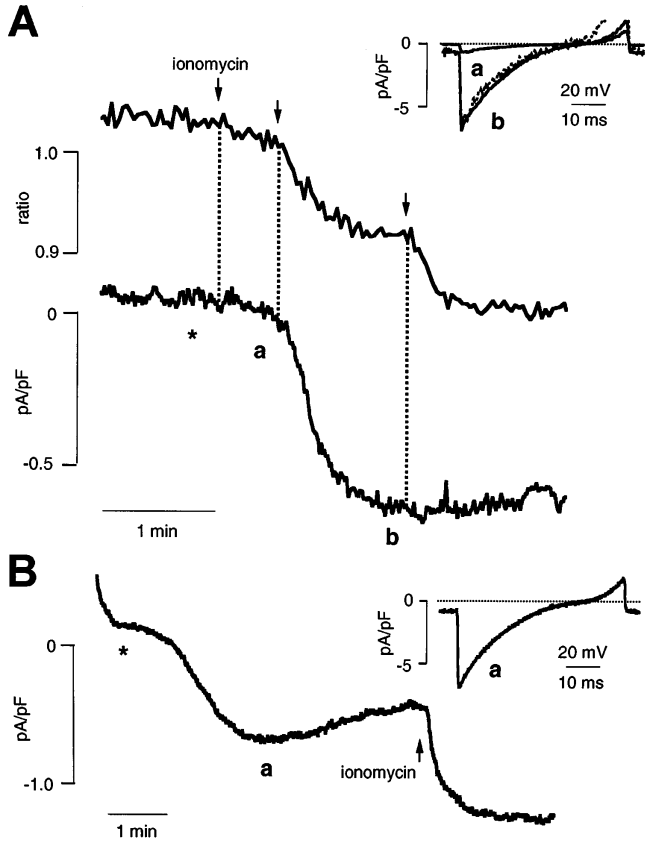

Figure 2. Is there a threshold level of store $[Ca^{2+}]$ depletion that results in the activation of I_{CRAC}? (*A*) Using the same protocol shown in Fig. 1, graded puffs of ionomycin were administered from an application pipette to produce stepwise drops in stored Ca^{2+} (*arrows*). The first puff produced a barely detectable decrease in the mag-fura-2 ratio, but after some delay, a very small inward current could be discerned, better resolved in current ramp *a* (*top inset*). The dotted line indicates ninefold amplification of ramp *a* to permit better comparison with ramp *b*, which shows the apparent maximal activation of I_{CRAC} after further depletion of stores with a second application of ionomycin. I–V relationships are background subtracted using ramps obtained from the time point marked by the asterisk in the whole-cell current recording. (*B*) Passive depletion of internal stores using Ca^{2+}-free pipette solution supplemented with 10 mM BAPTA produced a slow, partial activation of I_{CRAC} over a time course predicted to yield partial depletion of internal store Ca^{2+}. Additional current was elicited by ionomycin (1 μM) given from an application pipette.

tion of I_{CRAC} depended on a partial decrease of $[Ca^{2+}]_{ER}$, a full dose of ionomycin was given via an application pipette 5 min after breaking into the cell, resulting in a further increase in I_{CRAC} (typical of $n = 8$ experiments). Although we were obliged to conduct these experiments without the benefit of the mag-fura-2 measurement (because of the time required to dialyze the cytoplasmic indicator), parallel experiments using a variety of protocols to passively deplete stores (using Ca^{2+}-free medium or SERCA inhibitors) indicate that this length of time (5 min) is insufficient to completely empty internal Ca^{2+} stores (Hofer et al., 1995, 1996; and Hofer, A.M., and T. Pozzan, unpublished results in RBL cells).

A New Way to Reversibly Change the $[Ca^{2+}]$ in Internal Stores: Measurements in Permeabilized Mag-Fura-2–loaded Cells

$[Ca^{2+}]_{ER}$ can be depleted in many different ways: ionophores, sarco-endoplasmic reticulum Ca^{2+}-ATPase (SERCA) inhibitors, $InsP_3$, etc. Without exception, these treatments imply that the release into the cytoplasm of stored Ca^{2+} are difficult (or impossible) to grade and require tens of seconds or minutes to be reversed. The ideal way to study the quantitative correlation between $[Ca^{2+}]_{ER}$ and I_{CRAC} activation–inactivation, on the other hand, would be a method that (*a*) could be graded at will, (*b*) could be rapidly reversed, and (*c*) did not cause the loss of Ca^{2+} from the stores. The approach described below (which takes advantage of a compound introduced by our group several years ago, TPEN) was developed to fit as closely as possible to these requirements. TPEN is a membrane-permeant multivalent cation chelator, initially used for its ability to chelate heavy metals within intact cells (Arslan et al., 1985). However, the moderate affinity of TPEN for Ca^{2+} (K_d for Ca^{2+} ~130 μM) and the fact that it is membrane permeant in its uncomplexed form suggested to us that this compound could be used to rapidly and reversibly chelate Ca^{2+} within stores. It should be noted that TPEN binds Mg^{2+} ions very poorly at physiological concentrations. The rationale of the approach is quite simple: when TPEN is added to the medium, the uncomplexed form should diffuse rapidly across any cell membrane. Because of its low Ca^{2+} affinity, TPEN is predicted not to influence significantly the $[Ca^{2+}]$ in the cytoplasm or in any other cell compartment where the steady-state concentration is in the nanomolar or low micromolar range. On the contrary, in compartments where the $[Ca^{2+}]$ is comparable to its K_d, TPEN should bind Ca^{2+} and rapidly reduce its concentration. The opposite will happen when TPEN in the medium is washed off. The prediction therefore is that by grading the TPEN concentration in the bath it should be possible to cause graded and reversible decrease of the $[Ca^{2+}]_{ER}$, without interfering with either the release channels or the Ca^{2+} ATPases.

The effects of TPEN on intraluminal $[Ca^{2+}]$ were first tested in permeabilized mag-fura-2–loaded BHK-21 fibroblasts, a cell system that has been characterized extensively in previous studies (Hofer et al., 1995). We observed that, in general, mag-fura-2 measurements tended to be more sensitive in this cell type than in RBL-1 cells, possibly because the physical size of the agonist-sensitive store is larger in the BHK-21 fibroblast (or because of other differences in dye distribution or uptake between the two cell types). Furthermore, fluorescence signals from compartmentalized indicator can be calibrated in this cell type (for details see Hofer and Schulz, 1996) to give a good estimate of intraluminal free $[Ca^{2+}]$. According to the predictions, the experiment presented in Fig. 3 *A* shows that increasing doses of TPEN produced stepwise decreases in free $[Ca^{2+}]$. On average, 100 μM TPEN caused $[Ca^{2+}]$ to drop by 26 ± 3% ($n = 11$), 200 μM by 42 ± 3% ($n = 34$), 1 mM by 69 ± 5% ($n = 12$), and 2 mM reduced free $[Ca^{2+}]$ by 88 ± 2% ($n = 5$) in these cells. Lower doses of TPEN (1–10 μM), shown previously to be more than enough to chelate intracellular heavy metals completely (owing to its extremely high

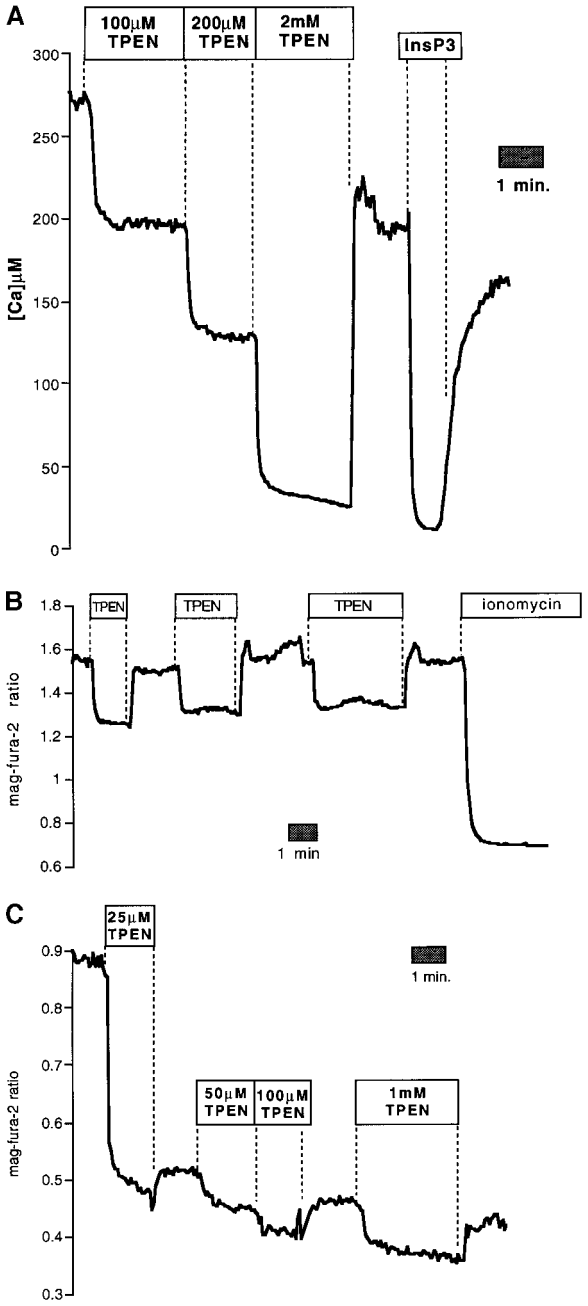

Figure 3. TPEN rapidly and reversibly chelates internal store Ca^{2+} as measured in permeablilzed mag-fura-2–loaded cells. (*A*) Steps in internal store $[Ca^{2+}]$ with increasing [TPEN] are clearly resolved in digitonin-permeabilized BHK-21 fibroblasts. Shown for comparison is the release of Ca^{2+} caused by $InsP_3$. (*B*) Three successive applications of 200 μM TPEN yield reproducible drops in store $[Ca^{2+}]$ that are rapidly reversed. (*C*) RBL-1 cells were frequently found to contain large quantities of stored heavy metals, making quantification of TPEN-induced $[Ca^{2+}]$ changes difficult in this cell type. Note the large ratio drop that occurs in RBL cells after treatment with 25 μM TPEN.

affinity for these cations, Arslan et al., 1985), did not produce any detectable fall in the mag-fura-2 ratio in BHK-21 cells, while 25 μM TPEN yielded very little change (not shown). The lack of effect of low [TPEN] indicates that, in this cell type, heavy metals do not interfere with the signal of organelle-trapped mag-fura-2. Removal of TPEN from the bathing solution caused free $[Ca^{2+}]$ to increase quickly because of rapid unbinding of the chelator from Ca^{2+} ions, as the free form was able to exit the compartment. Shown for comparison in the same cell is the response to 6 μM $InsP_3$. Fig. 3 *B* shows that a given TPEN concentration (in this case 200 μM) yielded reproducible drops in free $[Ca^{2+}]$ independent of the length of time that the chelator was present. Noteworthy is the rapidity of the TPEN effect, which required on average 18.2 ± 1.5 s to reach the maximal ratio drop, and about the same amount of time for a complete reversal of the effect (14.2 ± 1.9 s; $n = 11$). The reversal of the TPEN effect was not dependent on Ca^{2+} pumping, as it was also observed during treatment with tBHQ, a SERCA inhibitor ($n = 3$; data not shown).

We repeated the protocol of Fig. 3 *A* on mag-fura-2–loaded RBL-1 cells permeabilized with digitonin (Fig. 3 *C*). In contrast to the BHK-21 cells, a low dose of TPEN (25 μM) caused dramatic and essentially irreversible decreases in the mag-fura-2 ratio in over 80% of RBL cells. A similar effect, but with a slower time course, was observed at a concentration of TPEN as low as 2 μM. The effect of TPEN on fluorescence was evident as an increase in intensity at both the isoexcitation wavelength of 345 nm and at 375 nm (not shown). This response pattern is diagnostic of heavy metal interference on mag-fura-2 fluorescence. Therefore, a large proportion of RBL-1 cells appear to have substantial levels of heavy metals within subcellular compartments that can confound the measurement of free $[Ca^{2+}]$ with this method. After treatment with the low dose of TPEN, increasing [TPEN] to 50 μM, 100 μM, and 1 mM caused stepwise decreases in the mag-fura-2 ratio (due to chelation of Ca^{2+}, as there was no effect at the 345 nm excitation wavelength; typical of $n = 7$ experiments). Noteworthily, 1 μM ionomycin did not appear to release these interfering heavy metals in RBL-1 cells; ionomycin-induced ratio changes, such as those shown in Figs. 1 and 2, were largely unaffected by preincubation with 25 μM TPEN, and conversely, after release of stored Ca^{2+} by the ionophore, this low dose of TPEN caused further reduction of the mag-fura-2 ratio but did not increase I_{CRAC} (not shown).

TPEN Evokes Capacitative Ca^{2+} Entry as Measured in fura-2–loaded Intact Cells

The above experiments were carried out in permeabilized cells. Although the plasma membrane is known not to be a barrier to TPEN diffusion, we needed to verify whether TPEN could act as predicted on internal stores of intact cells. As a preliminary test, we again used BHK-21 cells since in this cell type conditions can be found under which even in intact cells the signal of mag-fura-2 trapped in the stores largely predominates over that in the cytoplasm (Hofer, A.M., B. Landolfi, L. Debellis, T. Pozzan, and S. Curci, manuscript submitted for publication). In the inset of Fig. 4, application of 800 μM TPEN (in the presence of 1 mM Ca^{2+} in the bathing solution) to an intact, live cell, resulted in a rapid drop of the $[Ca^{2+}]$ in the stores. Washout of TPEN from the medium resulted in a rapid return of the signal to slightly above the basal level; the fact that SERCAs continued to compensate for the drop in $[Ca^{2+}]_{ER}$ during TPEN addition likely accounts for this

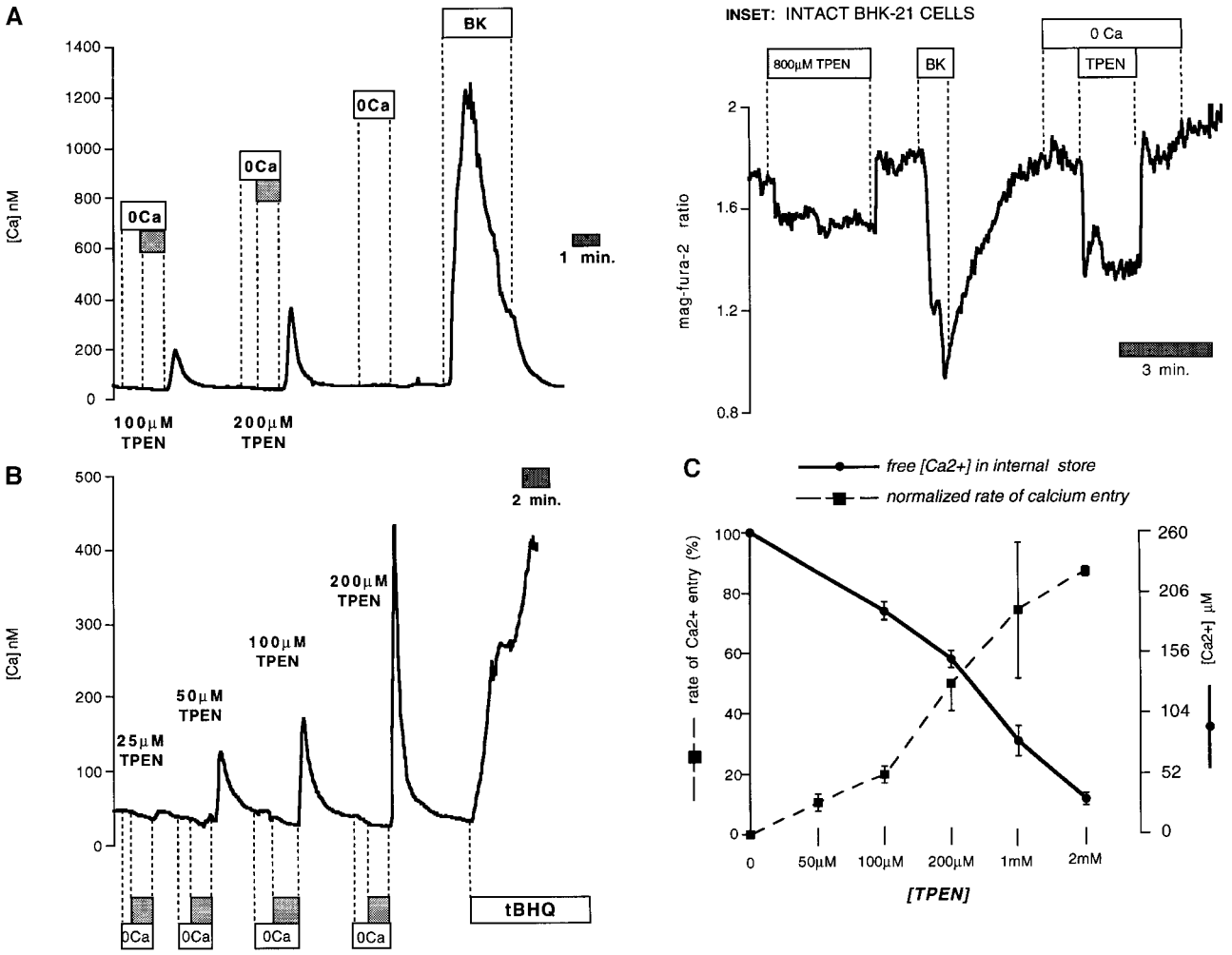

Figure 4. Chelating intrastore Ca^{2+} with TPEN activates Ca^{2+} entry in a dose-dependent manner. (*A*) TPEN activates Ca^{2+} entry in fura-2–loaded BHK-21 cells, but removal and readdition of external Ca^{2+} alone does not. Shown for comparison is the response to 100 nM bradykinin (*BK*). (*Inset*) Intact BHK-21 cell loaded with mag-fura-2-AM. Addition and removal of 800 μM TPEN (in the presence of 1 mM external Ca^{2+}; free [TPEN] ~170 μM) followed by brief stimulation with 100 nM bradykinin (*BK*). Intraluminal $[Ca^{2+}]$ dropped and was rapidly restored upon removal of 200 μM TPEN in the absence of bath Ca^{2+}. (*B*) Fura-2–loaded RBL-1 cells were treated with 25, 50, 100, or 200 μM TPEN in the absence of external Ca^{2+}. Upon Ca^{2+} readdition there was a graded increase in the rate and extent of Ca^{2+} entry. Shown at the end of the experiment is the effect of 100 μM tBHQ. (*C*) Initial rates of Ca^{2+} entry (which were closely correlated to the peak Ca^{2+}) after treatment with different [TPEN] (*dotted line*). Data are normalized in a given cell to the rate of influx after 100 μM TPEN treatment in that same cell (105 ± 19 nM/s). Overlaid for comparison is the free $[Ca^{2+}]$ in internal stores (calibrated according to Hofer and Schulz, 1996; *solid line*) for different [TPEN] from experiments such as those shown in Fig. 3 *A*.

small extra loading of Ca^{2+} into the store. Shown for comparison is the release of Ca^{2+} from internal stores evoked by 100 nM bradykinin. A second application of 200 μM TPEN in a Ca^{2+}-free medium produced a similar response; note that external Ca^{2+} was not necessary for the recovery of luminal Ca^{2+} upon washout of TPEN.

We next tested whether the fall in intraluminal $[Ca^{2+}]$ resulting from TPEN addition could stimulate capacitative Ca^{2+} entry in both BHK-21 and RBL-1 cells as measured by fura-2 measurements of cytoplasmic $[Ca^{2+}]$. In Fig. 4 *A*, BHK-21 cells were briefly superfused with a nominally Ca^{2+}-free solution and then with Ca^{2+}-free solution containing 100 μM TPEN. The absence of external Ca^{2+} ensured that the effective concentration of the membrane-permeant free form of TPEN remained sufficiently high. By itself, TPEN caused no effect on the cytoplasmic Ca^{2+} signal, but when Ca^{2+} was reintroduced in the perfusion medium, a transient cytoplasmic elevation was immediately observed. Increasing the [TPEN] to 200 μM resulted in a larger Ca^{2+} transient (n = 3 experiments; 22 cells). Similar results were also obtained in a primary culture of bovine aortic endothelial cells (not shown). These Ca^{2+} transients were not a consequence of simply removing and readding extracellular Ca^{2+}, as this maneuver alone in the absence of TPEN did not result in Ca^{2+} entry in any of the cell types used in this study. Shown for comparison in the same BHK-21 cell is the response to 100 nM bradykinin, an agonist that both releases Ca^{2+} from internal stores and causes Ca^{2+} influx via the plasma membrane.

Similar experiments were performed on intact fura-2–loaded RBL-1 cells using increasing TPEN concentrations of 25, 50, 100, and 200 μM (applied in Ca^{2+}-free solu-

tions; Fig. 4 *B*). Simultaneous Ca^{2+} readdition and TPEN removal produced transient peaks of cytoplasmic Ca^{2+} that were larger and faster with increasing [TPEN] (typical of $n = 6$ experiments; 59 cells). The conclusion from the experiments shown in Fig. 4, *A* and *B*, is that the magnitude and rate of the Ca^{2+} influx is closely linked to the degree of store emptying. Even 25 μM TPEN, which is calculated to lower $[Ca^{2+}]$ within stores by at most 16 μM (a 6% drop, assuming a resting $[Ca^{2+}]_{ER}$ of 260 μM), yielded a small Ca^{2+} entry as measured by fura-2 in RBL-1 cells, and 50 μM produced clear increases in Ca^{2+} influx. Fig. 4 *C* compares the extent of store depletion as measured by compartmentalized mag-fura-2 in BHK-21 cells upon addition of varying [TPEN] (derived from experiments such as those shown in Fig. 3 *A*) with the initial rate of Ca^{2+} entry (which was also closely correlated to the peak Ca^{2+} entry) in BHK-21 and RBL cells obtained using the protocol of Fig. 4 *B*.

The magnitude of capacitative Ca^{2+} entry appears to depend principally on the extent and not on the duration of the store depletion, as demonstrated in Fig. 5 *A*. RBL-1 cells were treated with 100 μM TPEN for different periods of time (1 vs. 3 min), but the amplitude and the rate of the Ca^{2+} entry (upon Ca^{2+} readdition) remained the same ($n =$ 3 experiments, data from 33 cells). Increasing the [TPEN] to 2 mM, however, produced a much larger peak of Ca^{2+}. Very brief pulses of 200 μM TPEN (20-s duration, not shown) produced a substantial, albeit smaller, influx of Ca^{2+} (peak upon Ca^{2+} readdition was 32 ± 3% of the control elevation elicited by a 2-min application of 200 μM TPEN; $n = 4$ experiments, data from 50 cells). Given that at least 10 s are required for completion of the TPEN-induced Ca^{2+} drop (Fig. 3 *B*), this indicates that the entry process can be activated by relatively short periods of store depletion (10 s), consistent with the kinetics of I_{CRAC} activation shown in Figs. 1 and 2, which show substantial activation within this period of time.

If internal store $[Ca^{2+}]$ is lowered (as when TPEN is applied) and then abruptly restored to its resting value (as occurs when TPEN is removed), it is expected that the mechanisms that signal the entry of Ca^{2+} will have been activated. How much time must elapse after store repletion before the signal is turned off? We examined this issue using the experimental protocol shown in Fig. 5 *B*. Cells were given 200 μM TPEN in a Ca^{2+}-free solution for at least 1 min and then washed in Ca^{2+}-free solution for varying times before Ca^{2+} readdition. In the first 10–15 s after TPEN removal, Ca^{2+} in the store returns to its original resting level as free TPEN exits the compartment, leaving behind Ca^{2+} that had been complexed. (See inset of Fig. 4; these conclusions have been verified thoroughly as part of a separate study in progress; Hofer, A.M., and T. Pozzan, manuscript in preparation).

External Ca^{2+} was then readded to probe the status of capacitative Ca^{2+} entry after varying times. When 90 s were allowed to elapse between TPEN removal and Ca^{2+} readdition, there was no detectable increase in cytoplasmic Ca^{2+}, indicating that the mechanisms responsible for sustaining Ca^{2+} entry had already been turned off. When the interval between TPEN removal was shortened to 60 s, a small Ca^{2+} entry was detected (5% of control), whereas at 30 s the entry was 36% of control. These data are summarized in the inset of Fig. 5 *B*. The ability of TPEN to stimu-

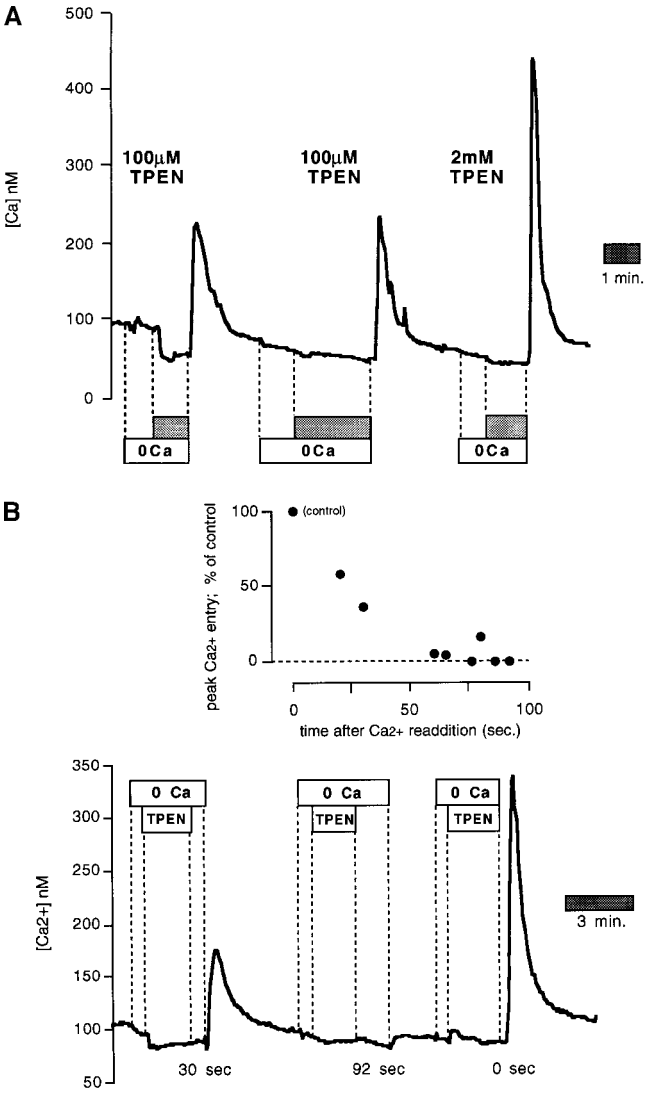

Figure 5. (*A*) The magnitude of capacitative Ca^{2+} entry depends on the extent of store depletion and not on duration. Fura-2–loaded RBL-1 cells were treated with 100 μM TPEN in Ca^{2+}-free solution for different periods of time, but the size of the Ca^{2+} entry upon Ca^{2+} readdition remained the same. (*B*) TPEN (200 μM), given in Ca^{2+}-free medium and then washed out in the absence of Ca^{2+} after 30 s, resulted in Ca^{2+} entry. The protocol was repeated, but the period in Ca^{2+}-free solution after TPEN removal was extended to 92 s. Readdition of Ca^{2+} did not produce detectable Ca^{2+} entry compared with control (Ca^{2+}-containing buffer superfused directly after TPEN). (*Inset*) Summary showing decay of Ca^{2+} entry for varying times after Ca^{2+} readdition. Each point represents averaged data from at least nine cells derived from one experimental run.

late Ca^{2+} entry was blocked completely when La^{3+} (10 μM) was given concurrently with Ca^{2+} after the washout of TPEN (not shown; $n = 3$ experiments, data from 39 cells).

TPEN Elicits I_{CRAC} as Measured by Whole-Cell Patch Clamp Technique

In whole-cell patch clamp experiments, chelation of store Ca^{2+} by TPEN also activated I_{CRAC} in RBL-1 cells. When the chelator was applied extracellularly as a large bolus via

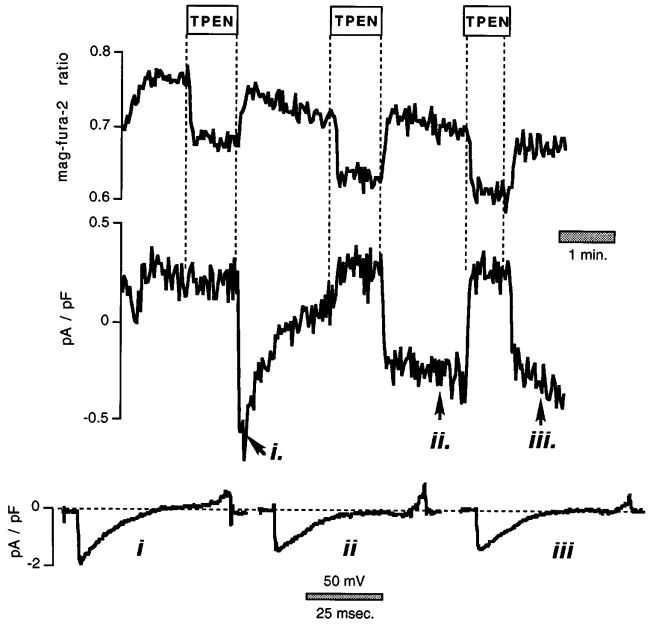

Figure 6. Chelation of store Ca^{2+} by TPEN activates I_{CRAC}. RBL-1 cells in the whole-cell patch clamp configuration were continuously superfused with solution containing 10 mM external Ca^{2+} and 25 μM TPEN to chelate contaminating heavy metals. Brief puffs of TPEN (5 mM in nominally Ca^{2+}-free external solution with 3 mM $MgCl_2$) administered by an application pipette caused a rapid drop of the mag-fura-2 ratio, which was quickly reversed when the application pipette was removed. I_{CRAC} (shown at −40 mV; holding potential 0 mV) was then apparent when the bath Ca^{2+} was restored as a consequence of rapid superfusion. After the removal of TPEN, stores were rapidly repleted with Ca^{2+} as TPEN exited the store, and the current decayed back to baseline. A second application of TPEN elicited an inward current but with dramatically reduced rate of current turn off. (*i–iii*) Background-subtracted current–voltage ramps are shown from the indicated portion of the current trace.

the application pipette, an inward current was observed (n = 24; Fig. 6). In these experiments, cells were rapidly superfused with external solution containing 10 mM Ca^{2+}, 3 mM $MgCl_2$, and 25 μM TPEN to chelate contaminating heavy metals. Brief puffs of TPEN (5 mM) in Ca^{2+}-free external solution (with 3 mM $MgCl_2$ to maintain stable seals and prevent transitions of the channel to a nonselective mode; Hoth and Penner, 1993) were given via an application pipette. 5 mM TPEN caused a rapid drop of the mag-fura-2 ratio, which was quickly reversed when pressure to the application pipette was discontinued. I_{CRAC} was then revealed when the external Ca^{2+} was restored as a consequence of rapid turnover of the bath. In control experiments using the same experimental conditions but with no TPEN in the application pipette, puffing 0 nM Ca^{2+}/3 nM Mg solution onto the cells had no effect on either the mag-fura-2 ratio or the whole-cell current (not shown; n = 3). These results indicate that the Ca^{2+} entry measured in fura-2 experiments after TPEN treatment is at least in part accounted for by the Ca^{2+} current measured in patch clamp experiments. After the removal of TPEN, stores were rapidly repleted with Ca^{2+} as TPEN exited the store, but the current decayed with a much slower time course (average $t_{1/2}$ = 23 s for the first TPEN application). This decay can be considered as the "intrinsic" turn-off rate of the current and is in good agreement with the fura-2 data shown in Fig. 5 *B*. Since stores were completely full (a situation rarely seen after recovery from agonist-stimulated release, which generally requires several minutes for completion), this rate of current inactivation is largely independent of the rate of refilling of the store.

It is well established that there is loss of I_{CRAC} activity after extended periods in the whole-cell configuration (Fasolato et al., 1993; Hoth and Penner, 1993). Fig. 6 indicates that prolonged dialysis results not only in reduced activation but also in a loss of the mechanism(s) responsible for inactivating I_{CRAC}. Each of the three sequential TPEN treatments were able to elicit the current, but the recovery back to baseline was diminished after each stimulation (n = 14 cells); in other experiments, the recovery was lost more gradually. One explanation for these data is that factors necessary for both the activation and inactivation of the current were lost with time when the cell was maintained in the whole-cell configuration.

Discussion

From their inception, early measurements of agonist-stimulated Ca^{2+} signaling events have revealed a high degree of spatiotemporal complexity (Bootman and Berridge, 1995). However, the notion that these dynamic patterns encode specific information that can be translated into physiological responses has only lately begun to find experimental support. For example, Dolmetsch et al. (1997) recently demonstrated that the altered amplitude of the plateau phase of Ca^{2+} entry with respect to the spike of Ca^{2+} release elicits distinct actions on particular Ca^{2+}-binding effectors, resulting in the differential expression of genes in immune cells. Similarly, mitochondria appear to accumulate Ca^{2+} effectively and activate their matrix dehydrogenases during the spike of a Ca^{2+} transient but not during the plateau phase (Rizzuto et al., 1994; Hajnóczky et al., 1995). Therefore, the pharmacological dissection of the Ca^{2+} peak from the plateau could ultimately prove to be of considerable biological and clinical utility. To date, however, concrete information regarding the specific proteins that mediate the entry of Ca^{2+} and couple the influx to store depletion is lacking.

In an effort to characterize this phenomenon more fully, we have probed the relationship between the filling state of internal Ca^{2+} stores and Ca^{2+} entry using two new tools: (*a*) simultaneous measurements of internal store [Ca^{2+}] and the store-operated Ca^{2+} current, I_{CRAC}, and (*b*) the use of a membrane-permeant Ca^{2+} chelator, TPEN, applied to both patch clamp experiments and imaging studies of fura-2–loaded cells.

Simultaneous measurements of store [Ca^{2+}] and I_{CRAC} showed that the release of Ca^{2+} by ionomycin not only occurred with a faster time course than did the activation of the current, but it also preceded the onset of I_{CRAC} by about 5 s. This lag time does not preclude the existence of a complex metabolic cascade for generating a message that signals Ca^{2+} entry at the plasma membrane. This is considerably longer than the lag time between the binding of an extracellular agonist to its receptor and the release of

intracellular Ca^{2+}, a process requiring several protein–protein interactions, enzymatic cleavage, and diffusion of a small second messenger ($InsP_3$) from the plasma membrane to the internal store.

Release of a minor portion of the store using submaximal doses of ionomycin resulted in the activation of a small current. The relationship between store emptying and I_{CRAC} appeared, however, to be nonlinear, as has been implicated by a number of previous studies (Petersen and Berridge, 1994; Mathes and Thompson, 1995; Zweifach and Lewis, 1996; Parekh et al., 1997). We observed that partial release of the store stimulated an inward current, the activation of which was initially very slow but accelerated with time, even though the additional drop in store $[Ca^{2+}]$ had been marginal during that period. Other modes of slow store depletion relying on the passive leak of Ca^{2+} from this compartment (removal of Ca^{2+} from the pipette solution as in Fig. 2 *B*, or treatment with SERCA blockers; not shown) consistently yielded a similar current profile. Thus, there appears to be a range of intraluminal $[Ca^{2+}]$ over which the current is turned on most effectively.

TPEN was shown to be a convenient tool for reversibly manipulating luminal $[Ca^{2+}]$ without interfering with other aspects of Ca^{2+} homeostasis, or activating other components of the signaling cascade (such as protein kinase C). Although TPEN also scavenges heavy metals that are of potential biological importance, it should be remembered that most of the commonly used Ca^{2+} indicators and chelators, such as fura-2, EGTA, and BAPTA, also have extraordinary affinities for these ions. Used at intracellular concentrations exceeding 100 μM, these dyes and buffers would also be expected to perturb cell functions dependent on heavy metals, and unlike the use of TPEN in this study, cells are generally exposed to these substances for prolonged periods without apparent adverse effects. While all other treatments previously used to elicit I_{CRAC} or Ca^{2+} influx (SERCA inhibitors, ionophores, agonists, $InsP_3$, etc.) rely on the release of Ca^{2+} from the store into the cytoplasm, TPEN rapidly permeates and binds the cation, thereby lowering the free $[Ca^{2+}]$. This approach should find widespread application. Because of its relatively high K_d for Ca^{2+}, the compound does not buffer cytoplasmic Ca^{2+}. Chelating free luminal $[Ca^{2+}]$ with TPEN was shown to evoke both I_{CRAC} and La^{3+}-sensitive Ca^{2+} influx as measured by fura-2, demonstrating for the first time that the $[Ca^{2+}]$ drop per se in the ER lumen, and not fluxes of the cation across membranes, is sufficient to stimulate Ca^{2+} entry.

A recent paper by Parekh et al. (1997) concluded that $InsP_3$-induced activation of I_{CRAC} is a threshold-dependent, all-or-none phenomenon; in other words, once initiated by a threshold level of store depletion, the current continues to develop to its maximum. This group proposed the existence of a specialized subcompartment of the store with differential sensitivity to $InsP_3$ to account for the finding that Ca^{2+} release into the cytoplasm could be observed without concomitant activation of Ca^{2+} influx. Our experiments on I_{CRAC} showed that the current was already evident after very small drops in store $[Ca^{2+}]$ and that partial release of the store resulted in submaximal I_{CRAC}. Furthermore, the imaging experiments on fura-2–loaded cells clearly indicate that the magnitude of Ca^{2+} entry is closely coupled to the extent of store depletion and, for store depletion exceeding 60 s, does not depend on the length of time that stores are empty.

As summarized in Fig. 4 *C*, progressively larger influxes occurred as the [TPEN] was raised (and correspondingly, the luminal $[Ca^{2+}]$ lowered). The possibility of carefully grading the extent of store depletion with TPEN should also (in principle) allow one to estimate directly and quantitatively the relationship between $[Ca^{2+}]_{ER}$ and I_{CRAC} activation. However, several uncertainties plague the absolute calibration of $[Ca^{2+}]_{ER}$ using compartmentalized indicators in cells such as RBL, T-lymphocytes, and mast cells, where the small amount of ER-trapped dye and heavy metal contamination produce significant complications. On the other hand, these cell types are notable for their robust store-operated Ca^{2+} currents and capacitative Ca^{2+} entry, which can be measured with relatively high precision. As such, assuming that a given [TPEN] elicits a similar drop in luminal $[Ca^{2+}]$ in both BHK-21 cells and RBL cells, we can conclude that a 65 μM (25%) reduction in store [Ca] (as elicited by 100 μM TPEN) results in ~20–25% activation of capacitative Ca^{2+} influx. Low doses of TPEN (25 and 50 μM), which yield marginal drops in store $[Ca^{2+}]$, also produced detectable Ca^{2+} influx (Fig. 4 *B*). These experiments indicate that if a threshold depletion is required to initiate Ca^{2+} entry, it is likely to be less than 10% of the resting $[Ca^{2+}]_{ER}$. Given that it has been firmly established that store-operated Ca^{2+} entry is essential for effective replenishment of Ca^{2+} stores, a mechanism incorporating graded Ca^{2+} entry seems logical from an energetic point of view, as the supply of Ca^{2+} for refilling is closely matched to the extent of depletion.

An aspect of the store-operated Ca^{2+} entry phenomenon that has received little consideration in the past is the mechanism by which this process inactivates (but see Montero et al., 1992). During physiological stimulation with Ca^{2+} releasing agonists, the complete release of Ca^{2+} from stores is fast (less than 60 s for completion) relative to the refilling of stores when the agonist is removed (which requires several minutes). Faster rates of release can be obtained with ionomycin (23 s in this study), while TPEN addition causes luminal $[Ca^{2+}]$ to fall to its minimum even more quickly.

We took advantage of the fact that free Ca^{2+} is rapidly (within 15 s) restored to resting levels after TPEN removal to look at the intrinsic turn off of I_{CRAC} and Ca^{2+} entry independent of SERCA-mediated refilling. Our data indicate that the cessation of I_{CRAC} requires about 70 s for completion. Therefore, once Ca^{2+} entry is activated by store depletion, drugs or other agents that interfere directly with the putative intraluminal ER Ca^{2+} detector will be expected to turn off I_{CRAC} and capacitative entry with a relatively slow time course, while agents acting directly at the plasma membrane channels should act more quickly. Our data hint that the turn off of the current may itself depend on a diffusible factor, as the inactivation time was progressively longer as the time in whole-cell configuration was increased. The time course for the inactivation of the entire entry process (channel inactivation and reversal of the signaling cascade responsible for stimulating entry, including the possible degradation of putative diffusible messengers) in the absence of Ca^{2+} influx was also probed in complementary experiments using fura-2–loaded cells

(Fig. 5 *B*). Our results show that ~60 s must elapse between the time that stores are refilled and the entry process is completely turned off in intact cells. Recent data from *Xenopus* oocytes have shown that internal Ca^{2+} release sites and regions of Ca^{2+} entry in the plasma membrane are colocalized (Petersen and Berridge, 1996; Jaconi et al., 1997). One interpretation of these results has been that direct physical coupling of the ER and plasma membrane (the conformational-coupling hypothesis) are necessary to activate Ca^{2+} influx. However, our data place an upper limit on the time required for the complete degradation of putative diffusible messenger(s) produced by empty stores (Randramampita and Tsien, 1993), and it remains to be established whether the lifetime of such molecules is sufficiently long to activate Ca^{2+} entry at sites distant from messenger production in these very large cells.

The ability to activate influx quickly and inactivate relatively slowly may be a mechanism that ensures that Ca^{2+} signals, once initiated, are sustained, and that Ca^{2+} entering the cytoplasm is adequate for effective refilling of stores.

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