Substantial depletion of the intracellular Ca^{2+} stores is required for macroscopic activation of the Ca^{2+} release-activated Ca^{2+} current in rat basophilic leukaemia cells

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1. Tight-seal whole-cell patch clamp experiments were performed to examine the ability of different intracellular Ca^{2+} mobilising agents to activate the Ca^{2+} release-activated Ca^{2+} current (I_{CRAC}) in rat basophilic leukaemia (RBL-1) cells under conditions of weak cytoplasmic Ca^{2+} buffering.

2. Dialysis with a maximal concentration of inositol 1,4,5-trisphosphate (IP_3) routinely failed to activate macroscopic I_{CRAC} in low buffer (0·1 mM EGTA, BAPTA or dimethyl BAPTA), whereas it activated the current to its maximal extent in high buffer (10 mM EGTA). Dialysis with a poorly metabolisable analogue of IP_3, with ionomycin, or with IP_3 and ionomycin all failed to generate macroscopic I_{CRAC} in low Ca^{2+} buffering conditions.

3. Dialysis with the sarco/endoplasmic reticulum Ca^{2+}-ATPase (SERCA) pump blocker thapsigargin was able to activate I_{CRAC} even in the presence of low cytoplasmic Ca^{2+} buffering, albeit at a slow rate. Exposure to IP_3 together with the SERCA blockers thapsigargin, thapsigargicin or cyclopiazonic acid rapidly activated I_{CRAC} in low buffer.

4. Following activation of I_{CRAC} by intracellular dialysis with IP_3 and thapsigargin in low buffer, the current was very selective for Ca^{2+} (apparent K_D of 1 mM). Sr^{2+} and Ba^{2+} were less effective charge carriers and Na^+ was not conducted to any appreciable extent. The ionic selectivity of I_{CRAC} was very similar in low or high intracellular Ca^{2+} buffer.

5. Fast Ca^{2+}-dependent inactivation of I_{CRAC} occurred at a similar rate and to a similar extent in low or high Ca^{2+} buffer. Ca^{2+}-dependent inactivation is not the reason why macroscopic I_{CRAC} cannot be seen under conditions of low cytoplasmic Ca^{2+} buffering.

6. I_{CRAC} could be activated by combining IP_3 with thapsigargin, even in the presence of 100 μM Ca^{2+} and the absence of any exogenous Ca^{2+} chelator, where ATP and glutamate represented the only Ca^{2+} buffers in the pipette solution.

7. Our results suggest that a threshold exists within the IP_3-sensitive Ca^{2+} store, below which intraluminal Ca^{2+} needs to fall before I_{CRAC} activates. Possible models to explain the results are discussed.

In rat basophilic leukaemia (RBL-1) cells, an experimental model for mucosal mast cells, calcium (Ca^{2+}) influx is a central event in the secretion of inflammatory mediators (Ali *et al.* 1990; Kim *et al.* 1997). Activation of cell-surface receptors that couple to inositol 1,4,5-trisphosphate (IP_3) production evokes a biphasic increase in intracellular Ca^{2+}: an initial Ca^{2+} release phase is followed by a smaller but sustained Ca^{2+} influx component (Berridge, 1993). In RBL-1 cells, like other non-excitable cells, emptying of the intracellular Ca^{2+} stores activates a Ca^{2+} current called I_{CRAC} (Ca^{2+} release-activated Ca^{2+} current; Hoth & Penner, 1992; Parekh & Penner, 1997). The relationship between IP_3-evoked Ca^{2+} release and subsequent activation of I_{CRAC} is complex. A partial dissociation between Ca^{2+} release and store-operated Ca^{2+} influx has been found in several cells including RBL-1 cells (Parekh *et al.* 1997; Hartmann & Verkhratsky, 1998; Liu *et al.* 1998). Despite its importance, the mechanisms underlying this widespread phenomenon are not known.

When I_{CRAC} is studied under conditons of physiological cytoplasmic Ca^{2+} buffering and IP_3 is used to deplete the intracellular stores, the current is not detectable, although intracellular fluorescent dyes reveal modest activation of Ca^{2+} influx following IP_3 elevation (Parekh *et al.* 1997; Huang *et al.* 1998). It is widely accepted that the stores are fully depleted under these conditions, and the inability to record any macroscopic I_{CRAC} reflects Ca^{2+}-dependent

Table 1. A summary of the several different conditions involving IP_3-evoked Ca^{2+} release and/or ionomycin that were used to activate I_{CRAC} is presented

Condition	+ (pA pF^{-1})	n	− (%)	n
(1) 30 μM IP_3–10 mM $[Ca^{2+}]_o$	−1·4	1	83	5/6
(2) 30 μMIP_3 + 2 mM ATP–10 mM $[Ca^{2+}]_o$	−1·1	1	93	14/15
(3) 30 μM IP_3 + 2 mM ATP–2 mM $[Ca^{2+}]_o$	—	—	100	5/5
(4) 30 μM IP_3-F + 2 mM ATP–10 mM $[Ca^{2+}]_o$	—	—	100	9/9
(5) 30 μM IP_3 + 2 mM ATP + 100 μM $[Ca^{2+}]_i$–10 mM $[Ca^{2+}]_o$	—	—	100	6/6
(6) 30 μM IP_3 + 2 mM ATP + 10 μM ionomycin–10 mM $[Ca^{2+}]_o$	−1·2	1	89	8/9
(7) 30 μM IP_3 + 2 mM ATP + 20 nM thapsigargin–10 mM $[Ca^{2+}]_o$	—	—	100	7/7
(8) 30 μM IP_3 + 2 mM ATP + 10 mM EGTA–10 mM $[Ca^{2+}]_o$	−2·19 ± 0·13	16	11	2/18
(9) 30 μM IP_3 + 2 mM ATP + 2 μM thapsigargin–10 mM $[Ca^{2+}]_o$	−1·96 ± 0·23	12	14	2/14
(10) 10 μM ionomycin + 2 mM ATP–10 mM $[Ca^{2+}]_o$	−0·9	1	89	8/9

For all conditions 0·1 mM EGTA or BAPTA was used unless otherwise is stated. '+' indicates that macroscopic currents could be detected and the corresponding mean ± S.E.M. values (wherever possible) and number of cells (n) are indicated. '−' means that no macroscopic current was detected. The percentage (%) of failures and number of cells (n) are indicated. $[Ca^{2+}]_o$ indicates extracellular calcium. No Ca^{2+} chelator was used in condition (5).

negative feedback mechanisms that maintain very low channel activity (Huang *et al.* 1998). Our results demonstrate that this explanation is not correct and instead provide evidence that Ca^{2+} release in response to a maximal concentration of IP_3 is unable to deplete the stores sufficiently to activate macroscopic I_{CRAC}. Instead, a threshold exists below which intraluminal Ca^{2+} has to fall before I_{CRAC} activates. The results also provide a mechanistic explanation for selective activation of Ca^{2+}-dependent processes including Ca^{2+} influx-dependent exocytosis in these cells.

METHODS

Rat basophilic leukaemia cells (RBL-1) cells, which were bought from the American Type Culture Collection, were cultured as previously described (Fierro & Parekh, 1999*a*,*b*).

Patch-clamp experiments were conducted in the tight-seal whole-cell configuration at room temperature (18–25 °C) as previously described (Hamill *et al.* 1981; Fierro & Parekh, 1999*a*). Sylgard-coated, fire-polished patch pipettes had DC resistances of 2·5–4 MΩ when filled with standard internal solution containing (mM): caesium glutamate 145, NaCl 8, $MgCl_2$ 1, Hepes 10 (pH 7·2 with CsOH). Depending on the experiment (described in the text), the Ca^{2+} chelators EGTA (Sigma), BAPTA tetracaesium salt (BAPTA) or dimethyl BAPTA tetrapotassium salt (both from Molecular Probes) were added to this solution at the specified concentrations, as sometimes was 30 μM IP_3, 2 μM thapsigargin (from three independent sources: Sigma, Calbiochem, Alomone Labs), 100 μM cyclopiazonic acid, or 2 μM thapsigargicin (Calbiochem). All chemicals were purchased from Sigma unless otherwise noted. A correction of +10 mV was applied for the subsequent liquid junction potential that arose from this glutamate-based internal solution. Extracellular solution contained (mM): NaCl 145, KCl 2·8, $CaCl_2$ 10, $MgCl_2$ 2, CsCl 10, glucose 10, Hepes 10 (pH 7·4 with NaOH). In some experiments, external Ca^{2+} was simply lowered to the desired concentration. High resolution current recordings were acquired and I_{CRAC} was measured as described previously. Data are presented as means ± S.E.M., and statistical evaluation was carried out using Student's unpaired t test.

RESULTS

IP_3 activates I_{CRAC} in high but not low intracellular Ca^{2+} buffer

Figure 1*A* compares the effect of dialysing individual RBL-1 cells with either a low or high concentration of EGTA (0·1 or 10 mM) together with a maximal concentration of IP_3 (30 μM) in the patch pipette. Most cells failed to generate any detectable I_{CRAC} in low Ca^{2+} buffer (IP_3 + 0·1 mM EGTA/BAPTA, panel *A*1 of Fig. 1*A* and Table 1; we can detect with confidence a macroscopic current of around −0·2 pA pF^{-1} at a bandwidth of 2·3 kHz) whereas a macroscopic current was routinely activated in the presence of high EGTA (panel *A*1 of Fig. 1*A*). Because IP_3 can be converted to IP_4 in a Ca^{2+}-dependent manner (Shears, 1992), we dialysed cells instead with IP_3-F (a poorly metabolisable analogue, Kozikowski *et al.* 1990) together with 0·1 mM EGTA. I_{CRAC} still routinely failed to be detected (panel *A*3 of Fig. 1*A*; Table 1). Dialysis with the Ca^{2+} ionophore ionomycin (10–15 μM) in 0·1 mM EGTA, alone or together with IP_3, also failed to activate any macroscopic current (Fig. 1*A* panels *A*4 and *A*5; Table 1). These results are somewhat unexpected, because numerous previous studies using fluorescent dyes have shown that IP_3 substantially reduces the Ca^{2+} content of the intracellular IP_3-sensitive Ca^{2+} stores in RBL-1 cells (Ali *et al.* 1995; Parekh *et al.* 1997; Huang *et al.* 1998). One would have

therefore expected I_{CRAC} to fully activate following exposure to IP_3 in low Ca^{2+} buffer. Because the fluorescence measurements were all done using external Ca^{2+} concentrations around 2 mM, whereas the I_{CRAC} recordings are carried out in 10 mM Ca^{2+} (which gives the maximum current), we speculated that it might be harder for IP_3 to deplete the stores in 10 mM Ca^{2+} due to their potentially greater loading. However, IP_3 was still unable to activate a detectable I_{CRAC} in low Ca^{2+} buffering conditions when external Ca^{2+} was reduced to 2 mM (Table 1).

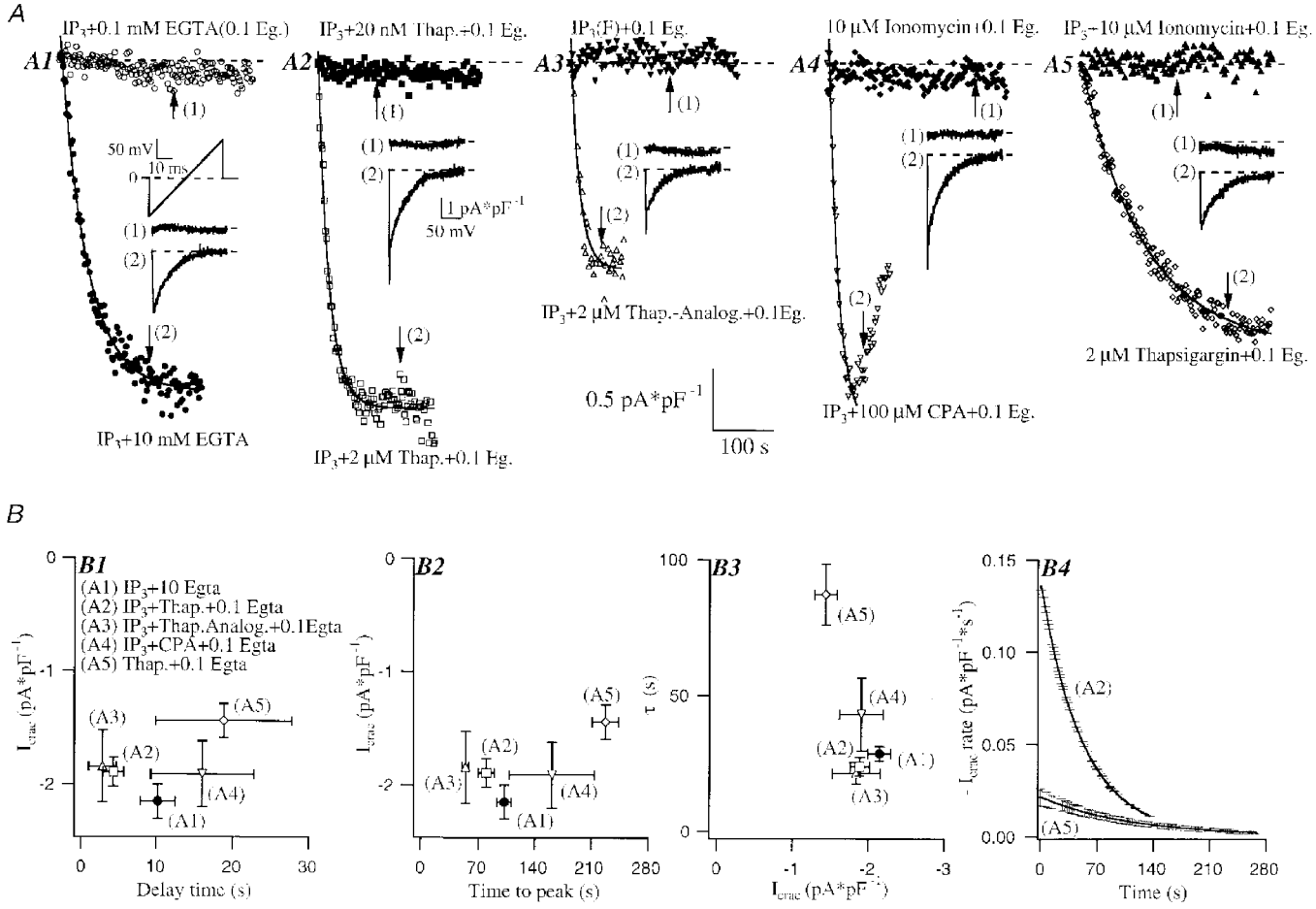

Figure 1. Block of the SERCA pumps rather than IP_3 (IP_3)-evoked Ca^{2+} release is sufficient to observe macroscopic I_{CRAC} under physiological conditions of low cytoplasmic Ca^{2+} buffering

A, the effect of dialysing individual cells with either 0·1 mM (low buffer) or 10 mM EGTA (high buffer) together with 30 μM IP_3 and 2 mM ATP is shown. I_{CRAC} amplitude (measured at −80 mV from voltage ramps from −100 to + 100 mV over 50 ms from a holding potential of 0 mV) is plotted against time after the onset of whole-cell recording. Whilst most of the cells dialysed with low buffer and IP_3 (*A*1), IP_3-F (*A*3), ionomycin (*A*4) or IP_3 and ionomycin (*A*5) failed to generate any detectable I_{CRAC}, the current was routinely activated in the presence of the SERCA pump blocker thapsigargin alone (*A*5). Dialysis with IP_3-F and 10 mM EGTA activated I_{CRAC} rapidly and to the maximal extent and the current was indistinguishable from that seen with IP_3 and 10 mM EGTA (data not shown). IP_3 interacted synergistically with the structurally distinct SERCA blockers thapsigargin (*A*2), thapsigargicin (*A*3) and cyclopiazonic acid (CPA) (*A*4) to generate I_{CRAC}. The effect of SERCA pump blockers was dose dependent, as is shown in *A*2. All traces where macroscopic I_{CRAC} is detected are fitted by a single exponential function. Insets show the *I–V* relationship of the current for each condition. The arrows in the plot of I_{CRAC} development against time indicate where the inset *I–V* relationships were taken. *B*, I_{CRAC} amplitude is plotted as a function of delay (*B*1), time to peak (*B*2) and the time constant (τ) of the exponential fits (*B*3). The different conditions are stated in panel *B*1. Each point represents mean ± S.E.M. of more than five cells. Delay was not significantly different between the conditions ($P > 0{\cdot}05$). Time to peak and τ were significantly different depending whether (*A*1–*A*4) or not (*A*5) IP_3 was present in the dialysing solution ($P < 0{\cdot}0001$). In panel *B*4, the rate of current development is plotted as a function of time for the conditions where I_{CRAC} was activated by IP_3 and thapsigargin (*A*2, $n = 16$) or thapsigargin alone (*A*5, $n = 6$). The first derivative of the exponential fit for each trace in either condition was calculated, multiplied by −1 and then pooled to calculate mean ± S.D. of the I_{CRAC} rate.

Block of SERCA pumps activates I_{CRAC} in low Ca^{2+} buffer

We considered two explanations for this inability of IP_3-evoked Ca^{2+} release to activate detectable I_{CRAC} despite substantial store depletion. First, the current activates only after the IP_3-sensitive stores have been depleted below a certain level, and a sizeable macroscopic current subsequently requires further and rather extensive emptying of the stores or, second, the current rapidly inactivates due to Ca^{2+}-dependent negative feedback mechanisms in the presence of the low Ca^{2+} chelator concentration in the cytoplasm.

If the first hypothesis is correct, then it predicts that sarco/endoplasmic reticulum Ca^{2+}-ATPases (SERCA)-type blockers (which can, in contrast to IP_3, fully empty IP_3-sensitive stores in many cells; Tse *et al.* 1994; Chatton *et al.* 1995; Montero *et al.* 1995, 1997) should be able to activate macroscopic I_{CRAC} in physiological cytoplasmic Ca^{2+} buffering. We therefore dialysed cells with 2 μM thapsigargin, a SERCA pump blocker (Thastrup *et al.* 1990), and 0·1 mM EGTA. Whereas IP_3 and ionomycin had failed to activate I_{CRAC}, we found that 2 μM thapsigargin alone was able to activate a sizeable inward Ca^{2+} current (Fig. 1*A*, panel *A*5). The current developed slowly, but was clearly I_{CRAC} from its biophysical features (ramp inset in panel *A*5 of Fig. 1*A*; reversal potential $>+50$ mV, inward rectification, Ca^{2+} selective, see below). Addition of IP_3 to the 2 μM thapsigargin–0·1 mM EGTA solution generated I_{CRAC} (Fig. 1*A* panel *A*2) after a similar delay to that seen with thapsigargin alone. This delay was $9{\cdot}84 \pm 2{\cdot}23$ s ($n = 62$, data pooled for all cells analysed in Fig. 1*B*, panel *B*1, because there was no difference between them, $P > 0{\cdot}05$). The panels *B*2–*B*4 of Fig. 1*B* show the striking difference in the rate of development of the current for IP_3 and thapsigargin together compared with thapsigargin alone (time to peaks were $92{\cdot}9 \pm 8{\cdot}5$; $n = 49$, data pooled from all cells in Fig. 1*B*, panels *B*2 and *B*3, that were dialysed with IP_3 because there was no significant difference between them) *vs.* $227{\cdot}85 \pm 16{\cdot}1$ ($n = 13$); activation time constants (τ) were $27{\cdot}1 \pm 2{\cdot}4$ and $87{\cdot}2 \pm 11{\cdot}3$ s ($n = 8$), respectively ($P < 0{\cdot}0001$ for both cases; Fig. 1*B*, panels *B*2 and *B*3). Although the current developed much more slowly in thapsigargin alone (panel *B*4), its overall extent was not significantly different from that seen in IP_3 and thapsigargin ($P > 0{\cdot}05$; Fig. 1*B*). Replacing 0·1 mM EGTA with dimethyl BAPTA or BAPTA did not affect the size or rate of development of the current in response to IP_3 and thapsigargin (data not shown).

We could mimic the effects of thapsigargin by using the different SERCA pump blockers cyclopiazonic acid (Inesi & Sagra, 1994; 7/7 cells; Fig. 1*A*, panel *A*4) and thapsigargicin (which is almost as potent as thapsigargin but less hydrophobic; Fig. 1*A*, panel *A*3). Furthermore, the effect of thapsigargin was concentration dependent because dialysis with IP_3 and 20 nM thapsigargin routinely failed to activate a detectable current (Fig. 1*A*, panel *A*2; Table 1). By blocking SERCA pumps, thapsigargin will raise intracellular Ca^{2+} in the presence of a low Ca^{2+} buffer. However, intracellular dialysis with IP_3 and approximately 1 μM free Ca^{2+} (only exogenous buffers present were 3·3 mM Mg-ATP and 145 mM glutamate in the presence of 100 μM $CaCl_2$ and 1 mM $MgCl_2$) failed to mimic the effect of IP_3 and thapsigargin (Table 1, condition (5), 6/6 cells). Block of the SERCA pumps is therefore sufficient for macroscopic I_{CRAC} to be recorded under conditions of physiological Ca^{2+} buffering.

Comparison of the ionic conductivity profile of I_{CRAC} in low and high intracellular Ca^{2+} buffer

We next compared the conductivity of I_{CRAC} to different external divalent and monovalent cations in the presence of either high or low cytoplasmic Ca^{2+} buffering. Figure 2*A* compares the effects of changing external Ca^{2+} on the size of I_{CRAC} for the combination of IP_3 and thapsigargin in low Ca^{2+} buffer (0·1 mM EGTA or dimethyl BAPTA) with that seen in high Ca^{2+} buffer (10 mM EGTA). Reducing external Ca^{2+} reduced the size of the current for all conditions equally. I_{CRAC} had quite a high affinity for Ca^{2+} with apparent K_D values of 0·84, 1·17 and 1·03 mM for IP_3 and 10 mM EGTA, IP_3 and thapsigargin in 0·1 mM EGTA, and IP_3 and thapsigargin in 0·1 mM dimethyl BAPTA, respectively. These values are very similar and indicate that the current has the same external Ca^{2+} dependence for the different buffering conditions. The high affinity for Ca^{2+} in RBL-1 cells is different from a previous report in mast cells and markedly distinct from that in *Xenopus* oocytes (apparent K_D values of 3·3 and 11·5 mM, respectively; Hoth & Penner, 1993; Yao & Tsien, 1997). I_{CRAC} in RBL-1 cells is permeable to Ba^{2+} and Sr^{2+}, although to lesser extents than Ca^{2+} (Fierro & Parekh, 1999*a*). As expected, the current activated by the combination of IP_3 and thapsigargin in low cytosolic Ca^{2+} buffering conditions was also less permeable to Ba^{2+} and Sr^{2+} and the conductivity profile for both low and high Ca^{2+} buffering was $Ca^{2+} > Sr^{2+} \geqslant Ba^{2+}$ (Fig. 2*B*). We did find that Ba^{2+} was conducted better than we have previously found for high Ca^{2+} buffering conditions (Fierro & Parekh, 1999*a*), although still less so than Ca^{2+}. Furthermore, the current carried by Ba^{2+} had a more negative reversal potential and approached zero at $\leqslant -35$ mV (see ramp in Fig. 2*B*). This may be related to an anomalous mole fraction effect (Hoth, 1995). There was a tendency for the Sr^{2+} and Ba^{2+} currents (relative to Ca^{2+}) to be smaller in low rather than high Ca^{2+} buffer (Fig. 2*B*), which might indicate that the channel selectivity depends on the intracellular Ca^{2+} buffering, as previously noted (Zhang & McCloskey, 1995). However, to address this issue thoroughly will require detailed single-channel recordings, which are not feasible yet for the CRAC channel.

We then examined whether CRAC channels were permeable to Na^+ in the presence of 1 mM external Ca^{2+} under both low and high Ca^{2+} buffer conditions. Reducing external Na^+ 14·5-fold failed to alter the *I–V* relationship and the size of the current for either condition (Fig. 2*C*). Hence I_{CRAC} is very selective for Ca^{2+}, even under physiological Ca^{2+}

buffering conditions. We also tested whether external Mg^{2+} could affect Ca^{2+} permeability through I_{CRAC} under the conditions of both low cytosolic Ca^{2+} buffering and physiological external Ca^{2+} concentrations. Lowering external Mg^{2+} from 2 mM to 0 (nominally Mg^{2+} free) did not affect the size of the current (in 2 mM Ca^{2+}) nor its *I–V* relationship for both low and high Ca^{2+} buffering conditions (Fig. 2*D*). Taken together, the conductivity results demonstrate that I_{CRAC} activated by the combination of IP_3 and thapsigargin in low cytoplasmic Ca^{2+} buffer is very similar to the current seen in the presence of high Ca^{2+} buffer, and therefore the two are one and the same. The divalent and monovalent cation conductivity profile of I_{CRAC} in native RBL-1 cells is strikingly similar to the profile seen for the recombinant mammalian trp homologue bCCE1 expressed in Chinese hamster ovary cells under conditions of high Ca^{2+} buffering (Warnat *et al.* 1999). I_{CRAC} could be related therefore to bCCE1 and the latter might contribute to the CRAC channel pore.

Ca^{2+}-dependent inactivation does not affect I_{CRAC} development in low Ca^{2+} buffer

Although we have provided evidence to support our first explanation, namely that a threshold exists before I_{CRAC} can activate, we set about testing the currently accepted view

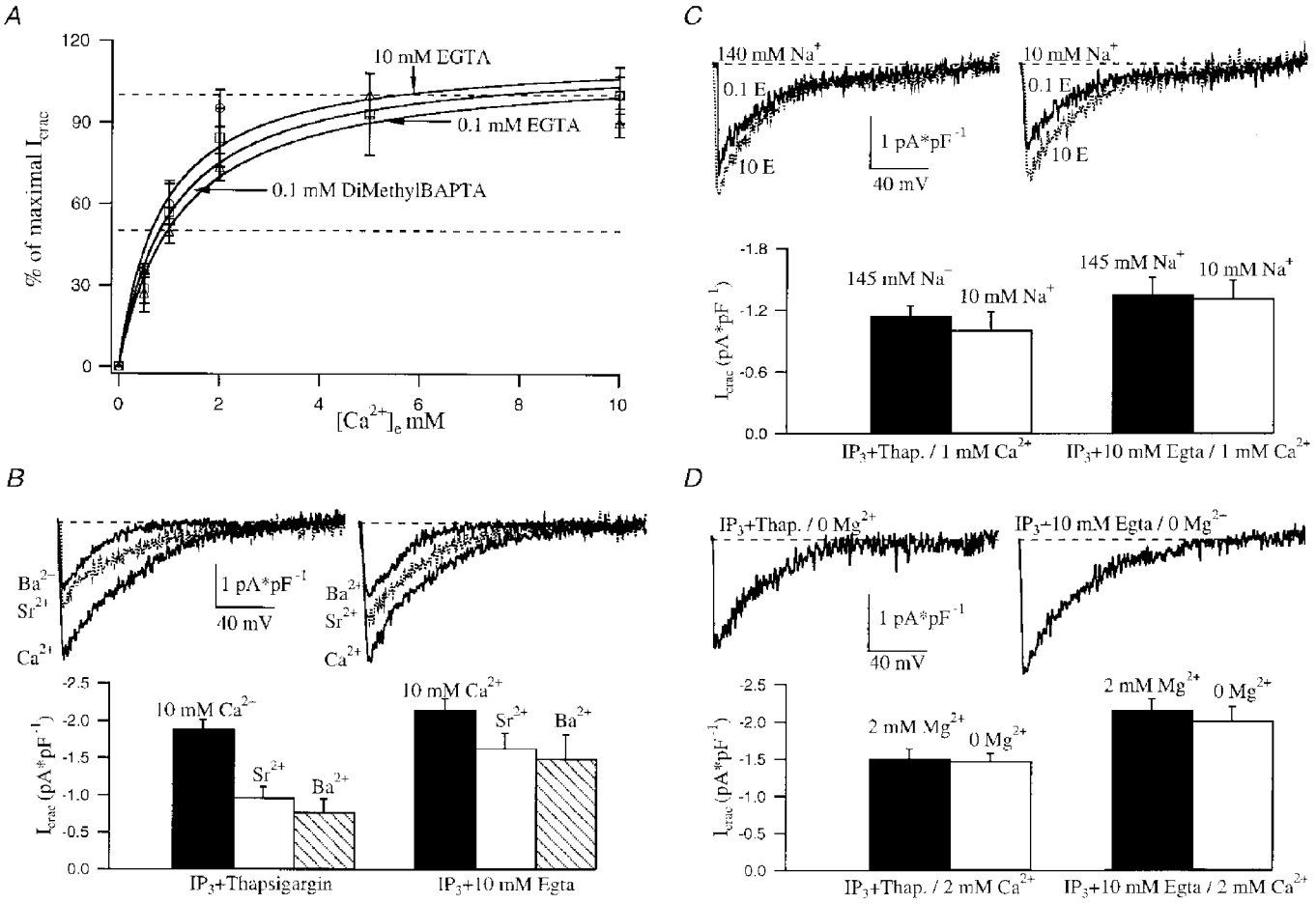

Figure 2. I_{CRAC} activated in the presence of either high or low cytoplasmic Ca^{2+} buffering is highly selective for Ca^{2+} ions

A, effect of extracellular Ca^{2+} concentration on I_{CRAC} amplitude. Each point represents the mean ± S.E.M. of more than six cells. Data for the three different conditions were fitted with a Michaelis-Menten type equation and a K_D of around 1 mM external Ca^{2+} was obtained for each condition (see text). Cells were directly placed in an external solution containing the desired Ca^{2+} concentration from the onset. *B*, I_{CRAC} profile in high and low cytoplasmic Ca^{2+} buffering is compared for cells bathed with three different extracellular divalent cations (Ca^{2+}, Sr^{2+}, Ba^{2+}). *I–V* relationships are shown in the upper panel and averaged data below as a histogram. Each bar represents the mean ± S.E.M. of more than seven cells. *C*, reducing external Na^+ 14·5-fold failed to alter the *I–V* relationship and the size of I_{CRAC} for both high and low cytoplasmic Ca^{2+} buffering. Each bar represents the mean ± S.E.M. of more than five cells. *D*, lowering external Mg^{2+} from 2 mM to 0 (nominally Mg^{2+} free) did not affect the size of the current (in 2 mM Ca^{2+}) nor its *I–V* relationship for both Ca^{2+} buffering conditions. Each bar represents the mean ± S.E.M. of more than four cells.

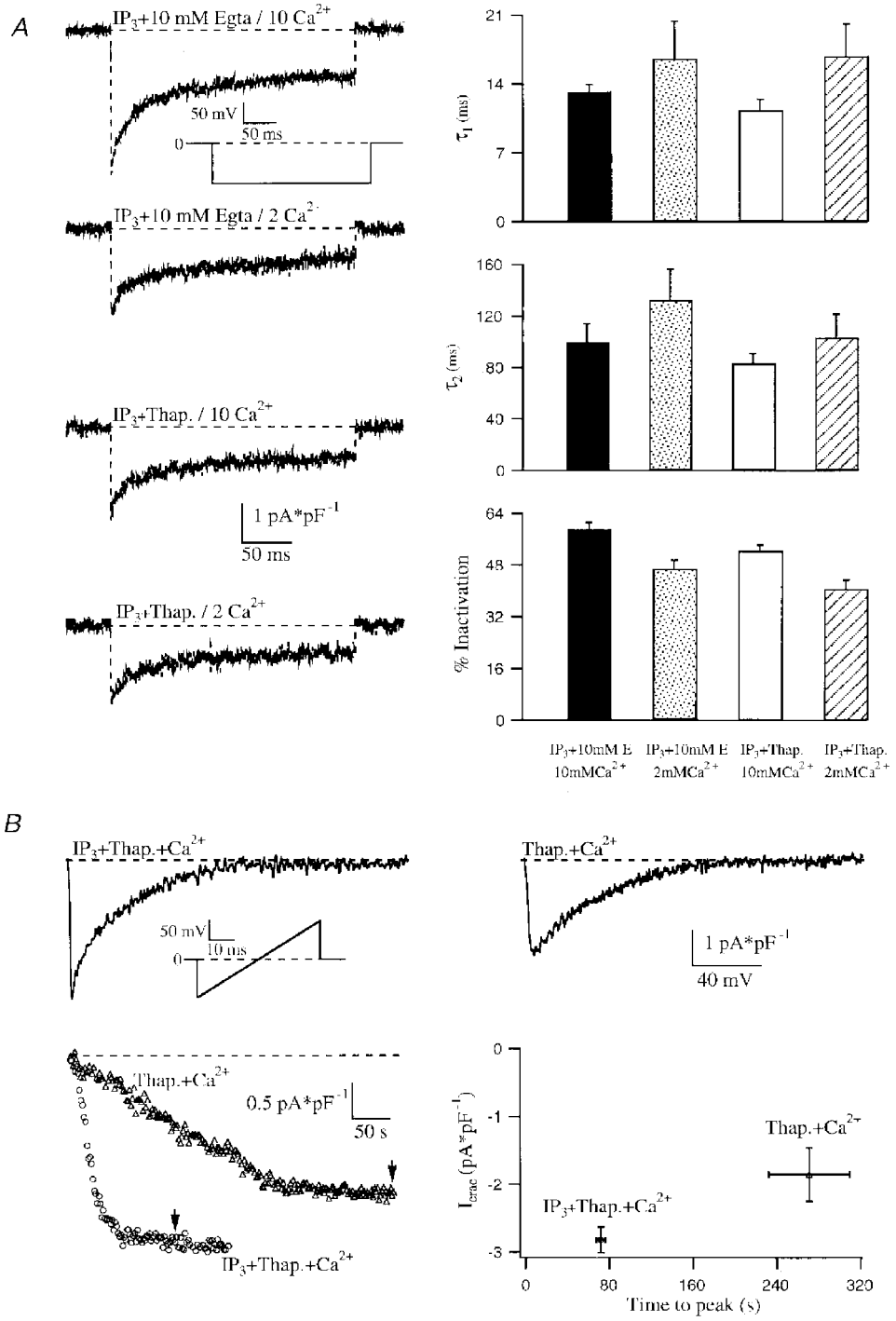

Figure 3. Ca^{2+}-dependent negative feedback mechanisms do not prevent macroscopic activation of I_{CRAC} in physiological cytoplasmic Ca^{2+} buffering conditons

A, Ca^{2+}-dependent fast inactivation of I_{CRAC} is compared under high and low cytoplasmic Ca^{2+} buffering conditions. The inactivation phase of each trace could be fitted with a double exponential function (not shown). Average time constants from these fits and the extent of inactivation for each condition are shown in the right panel. Each bar represents the mean ± S.E.M. of more than seven cells. *B*, effects of dialysing cells with 100 μM Ca^{2+} and no added Ca^{2+} chelator on I_{CRAC}. I_{CRAC} was activated by inclusion of either IP_3 and thapsigargin (left ramp) or thapsigargin alone (right ramp) in the pipette solution. Ca^{2+} would be weakly buffered by ATP (3 mM total Mg^{2+}), glutamate and the endogenous cytoplasmic Ca^{2+} buffers. Each point represents the mean ± S.E.M. of more than five cells. Current amplitude ($P < 0{\cdot}05$) and time to peak ($P < 0{\cdot}0001$) were significantly different for these two groups of cells.

that Ca^{2+}-dependent inactivation prevents macroscopic I_{CRAC} from developing in low Ca^{2+} buffer. I_{CRAC} is subjected to Ca^{2+}-dependent inactivation. A fast process which operates on a millisecond time scale (Hoth & Penner, 1993; Zweifach & Lewis, 1995*a*; Fierro & Parekh, 1999*a*) and a slower inactivation occurring over tens of seconds/minutes (Zweifach & Lewis, 1995*b*; Parekh, 1998) both reduce CRAC channel activity. Fast inactivation is widely believed to account for the inability to measure macroscopic I_{CRAC} under physiological cytosolic Ca^{2+} buffering during whole-cell recordings (Huang *et al.* 1998). However, this is an unlikely explanation for several reasons. First, fast inactivation only occurs in RBL-1 cells at potentials negative to −40 mV and, at −80 mV, it reduces the current by less than 40 %; fast inactivation develops with time constants in the range of 10 and 120 ms and recovers with time constants of 34 and 233 s (Fierro & Parekh, 1999*a*). When the membrane potential is clamped at 0 mV and voltage ramps spanning −100 to +100 mV over 50 ms are applied every 2 s, fast inactivation would therefore not be present during the initial negative portion of the ramp, and hence the CRAC channels should be functional and thus give rise to a clear current. Second, we have found that fast inactivation is reduced when Sr^{2+} replaces Ca^{2+} as the charge carrier (Fierro & Parekh, 1999*a*). However, even with Sr^{2+} as the charge carrier instead of Ca^{2+}, we generally failed to record a detectable I_{CRAC} in response to IP_3 and 0·1 mM EGTA (only 3 of 13 cells responded and these gave small I_{CRAC}; data not shown). Third, we clamped cells at +50 mV (to reduce the driving force for Ca^{2+} influx) and administered voltage ramps both at a low frequency (1 ramp per 4 s instead of 2 s) and under conditions where less Ca^{2+} influx would occur during the ramps (now spanning −80 to +80 mV over 20 ms instead of −100 to +100 mV over 50 ms). However, IP_3 still routinely failed to activate I_{CRAC} in 0·1 mM EGTA (7/9 cells; data not shown). Taken together, the absence of macroscopic I_{CRAC} in the presence of 0·1 mM chelator and IP_3 is unlikely to be due solely to fast inactivation. In Fig. 3*A* we compared the rate and extent of fast inactivation for I_{CRAC} following activation by IP_3 and thapsigargin in 0·1 mM EGTA with that for IP_3 and 10 mM EGTA for 2 and 10 mM external Ca^{2+}. The fast and slow rates, as well as the extent of inactivation, were not significantly different for each external Ca^{2+} concentration for low or high Ca^{2+} buffer. The extent of inactivation was less in the lower external Ca^{2+} concentration, in agreement with a previous report from T-cells (Zweifach & Lewis, 1995*a*). The fact that fast inactivation is the same under the two conditions provides additional evidence that, first, the inability to measure macroscopic I_{CRAC} induced in response to IP_3 in low Ca^{2+} buffer is unlikely to be due to this inactivation mechanism and, second, the channels underlying the current under the two conditions are the same.

Slow inactivation develops over a time course of several tens of seconds and should not prevent macroscopic development of the current. Furthermore, the combination of IP_3 and thapsigargin would raise intracellular Ca^{2+} to a higher level than IP_3 alone and maintain it for longer at an elevated level, yet the current still developed. This argues against activation of Ca^{2+}-dependent inhibitory feedback mechanisms that prevent I_{CRAC} from being measured under low Ca^{2+} buffering conditions. This also provides evidence against an inhibitory action of IP_3 itself or a metabolite thereof, since one would expect such an action to also function in the presence of thapsigargin. In Fig. 3*B* we examined the intracellular Ca^{2+} dependence of I_{CRAC} activation. The upper panel shows the current in response to a voltage ramp following dialysis with IP_3, thapsigargin, 100 μM Ca^{2+} and no added Ca^{2+} chelator. Ca^{2+} was weakly buffered only, by the ATP and glutamate in the recording pipette. Astonishingly, the current could be activated at the normal rate (Fig. 3*B*, lower right panel). Dialysis with thapsigargin and 100 μM Ca^{2+} (in the absence of IP_3) also activated I_{CRAC}, with the typical slow kinetics expected for thapsigargin alone. Although we do not know exactly what the free Ca^{2+} concentration is in the cells under these conditions (due to endogenous Ca^{2+} buffering/removal), in these latter experiments the slow development of I_{CRAC} would ensure that reasonable dialysis with micromolar Ca^{2+} concentrations would have occurred as I_{CRAC} activated. Again, both the final extent of the current as well as the time to peak were similar to that seen with thapsigargin and 0·1 mM chelator (compare graphs in Figs 1*B* and 3*B*). This unexpected result challenges current views that I_{CRAC} cannot activate when cytosolic Ca^{2+} is high and that intracellular Ca^{2+} buffers are required for the current to activate.

DISCUSSION

Our results suggest that partial depletion of the IP_3-sensitive stores is not sufficient to activate I_{CRAC} to a detectable level in whole-cell recording. Only when stores are substantially depleted of Ca^{2+}, as seen with the SERCA blockers, can the macroscopic current develop. Although SERCA block alone can activate I_{CRAC}, IP_3 accelerates the rate at which the current develops. At first glance, one might be surprised that IP_3 or ionomycin is unable to fully deplete the stores whereas SERCA blockers can. Recently, we have found that the SERCA pumps in RBL-1 cells are able to operate efficiently when cytosolic Ca^{2+} is heavily clamped at < 10 nM, even in the absence of ATP from the pipette solution (Fierro & Parekh, 1999*b*). In fact, SERCA pumps could fully prevent I_{CRAC} from activating in the presence of both a significant passive leak of Ca^{2+} from the stores and a cytosolic Ca^{2+} binding ratio of 6000. Under conditions of low cytosolic Ca^{2+} buffering, SERCA pump activity will be even higher, both because of the elevated cytosolic Ca^{2+} following IP_3-evoked Ca^{2+} release and the fact

that a fall in luminal Ca^{2+} strongly stimulates pump activity (Favre *et al.* 1996; Mogami *et al.* 1998). In addition, both cytosolic and luminal Ca^{2+} have been reported to reduce the Ca^{2+} flux through IP_3-gated channels that span the stores, thereby enabling the pumps to partially reload the stores (Berridge, 1993). However, our results are not compatible with the notion that Ca^{2+} influx through CRAC channels causes sizeable inhibition of the IP_3 receptor, thereby

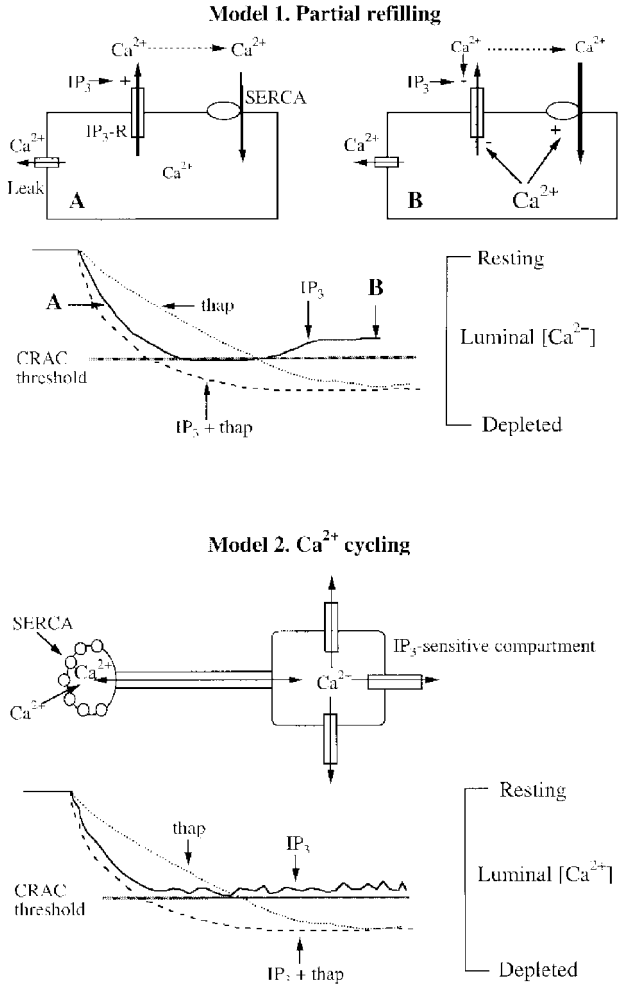

Figure 4. Two models to explain how the threshold can account for the inability of IP_3 to activate macroscopic I_{CRAC} in low Ca^{2+} buffering, and the effectiveness of SERCA blockers under the same conditions

For both models, the rate and extent of store depletion are arbitrary and not based on empirical data. Model 1, 30 μM IP_3 is initially quite effective in depleting the stores, but the negative feedback mechanisms start to operate and curtail Ca^{2+} efflux through the IP_3-gated channel. Simultaneously, SERCA pump activity increases. The combination of reduced Ca^{2+} efflux and enhanced Ca^{2+} uptake ensure that the threshold is not crossed. Negative feedback mechanisms on the IP_3 receptor might include, alone or in combination, the potential effects of a fall in luminal Ca^{2+} (Berridge, 1993), inhibition by cytosolic Ca^{2+} directly (Berridge, 1993), or via calmodulin (Missiaen *et al.* 1999) and inactivation by IP_3 itself (Hajnockzy & Thomas, 1994). Such feedback mechanisms might not operate when the potent IP_3 receptor agonist adenophostin A is used to deplete the stores (Huang *et al.* 1998). SERCA pump activity would be enhanced by both the fall in luminal Ca^{2+} (Favre *et al.* 1996; Mogami *et al.* 1998) and the increase in cytosolic Ca^{2+} in low Ca^{2+} buffer (see text). Model 2, this model differs from model 1 in that no inhibitory mechanisms are necessary to reduce Ca^{2+} efflux in response to a supramaximal concentration of IP_3. Instead, Ca^{2+} uptake and release mechanisms are spatially separate. IP_3 effectively depletes its own compartment, but a standing Ca^{2+} gradient exists whereby Ca^{2+}, taken up by the pumps, diffuses to the release sites and then enters the cytosol. The threshold here would reflect to a large extent the Ca^{2+} in the region of the uptake sites. Only when this falls below a certain level will I_{CRAC} activate. In this scheme, CRAC activation will be determined to a greater extent by the Ca^{2+} content in the uptake sites. A recent study using differential centrifugation in RBL cells reported that IP_3 receptors were not uniformly distributed in the endoplasmic reticulum but were localised instead to certain regions (Vanlingen *et al.* 1997).

favouring store refilling, for two reasons. First, manoeuvres that reduced the extent of Ca^{2+} influx (clamping at +50 mV, reduced duration and size of voltage ramps) still failed to enable IP_3 to activate I_{CRAC} in low Ca^{2+} buffer. Second, although Sr^{2+} is an effective charge carrier through CRAC channels (Fierro & Parekh, 1999*a*), it is at least two orders of magnitude less effective than Ca^{2+} in inducing inhibition of the IP_3 receptor (Hannaert-Merah *et al.* 1995). One might therefore have expected I_{CRAC} to activate substantially in low Ca^{2+} buffer in the presence of external Sr^{2+}. However, we routinely failed to detect any macroscopic current under these conditions.

Previous studies have shown that low concentrations of IP_3 can partially empty Ca^{2+} stores yet fail to activate any Ca^{2+} influx (Parekh *et al.* 1997; Hartmann & Verkhratsky, 1998; Liu *et al.* 1998). It has been suggested that either functionally distinct IP_3-sensitive stores exist in RBL-1 cells and that the one with lower sensitivity for IP_3 activates I_{CRAC}, or there may be one homogeneous IP_3-sensitive store, but partial depletion does not partially activate I_{CRAC} (Parekh *et al.* 1997). Our results using a high dose of IP_3 (which would not discriminate between the various IP_3-sensitive stores) extends these studies and provides an explanation for this widespread phenomenon. Intraluminal Ca^{2+} must fall below a certain level in the specific IP_3-sensitive store that controls I_{CRAC} before the current can activate. Only when SERCA pumps are blocked is this threshold readily overcome. Our results suggest that I_{CRAC} activation probably requires quite a substantial depletion of the stores because the combination of IP_3 and 20 nM thapsigargin failed to generate a macroscopic current, despite this concentration of thapsigargin evoking Ca^{2+} release in fura 2-AM-loaded cells. Two models that could account for our results are shown in Fig. 4 (see legend for details).

Our conclusions differ from those of a previous study in which intraluminal Ca^{2+} was measured directly using mag-fura (Hofer *et al.* 1998). It was reported that Ca^{2+} influx was tightly coupled to the extent of store depletion and no apparent threshold was observed. However, such experiments require that cells are dialysed for several minutes in order to remove non-compartmentalised dye from the cytosol and with a solution in which Ca^{2+} is strongly buffered close to 100 nM with 10 mM BAPTA, so that I_{CRAC} does not spontaneously activate. Under these conditions, because Ca^{2+} is continuously cycling across the stores membrane, as suggested by our previous results (Fierro & Parekh, 1999*b*), then the amount of Ca^{2+} that is taken back up into the stores by SERCA pumps will be less than in conditions where no external added buffer is present. Assuming a constant Ca^{2+} leak from the stores (Mogami *et al.* 1998), then the latter would be slowly but continuously drained of their Ca^{2+}. Over a time course of several minutes, one might expect that the stores content would be reduced to a level close to the threshold where I_{CRAC} activates, so that it would not have been observed. Furthermore, substantial dye loading is required to obtain a resolvable signal from the small Ca^{2+} organelles and it is likely that the strong intraluminal Ca^{2+} buffering introduced by the dye also lowered intraluminal Ca^{2+} close to the threshold.

Our results challenge contemporary views on Ca^{2+} signalling and have implications for both the physiological role of I_{CRAC} and its activation mechanism. First, the fact that I_{CRAC} can be measured in 1–2 mM external Ca^{2+} and in the presence of low cytosolic Ca^{2+} buffering convincingly demonstrates that the CRAC channels are fully functional under physiological conditions and therefore contribute directly to Ca^{2+}-dependent processes. Second, contrary to what is widely thought, the inability of IP_3 to activate macroscopic I_{CRAC} in low Ca^{2+} buffer is not due to Ca^{2+}-dependent feedback mechanisms operating on the current. Instead it seems to reflect incomplete store depletion after Ca^{2+} release evoked by a maximal concentration of IP_3. Third, our characterisation of the ionic selectivity of the CRAC channels under physiological conditions reinforces the notion that I_{CRAC} is highly selective for Ca^{2+} and the results will be helpful in identifying putative CRAC channel clones. Finally, the threshold phenomenon endows I_{CRAC} with a unique ability to regulate a broad spectrum of Ca^{2+}-dependent processes with different affinities/spatial distributions. Small Ca^{2+} release events (or larger events at low frequency) fail to reach the threshold and do not activate store-operated Ca^{2+} influx at all (Parekh *et al.* 1997). Processes that require only Ca^{2+} release can therefore be activated separately from those that require Ca^{2+} entry. Further Ca^{2+} release that then reaches the threshold activates a small amount of Ca^{2+} influx through I_{CRAC} and this underlies the plateau seen in most non-excitable cells following receptor stimulation. Changes in membrane potential will play a particularly important role during this Ca^{2+} plateau by determining the rate and extent of Ca^{2+} influx through CRAC channels, especially since the latter's *I–V* relationship is inwardly rectifying (Hoth & Penner, 1992; Parekh & Penner, 1997). The plateau will be able to activate processes with moderate affinity for Ca^{2+}, as well as raising the possibility of creating spatial Ca^{2+} gradients from the cell periphery. More extensive stimulation (high frequency of large amplitude Ca^{2+} spikes) will gradually activate I_{CRAC} further (since the released Ca^{2+} will be extruded across the plasma membrane as well as resequestrated into other stores) and this will result in a large and sustained global Ca^{2+} increase and hence activation of processes with a low affinity for Ca^{2+} such as exocytosis. Antigen stimulation generates initially large cytosolic sinusoidal Ca^{2+} oscillations which develop into a plateau reflecting Ca^{2+} entry. Exocytosis starts only when the latter occurs (Ali *et al.* 1990; Kim *et al.* 1997). Presumably, the initial oscillations are not sufficient to exceed the threshold for significant I_{CRAC} activation. In contrast, thapsigargin generates a plateau quickly, and robust secretion occurs almost immediately (Kim *et al.* 1997). This model also explains why IP_3 alone was unable to evoke capacitance increases in RBL-1 cells (indicative of

vesicular fusion) in low Ca^{2+} buffer whereas the combination of IP_3 and thaspigargin was very potent (Artalejo *et al.* 1998).

Our results, on the other hand, indicate that SERCA pump activity appears to be one of the main determinants of whether or not I_{CRAC} activates under physiological conditions. Therefore they also raise the intriguing possibility that hormones that do not couple to the phosphatidylinositol pathway may still activate I_{CRAC} by inhibiting the pump, for example by protein phosphorylation. The SERCA pumps may therefore represent a means for stimulating Ca^{2+} influx without IP_3-mediated Ca^{2+} release.

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