Sarcoplasmic/endoplasmic-reticulum-Ca^{2+}-ATPase-mediated Ca^{2+} reuptake, and not Ins(1,4,5)P_3 receptor inactivation, prevents the activation of macroscopic Ca^{2+} release-activated Ca^{2+} current in the presence of physiological Ca^{2+} buffer in rat basophilic leukaemia-1 cells

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Whole-cell patch-clamp experiments were performed to examine the mechanism underlying the inability of intracellular Ins(1,4,5)P_3 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. Dialysis with Ins(1,4,5)P_3 in weak Ca^{2+} buffer did not activate any macroscopic I_{CRAC} even after precautions had been taken to minimize the extent of Ca^{2+} entry during the experiment. Following intracellular dialysis with Ins(1,4,5)P_3 for > 150 s in weak buffer, external application of the sarcoplasmic/endoplasmic-reticulum Ca^{2+}-ATPase (SERCA) pump blocker thapsigargin activated I_{CRAC}, and the current developed much more quickly than when thapsigargin was applied in the absence of Ins(1,4,5)P_3. This indicates that the Ins(1,4,5)P_3 receptors had not inactivated much over this time-course. When external Ca^{2+} was replaced by Ba^{2+}, Ins(1,4,5)P_3 still failed to generate any detectable I_{CRAC} even though Ba^{2+} permeates CRAC channels and is not taken up into the intracellular Ca^{2+} stores. In strong Ca^{2+} buffer, I_{CRAC} could be activated by muscarinic-receptor stimulation, provided protein kinase C (PKC) was blocked. In weak buffer, however, as with Ins(1,4,5)P_3, stimulation of these receptors with carbachol did not activate I_{CRAC} even after inhibition of PKC. The inability of Ins(1,4,5)P_3 to activate macroscopic I_{CRAC} in weak Ca^{2+} buffer was not altered by inhibition of Ca^{2+}-dependent phosphorylation/dephosphorylation reactions. Our results suggest that the inability of Ins(1,4,5)P_3 to activate I_{CRAC} under conditions of weak intracellular Ca^{2+} buffering is not due to strong inactivation of the Ins(1,4,5)P_3 receptors. Instead, a futile Ca^{2+} cycle across the stores seems to be occurring and SERCA pumps resequester sufficient Ca^{2+} to ensure that the threshold for activation of macroscopic I_{CRAC} has not been exceeded.

Key words: Ca^{2+} store, I_{CRAC}, InsP_3, SERCA pump.

INTRODUCTION

The Ca^{2+} release-activated Ca^{2+} current (I_{CRAC}) is the best characterized and most widely distributed of the store-operated Ca^{2+} currents [1]. Although I_{CRAC} has been implicated in controlling a variety of physiological processes, including regulated exocytosis [2], gene transcription [3] and cell proliferation [4], elucidation of its precise role has been severely hampered by the inability to record the current under physiological conditions. I_{CRAC} can be observed routinely only after high concentrations (several millimolar) of Ca^{2+} chelators have been included in the recording pipette [1,5], and this is thought to be necessary for suppressing Ca^{2+}-dependent inactivation of the CRAC channels. Recently, however, we have demonstrated that I_{CRAC} can be recorded in rat basophilic leukaemia (RBL)-1 cells in the presence of weak intracellular Ca^{2+} buffer (0.1 mM chelator) simply by inhibiting sarcoplasmic/endoplasmic-reticulum Ca^{2+}-ATPase (SERCA) pumps. But the physiological signal, Ins(1,4,5)P_3 alone, generally failed to activate whole-cell (macroscopic) I_{CRAC} in weak Ca^{2+} buffer [6]. Considering that, in these cells, concentrations of Ins(1,4,5)P_3 which fail to activate any store-operated Ca^{2+} influx nevertheless substantially reduce the Ca^{2+} content of the intracellular stores [7] and that the SERCA pumps are remarkably powerful [8], we have suggested that the inability of Ins(1,4,5)P_3 to activate macroscopic I_{CRAC} in weak intracellular Ca^{2+} buffer probably reflects the existence of a threshold below which intraluminal Ca^{2+} must fall before macroscopic I_{CRAC} activates [6,7]. Because Ins(1,4,5)P_3 receptors have a high Ca^{2+} conductance (80 pS with sub-conductance levels of 20, 40 and 60 pS for the best characterized receptor from cerebellum [9]) whereas SERCA pumps transport Ca^{2+} much more slowly (200 Ca^{2+} ions/s in muscle [10]), one might have expected the stores to deplete in the continuous presence of high levels of Ins(1,4,5)P_3. The fact that this does not seem to be the case indicates that either the Ins(1,4,5)P_3 receptors inactivate in the continuous presence of Ins(1,4,5)P_3 or that SERCA pump activity is increased dramatically following Ins(1,4,5)P_3-mediated Ca^{2+} release. Discriminating between these possibilities is essential to our understanding of the regulation of I_{CRAC} under physiological conditions.

EXPERIMENTAL

RBL-1 cells were purchased from the Cell Bank at the Sir William Dunn School of Pathology, University of Oxford. Cell culture was performed as previously described [6,7].

Abbreviations used: I_{CRAC}, Ca^{2+} release-activated Ca^{2+} current; PKC, protein kinase C; RBL, rat basophilic leukaemia; SERCA, sarcoplasmic/endoplasmic-reticulum Ca^{2+}-ATPase.

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Patch-clamp experiments were conducted in the tight-seal whole-cell configuration at room temperature (20–25 °C) as previously described [6,11]. Sylgard-coated, fire-polished pipettes had d.c. resistances of 2.9–4 MΩ when filled with standard internal solution containing 10 mM Hepes/CsOH (pH 7.2), 145 mM Cs-glutamate, 8 mM NaCl, 1 mM $MgCl_2$ and 2 mM Mg-ATP. The Ca^{2+} chelator EGTA (Sigma, Poole, U.K.) was added to this solution at the specified concentrations. Ins(1,4,5)P_3 (added to the pipette solution) was obtained from Sigma. In experiments where muscarinic receptors were stimulated, 0.2 mM Li-GTP was added to the pipette solution. Thapsigargin was purchased from Alomone Laboratories (Jerusalem, Israel). All other chemicals were purchased from Sigma. A correction of +10 mV was applied for the liquid junction potential arising from this glutamate-based internal solution. Extracellular solution contained 10 mM Hepes/NaOH (pH 7.4), 145 mM NaCl, 2.8 mM KCl, 10 mM $CaCl_2$, 2 mM $MgCl_2$, 10 mM CsCl and 10 mM glucose. Where stated, $CaCl_2$ was replaced by equimolar $SrCl_2$ or $BaCl_2$. Voltage ramps (−100 to +100 mV in 50 ms) were applied at 0.5 Hz from a holding potential of 0 mV, and I_{CRAC} was measured at −80 mV from the ramps, as previously described [6,11]. In the experiments of Figure 2, ramps of 20 ms duration from −80 to +80 mV were applied once every 4 s at holding potentials of 0 to +50 mV, to minimize the extent of Ca^{2+} influx. In this experiment, I_{CRAC} was measured at −64 mV from the ramp. Currents were filtered using an 8-pole Bessel filter at 2.9 kHz and digitized at 100 μs, and I_{CRAC} was normalized for cell size by dividing the whole-cell current amplitude by cell capacitance. Capacitative currents were compensated before each ramp using the automatic compensation of the EPC 9-2 amplifier. All leak currents were subtracted by averaging the first 2–4 ramp currents, and then subtracting this from all subsequent currents. Data are presented as means ± S.E.M., and statistical evaluation was carried out using the Student's unpaired *t* test.

RESULTS

I_{CRAC} can be activated by Ins(1,4,5)P_3 in strong, but not weak, intracellular Ca^{2+} buffer

Figure 1 summarizes the effects of dialysing RBL-1 cells with 30 μM Ins(1,4,5)P_3 (a supramaximal concentration [7]) with either 10 mM or 0.1 mM EGTA (strong and weak buffer respectively). The time course of activation of I_{CRAC} is plotted in Figure 1(A). Whereas I_{CRAC} activated rapidly in the presence of strong buffer for all cells tested (24/24), in weak buffer only 7/23 cells responded, and in these 7 cells a current was obtained that was significantly smaller than that seen in cells heavily buffered with EGTA ($P < 0.001$; also see [6]). Leak-subtracted I–V relationships, taken at 100 s for the cell in strong buffer and at 78 s for the non-responder in weak buffer, are shown in Figure 1(B). The mean amplitude of I_{CRAC} for the two conditions is summarized in Figure 1(C), where all cells (responders and non-responders) have been included.

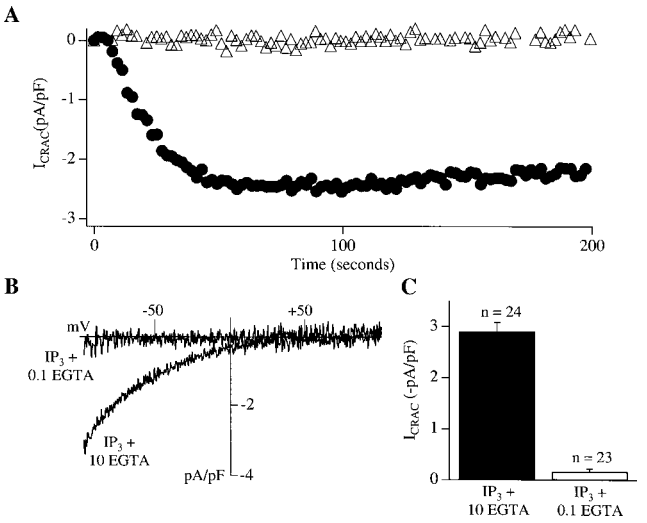

Figure 1 Ins(1,4,5)P_3 activates I_{CRAC} in strong, but not weak, Ca^{2+} buffer

(**A**) Time course of development of I_{CRAC} following dialysis with a pipette solution containing 30 μM Ins(1,4,5)P_3 and either 10 mM (●) or 0.1 mM (△) EGTA. (**B**) The I–V relationship is shown (taken at 100 s for strong buffer and 78 s for the non-responder in weak buffer). (**C**) The mean amplitude from several cells is summarized.

Do Ins(1,4,5)P_3 receptors inactivate in weak Ca^{2+} buffer?

The inability of Ins(1,4,5)P_3 to activate macroscopic I_{CRAC} in weak intracellular Ca^{2+} buffer could be accounted for either by inactivation of the Ins(1,4,5)P_3 receptors or by enhanced activity of the SERCA pumps [6]. We designed experiments to address whether the Ins(1,4,5)P_3 receptors indeed inactivate in the continuous presence of Ins(1,4,5)P_3 under these conditions. We compared the rate of development of I_{CRAC} in weak buffer with thapsigargin alone, with Ins(1,4,5)P_3 and thapsigargin together, and with Ins(1,4,5)P_3 followed 150–200 s later by thapsigargin. Ins(1,4,5)P_3 and thapsigargin together activate I_{CRAC} rapidly with a time constant similar to that seen following dialysis with Ins(1,4,5)P_3 and 10 mM EGTA [6]. Thapsigargin alone activates I_{CRAC} much more slowly at a rate determined mainly by the Ca^{2+} leak conductance of the ER, which may be carried in part by a few Ins(1,4,5)P_3 receptors opened by basal Ins(1,4,5)P_3. If the Ins(1,4,5)P_3 receptors inactivate, then application of thapsigargin after Ins(1,4,5)P_3 should result in I_{CRAC} developing at a rate similar to, or perhaps even slower than, thapsigargin alone, depending on the basal activity of Ins(1,4,5)P_3 receptors.

The left-hand panel of Figure 2(A) shows a typical recording following dialysis with weak Ca^{2+} buffer (open circles). After 150 s, 4 μM thapsigargin was applied locally to the cell. After a delay of approx. 100 s, I_{CRAC} started to develop slowly, before reaching steady-state after almost 300 s. Also shown is a recording from a cell dialysed with Ins(1,4,5)P_3 and thapsigargin (filled squares) in which I_{CRAC} activated within 2 s and developed rapidly. The delay, half-time (when the current had reached 50 % of its peak amplitude) and time-to-peak of I_{CRAC} activation were each significantly faster in cells dialysed with Ins(1,4,5)P_3 plus thapsigargin than in cells dialysed with thapsigargin alone ($P < 0.005$; Figure 2B). Current amplitudes were not significantly different, however ($P = 0.20$).

We then broke in with Ins(1,4,5)P_3 and applied thapsigargin 150–200 s later. The middle panel of Figure 2(A) shows a recording in which Ins(1,4,5)P_3 alone activated I_{CRAC} transiently in weak buffer (a relatively infrequent occurrence). Once the current had fully deactivated, we applied thapsigargin. I_{CRAC} reactivated with a short delay and developed very quickly. The right-hand panel of Figure 2(A) superimposes recordings from two further cells. The trace depicted by filled circles represents another cell dialysed with Ins(1,4,5)P_3 (with no initial transient I_{CRAC} activation), and the open circles correspond to a cell dialysed with weak buffer alone. Thapsigargin was applied as indicated. For the cell dialysed with Ins(1,4,5)P_3, I_{CRAC} activated much more rapidly, with a delay 2-fold less than when Ins(1,4,5)P_3 was omitted from the pipette, although this was not

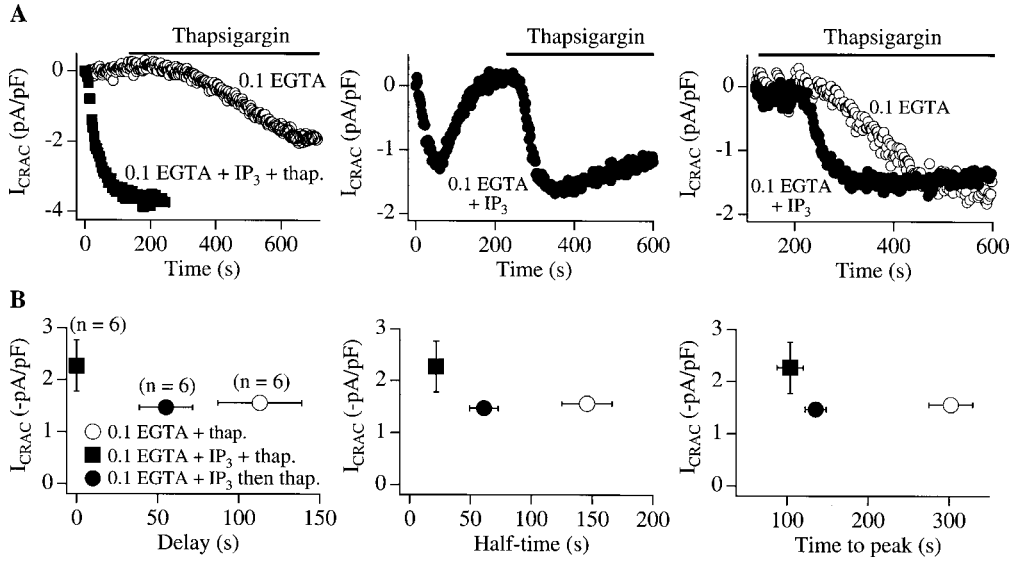

Figure 2 Continuous presence of intracellular Ins(1,4,5)P_3 accelerates the rate of activation of I_{CRAC} in weak Ca^{2+} buffer following subsequent application of thapsigargin

(**A**) Left-hand panel: shows a cell dialysed with 0.1 mM EGTA; thapsigargin was applied as indicated (○). I_{CRAC} developed slowly and after a sizeable delay. (■) A recording in which a cell was dialysed with Ins(1,4,5)P_3 + 2 μM thapsigargin (thap.; a treatment that routinely activates the current). I_{CRAC} developed much more rapidly than thapsigargin alone, reflecting the presence of Ins(1,4,5)P_3. Middle panel: shows a relatively rare recording in which Ins(1,4,5)P_3 activated a small and transient current in weak buffer. When the current had fully deactivated, application of thapsigargin resulted in the reactivation of I_{CRAC} but, importantly, the current developed quickly. Right-hand panel: compares data from two different cells. One was dialysed with Ins(1,4,5)P_3 (●) and the other was not (○). Ins(1,4,5)P_3 did not activate I_{CRAC} in this cell. Thapsigargin was applied to both cells after 160 s. I_{CRAC} activated much more rapidly in the cell continuously exposed to Ins(1,4,5)P_3. (**B**) Analysis of the various parameters obtained from experiments in (**A**). Ins(1,4,5)P_3 (IP_3) then thapsigargin (thap.) means dialysis with Ins(1,4,5)P_3 followed by local application of thapsigargin after 150–200 s. Ins(1,4,5)P_3 + thapsigargin represents the case where both Ins(1,4,5)P_3 and thapsigargin were included in the recording pipette. Note that Ins(1,4,5)P_3 then thapsigargin activated I_{CRAC} significantly faster than thapsigargin alone and closely resembled Ins(1,4,5)P_3 + thapsigargin. This indicates that the Ins(1,4,5)P_3 receptors had not inactivated appreciably despite continuous exposure to Ins(1,4,5)P_3 in weak Ca^{2+} buffer.

quite significant ($P = 0.07$, presumably reflecting the large variability in the controls). Half-times and time-to-peak values were significantly different between Ins(1,4,5)P_3-treated and control cells ($P < 0.01$). Strikingly, the half-time and time-to-peak values for I_{CRAC} activation in cells dialysed with Ins(1,4,5)P_3 and then treated with thapsigargin were quite similar to those in cells dialysed with Ins(1,4,5)P_3 plus thapsigargin (compare filled squares with filled circles in Figure 2B). Note that we are comparing the rate of development of I_{CRAC} following dialysis with Ins(1,4,5)P_3 and thapsigargin with the situation where thapsigargin is applied locally to the cell from the outside. Dialysis with thapsigargin will result in a more rapid increase in its intracellular concentration compared with local application; however, this effect is not very strong. The delay before I_{CRAC} activates is shorter when thapsigargin is included in the pipette but time-to-peak is similar (see Figure 1 in [6]). The key point is that I_{CRAC} activates much more quickly in response to thapsigargin when Ins(1,4,5)P_3 is also included in the pipette solution. For both cases, the means of applying thapsigargin is the same and hence does not affect the interpretation. Collectively, these results indicate that activation of I_{CRAC} by thapsigargin is considerably faster following exposure to Ins(1,4,5)P_3, even though Ins(1,4,5)P_3 does not generally activate I_{CRAC}. This might indicate that the Ins(1,4,5)P_3 receptor does not inactivate much over the time course of the experiment.

Positive holding potentials do not enable Ins(1,4,5)P_3 to activate I_{CRAC} in weak intracellular Ca^{2+} buffer

It has been suggested that the inability of Ins(1,4,5)P_3 to activate detectable I_{CRAC} in the presence of weak intracellular Ca^{2+} buffer reflects Ca^{2+} inactivation of the Ins(1,4,5)P_3 receptors by Ca^{2+} permeating the CRAC channels, followed by SERCA-pump-mediated store refilling [12]. If this were true, then decreasing the extent of Ca^{2+} entry between and during voltage ramps should reduce Ca^{2+}-inactivation of the Ins(1,4,5)P_3 receptors and unmask I_{CRAC}. To test this, we clamped RBL-1 cells at different voltages ranging from 0 to +50 mV (which reduces Ca^{2+} influx in RBL cells [7]; results not shown) and applied ramps of reduced voltage range, duration and frequency (−80 to +80 mV; 20 ms duration; 0.25 Hz). Figure 3(A) compares the time course and extent of I_{CRAC} activation at holding potentials of 0 and +40 mV for weak and strong buffers. Pooled data are summarized in Figure 3(B). With this protocol, Ins(1,4,5)P_3 in strong Ca^{2+} buffer activated robust I_{CRAC}, but consistently failed to activate a detectable current in weak buffer. In strong buffer, the rate of development of I_{CRAC} was slower and the amplitude of the steady-state current higher at +40 compared with 0 mV, as described previously ([13]; see the Discussion section).

These results indicate that I_{CRAC} does not consistently activate in weak Ca^{2+} buffer to Ins(1,4,5)P_3 even when precautions are taken to minimize Ca^{2+} entry between and during application of the voltage ramps.

Effects of replacing external Ca^{2+} with Ba^{2+} or Sr^{2+}

The group II alkali earth cations Ba^{2+} and Sr^{2+} permeate CRAC channels to appreciable extents [6], but interact differently with Ins(1,4,5)P_3 receptors and SERCA pumps. Ba^{2+} does not inactivate the Ins(1,4,5)P_3 receptor, and Sr^{2+} is more than two orders of magnitude less effective than Ca^{2+} [14,15]. Importantly, Ba^{2+} is also not taken up by SERCA pumps [16]. Sr^{2+} is

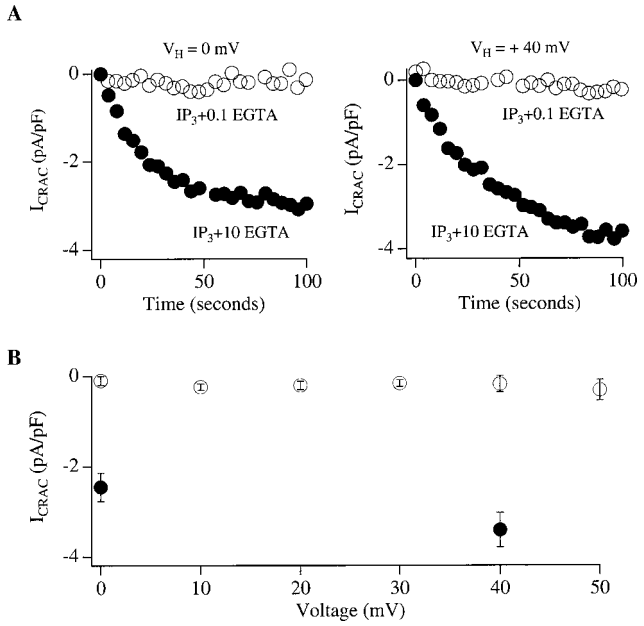

Figure 3 Reducing Ca^{2+} entry by changing the holding potential does not reveal the presence of I_{CRAC} in weak Ca^{2+} buffer

(**A**) Effect of 0 and + 40 mV holding potentials (V_H) on the activation of I_{CRAC} in weak compared with strong Ca^{2+} buffer. Ins(1,4,5)P_3 (IP_3; 30 μM) was always included in the recording pipette. Whereas I_{CRAC} in strong buffer tended to be larger at + 40 mV compared with 0 mV (also see [13]), the macroscopic current was still undetectable in weak buffer. (**B**) Summarizes pooled data from different holding potentials for weak (○) and strong (●) buffer. Over the entire voltage range tested, Ins(1,4,5)P_3 in weak buffer consistently failed to activate macroscopic I_{CRAC}. Each point is the mean of at least 5 cells. In these experiments, voltage ramps were applied at 0.25 Hz and spanned − 80 to + 80 mV in 20 ms. The amplitude of the current was measured at − 64 mV.

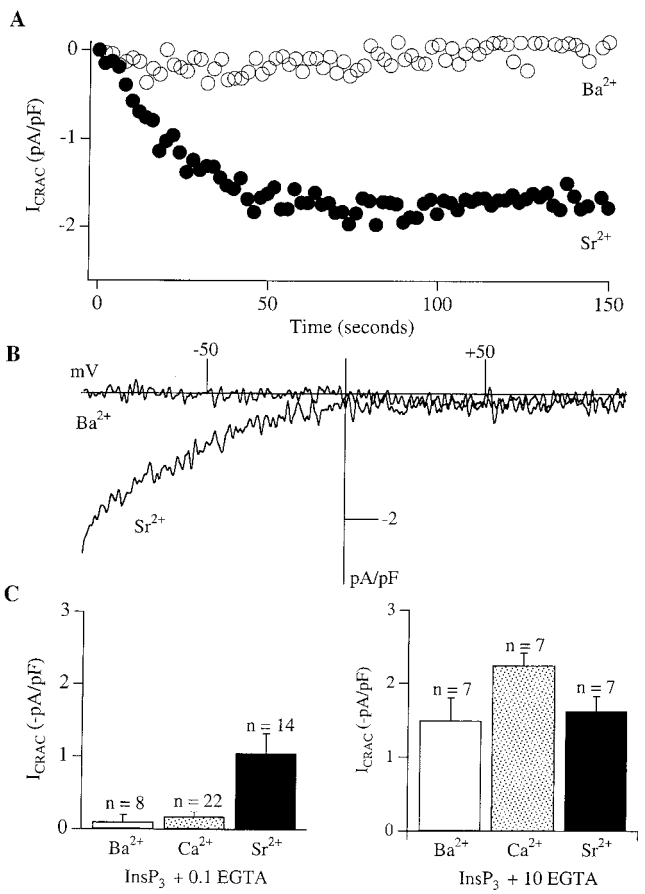

Figure 4 Effect of replacing external Ca^{2+} with Ba^{2+} or Sr^{2+} on the ability of Ins(1,4,5)P_3 ($InsP_3$) to activate I_{CRAC} in weak intracellular Ca^{2+} buffer

(**A**) Time course of I_{CRAC} in the presence of either 10 mM Ba^{2+} (○) or Sr^{2+} (●). (**B**) Corresponding I–V relationships are shown (48 s for Ba^{2+} and 70 s for Sr^{2+}). (**C**) The mean amplitudes for Ba^{2+}, Ca^{2+} and Sr^{2+} are summarized in the histogram on the left for 0.1 mM EGTA and on the right for 10 mM EGTA.

transported into the stores [16], albeit at a slower rate than Ca^{2+}. If the inability to record I_{CRAC} in weak buffer indeed reflected Ca^{2+}-inactivation of the Ins(1,4,5)P_3 receptors followed by SERCA-mediated store refilling, we would expect to observe a robust current with Ba^{2+} as the charge carrier, since this cation neither inactivates the Ins(1,4,5)P_3 receptor, nor is it taken up into the stores. With Sr^{2+}, we should see smaller I_{CRAC} since this cation can weakly inactivate Ins(1,4,5)P_3 receptors and is taken up into the stores. Hence, in weak buffer, I_{CRAC} should follow the cation profile $I_{Ba} > I_{Sr} > I_{Ca}$. As shown in Figure 4, the cation profile was very different from this. We failed to detect macroscopic I_{CRAC} with Ba^{2+} as the charge carrier (0/7 cells). A typical whole-cell recording is shown in Figure 4(A); Figure 4(B) depicts the ramp current taken 64 s after break-in. Pooled data are summarized in Figure 4(C). However, with Sr^{2+}, we were able to record robust I_{CRAC} in just over 50 % of cells (8/14; Figure 4B); moreover, I_{CRAC} was larger in responding cells than seen with Ca^{2+} as the charge carrier. A cation profile of $I_{Sr} > I_{Ca} > I_{Ba}$ is markedly different from that predicted if Ca^{2+} entry inactivated the Ins(1,4,5)P_3 receptors and SERCA pumps subsequently refilled the stores [12]. For comparison, the cation profile of I_{CRAC} in strong buffer is shown (Figure 4C, right-hand panel), where effects on Ins(1,4,5)P_3 receptors and SERCA pumps are reduced.

Receptor stimulation fails to activate detectable I_{CRAC} in weak Ca^{2+} buffer

Although dialysis with Ins(1,4,5)P_3 fails to evoke I_{CRAC} in weak Ca^{2+} buffer, we considered that receptor activation might be a more effective stimulus. Receptor stimulation would be expected to generate a variety of signals in addition to Ins(1,4,5)P_3 that might facilitate store depletion (e.g. via phosphorylation of SERCA pumps). Moreover, it has been suggested that a fraction of the Ins(1,4,5)P_3 receptors are bound to, and inhibited by, PtdIns(4,5)P_2, the precursor of endogenous Ins(1,4,5)P_3 [17]. Hence receptor agonists which hydrolyse PtdIns(4,5)P_2 might be able to remove this inhibition and activate Ins(1,4,5)P_3 receptors that are not accessible when the exogenous Ins(1,4,5)P_3 is dialysed into the cytoplasm. We locally applied 100 μM carbachol to RBL-1 cells stably transfected with the muscarinic m5-type receptor [18], which couples to phospholipase Cβ via a GTP-binding protein. However, we consistently failed to activate I_{CRAC} in cells dialysed with 0.1 mM EGTA (Figures 5A and 5B). Carbachol was only marginally more effective when cells were dialysed with strong Ca^{2+} buffer instead (10 mM EGTA, Ca^{2+} buffered at 120 nM to prevent spontaneous depletion of stores; Figure 5). Because protein kinase C (PKC) activation, following receptor stimulation, can inactivate I_{CRAC} [19] we exposed cells to carbachol together with the kinase blocker staurosporine. Nevertheless, the current could not be evoked in weak buffer (Figure 5), whereas it was consistently activated in strong buffer. There-

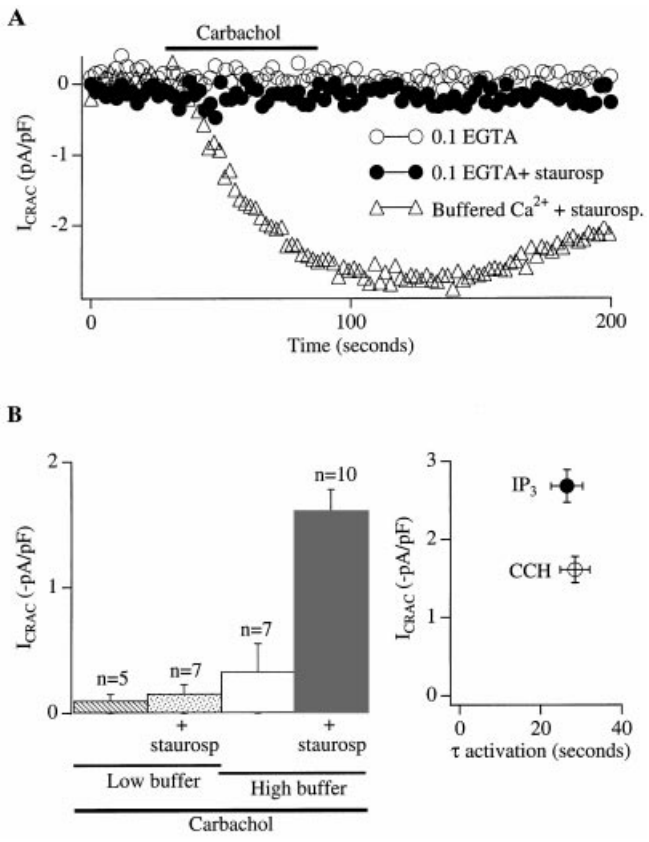

Figure 5 Stimulation of muscarinic receptors activates I_{CRAC} in strong, but not weak, intracellular Ca^{2+} buffer

(**A**) Shows a cell dialysed with 0.1 mM EGTA and application of carbachol after 40 s. I_{CRAC} failed to activate. Because of PKC-mediated inactivation of I_{CRAC}, cells were challenged with carbachol shortly after 2 μM staurosporine (staurosp) was applied externally. I_{CRAC} still failed to activate in weak buffer (●) whereas the current developed in strong buffer (120 nM Ca^{2+}, 10 mM EGTA; △). The pipette solution contained 0.2 mM GTP and 2 mM Mg-ATP for all of these recordings. (**B**) Summarizes the mean data for the different conditions (histogram on left). Weak buffer refers to 0.1 mM EGTA, and strong buffer refers to 120 nM Ca^{2+}/10 mM EGTA. The graph on the right compares the amplitude of I_{CRAC} and activation time-constant following application of carbachol (CCH) with Ins(1,4,5)P_3 (IP_3). The currents activated at similar rates, but I_{CRAC} evoked by receptor stimulation (in the presence of staurosporine) was less than that seen for Ins(1,4,5)P_3.

fore like Ins(1,4,5)P_3, receptor stimulation generally failed to activate I_{CRAC} in weak intracellular Ca^{2+} buffer.

Carbachol-evoked I_{CRAC} in strong buffer was significantly smaller (approx. 40%) than when the current was evoked by dialysis with 30 μM Ins(1,4,5)P_3 under the same conditions in the transfected cells (Figure 5B, right-hand panel). This may reflect the presence of additional inhibitory signals generated by receptor stimulation, but this was not examined further.

Effects of pharmacological intervention on the Ca^{2+}-dependent phosphorylation/dephosphorylation cycle

Because Ca^{2+}-dependent phosphorylation/dephosphorylation is believed to regulate SERCA pump activity [20], we tested whether I_{CRAC} could be activated in weak buffer in cells pretreated with 1 μM cyclosporin A, a selective inhibitor of the Ca^{2+}-dependent phosphatase calcineurin [21]. Under these conditions, I_{CRAC} did not activate in cells dialysed with Ins(1,4,5)P_3 plus 0.1 mM EGTA, but was unaffected when evoked by dialysis with 30 μM Ins(1,4,5)P_3 plus 10 mM EGTA (results not shown). Similarly, a 30 min pretreatment with 20 μM KN-62, a selective inhibitor of calmodulin-dependent kinase II, which has been shown to phosphorylate and inactivate Ins(1,4,5)P_3 receptors [22,23], consistently failed to allow Ins(1,4,5)P_3 to activate I_{CRAC} in weak buffer (results not shown). Cells treated with KN-62 routinely responded to Ins(1,4,5)P_3 plus 10 mM EGTA with robust I_{CRAC}, indicating that the CRAC channels themselves had not been compromised (results not shown).

DISCUSSION

The reason why, in weak Ca^{2+} buffer, Ins(1,4,5)P_3 generally fails to empty stores sufficiently for macroscopic I_{CRAC} activation is unclear. It is nevertheless important to elucidate, since it will provide insight into the regulation of the current under physiological conditions. Two possible mechanisms exist. One possibility is that Ins(1,4,5)P_3 receptors inactivate in weak buffer to curtail further Ca^{2+} release. SERCA pumps could then pump sufficient Ca^{2+} back into the stores to prevent the threshold for macroscopic I_{CRAC} activation from being exceeded. Alternatively, the Ins(1,4,5)P_3 receptors may not inactivate, but the activity of the SERCA pumps might increase substantially following Ca^{2+} release, again preventing the threshold for I_{CRAC} activation from being reached.

Ins(1,4,5)P_3 receptors do not seem to inactivate significantly under our conditions

Broad et al. [12] have suggested that Ca^{2+}-dependent inactivation of Ins(1,4,5)P_3 receptors accounts for the inability to detect I_{CRAC} following dialysis with Ins(1,4,5)P_3 in weak Ca^{2+} buffer. They proposed that, in the presence of Ins(1,4,5)P_3 in weak buffer, activation of store-operated Ca^{2+} influx inactivates the Ins(1,4,5)P_3 receptors, thereby terminating Ca^{2+} release. SERCA pumps subsequently refill the stores.

Our results are not readily reconcilable with this interpretation. Three pieces of evidence mitigate against it. First, local application of thapsigargin approx. 150–200 s after continuous dialysis with weak buffer and Ins(1,4,5)P_3 activates I_{CRAC} at a rate significantly faster than when Ins(1,4,5)P_3 is omitted from the pipette. Although interpretation is complicated by the fact that prior to thapsigargin application cells dialysed with Ins(1,4,5)P_3 will be closer to the threshold for activating macroscopic I_{CRAC}, our results nevertheless demonstrate that neither Ca^{2+}- nor ligand-dependent inactivation of the Ins(1,4,5)P_3 receptors occurs to a major extent over this time frame. Furthermore, although Ins(1,4,5)P_3 receptors (types I and II) are well known to exhibit Ca^{2+}-dependent inactivation, it has recently been established that this occurs only in the presence of sub-maximal concentrations of Ins(1,4,5)P_3 and is prevented in the presence of high concentrations of Ins(1,4,5)P_3 (K_D = 10 μM in cerebellum [24]). Because we used a supramaximal concentration of Ins(1,4,5)P_3 in this study (30 μM), and if the Ins(1,4,5)P_3 receptors in these cells behave like those in other systems, then significant Ca^{2+}-inactivation probably does not occur. In agreement with this, Oancea and Meyer [25] used caged Ins(1,4,5)P_3 to study Ca^{2+}-inactivation of Ins(1,4,5)P_3 receptors in RBL-1 cells. Inactivation of Ins(1,4,5)P_3 receptors following moderate increases of Ins(1,4,5)P_3 after flash photolysis could be overcome by further increases in Ins(1,4,5)P_3 using stronger flashes. These authors concluded that in the presence of high intracellular Ins(1,4,5)P_3, most Ins(1,4,5)P_3 receptors remain active [25].

Second, we consistently failed to detect macroscopic I_{CRAC} in response to Ins(1,4,5)P_3 in weak Ca^{2+} buffer when we shortened the voltage range, duration and frequency of ramps (−80 to

+80 mV; 20 ms; 0.25 Hz) and held the cells at +50 mV (close to the Ca^{2+} reversal potential) to reduce the extent of Ca^{2+} entry. We have recently described that the whole-cell CRAC conductance is voltage-dependent, and holding at +50 mV almost doubles the amplitude of I_{CRAC} compared with a holding potential of 0 mV [13]. Despite this increased 'gain', we still failed to detect I_{CRAC} in weak buffer.

Third, I_{CRAC} fails to activate in response to Ins(1,4,5)P_3 in weak Ca^{2+} buffer when Ba^{2+} is used as the charge carrier. If Ca^{2+}-dependent Ins(1,4,5)P_3 receptor inactivation of the Ins(1,4,5)P_3 receptors had occurred, we might have expected Ba^{2+} to support quite robust I_{CRAC} in weak buffer, since it is conducted through CRAC channels [6], does not inactivate the Ins(1,4,5)P_3 receptor [14,15], and, crucially, is not taken up into the stores by SERCA pumps [16]. However, no Ba^{2+} current was detected. We do not know why Sr^{2+} supports some I_{CRAC} in weak Ca^{2+} buffer. With Ins(1,4,5)P_3 and thapsigargin in weak Ca^{2+} buffer or Ins(1,4,5)P_3 in strong buffer $I_{Ca} > I_{Sr} > I_{Ba}$ [6] so we do not think that Sr^{2+} is having some additional effect on CRAC channel gating or on the activation mechanism itself.

Importance of SERCA pumps: Ca^{2+} cycling

The finding that Ins(1,4,5)P_3 receptors do not seem to inactivate is important, because it implies that Ca^{2+} is continuously cycling across the ER. Ca^{2+} efflux through (at least partially) open Ins(1,4,5)P_3 receptors must be countered by reuptake via SERCA pumps to prevent the threshold for macroscopic I_{CRAC} activation from being reached. Our results indicate that it is the activity of the SERCA pumps that prevents macroscopic I_{CRAC} from developing in weak Ca^{2+} buffer. It might seem surprising that SERCA pumps can maintain a sufficient Ca^{2+} store content to prevent macroscopic I_{CRAC} activation in spite of continuous Ca^{2+} release via open Ins(1,4,5)P_3 receptors. Under physiological conditions, the Ins(1,4,5)P_3 receptor has a mean open time of 3.7 ms [26]; for a typical unitary current amplitude of 0.5 pA, this would mean 5400 Ca^{2+} ions leaving the store per single-channel opening. Over the same time frame, only 1 Ca^{2+} ion would be transported by a single SERCA pump [10]. However, we have found that SERCA pumps are remarkably effective in RBL-1 cells and can prevent I_{CRAC} from activating, despite significant Ca^{2+} leakage from the stores and a cytosolic Ca^{2+} binding ratio of 6000 (300-fold greater than within the stores [27]), even when ATP is excluded from the recording pipette [6]. Importantly, small changes in ambient luminal and cytosolic Ca^{2+} can have marked effects on SERCA pump activity. Elegant studies in pancreatic acinar cells have clearly shown that a fall in luminal Ca^{2+}, which would occur following Ins(1,4,5)P_3-mediated Ca^{2+} release, increases the rate of SERCA-mediated Ca^{2+} reuptake [28]. Rises in cytosolic Ca^{2+} also stimulate SERCA pump activity. A detailed study of the Ca^{2+}-dependence of SERCA pumps in intact cells has revealed that pump activity increases 10-fold when cytosolic Ca^{2+} is doubled above the resting level [29]. Changes in SERCA-mediated reuptake will therefore have quite dramatic effects on the activation of I_{CRAC}. Reduced Ca^{2+} uptake into stores due to increased Ca^{2+} buffering by mitochondria can result in the activation of I_{CRAC} in weak buffer [30].

A further consideration is the distribution and density of SERCA pumps compared with Ins(1,4,5)P_3 receptors. RBL-1 cells express appreciable amounts of SERCA 2b and 3 isoforms [31], although the amount of protein relative to Ins(1,4,5)P_3 receptors is not known. We and others have suggested that a subcompartment of the ER may be intimately associated with the activation of I_{CRAC} in RBL-1 cells [7,32,33]. This putative specialized region is quite hard to deplete in that it requires quite high levels of Ins(1,4,5)P_3 [7], and may therefore be relatively devoid of Ins(1,4,5)P_3 receptors or rich in SERCA pumps. Such a dichotomy would permit some store refilling in spite of open Ins(1,4,5)P_3 receptors.

Physiological implications of our study

The inability to detect macroscopic I_{CRAC} in response to Ins(1,4,5)P_3 in weak intracellular buffer despite substantial store emptying does not mean that the current is physiologically unimportant. I_{CRAC} activation may occur, but to an extent which is currently undetectable. We are not confident of resolving a whole-cell current < 2 pA. A whole-cell I_{CRAC} of 2 pA at −80 mV reflects a minor fraction of the total number of available CRAC channels (approx. 4 %), but would nevertheless increase cytosolic Ca^{2+} at a rate of 50 nM·s^{-1} in a 15 pF cell (taking the Ca^{2+} binding ratio to be 75 [34]). Ca^{2+} entry through a relatively small number of open CRAC channels could clearly provide sufficient Ca^{2+} to regulate a variety of processes with high/moderate Ca^{2+} affinity. This should not be taken to mean that there is no threshold below which intraluminal Ca^{2+} needs to fall in order for macroscopic I_{CRAC} to activate. We and others have reported, using fluorescent Ca^{2+} dyes which are a more sensitive indicator of Ca^{2+} influx than electrophysiology, that the Ca^{2+} content of the stores can be reduced without any Ca^{2+} entry [7,35,36]. However, once the threshold has been reached, a few CRAC channels may activate, but not give rise to a detectable whole-cell current. Further depletion then results in a large increase in macroscopic I_{CRAC} [6].

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REFERENCES

1 Parekh, A. B. and Penner, R. (1997) Store-operated calcium influx. Physiol. Rev. **77**, 901–930
2 Artalejo, A. R., Ellory, J. C and Parekh, A. B. (1998) Ca^{2+}-dependent capacitance increases in rat basophilic leukaemia cells following activation of store-operated Ca^{2+} entry and dialysis with high Ca^{2+}-containing intracellular solution. Pfluegers Arch. **436**, 934–939
3 Dolmetsch, R. E., Xu, K. and Lewis, R. S. (1998) Calcium oscillations increase the efficiency and specificity of gene expression. Nature (London) **392**, 933–936
4 Partiseti, M., Le Deist, F., Hivroz, C., Fischer, A., Korn, H. and Choquet, D. (1994) The calcium current activated by T cell receptor and store depletion in human lymphocytes is absent in a primary immunodeficiency. J. Biol. Chem. **269**, 32327–32335
5 Hoth, M. and Penner, R. (1992) Depletion of intracellular calcium stores activates a calcium current in mast cells. Nature (London) **355**, 353–356
6 Fierro, L. and Parekh, A. B. (2000) Substantial depletion of the intracellular calcium stores is required for macroscopic activation of I_{CRAC} in RBL-1 cells. J. Physiol. (Cambridge, U.K.) **522**, 247–257
7 Parekh, A. B., Fleig, A. and Penner, R. (1997) The store-operated calcium current I_{CRAC}: Non-linear activation by $InsP_3$ and dissociation from calcium release. Cell (Cambridge, Mass.) **89**, 973–980
8 Fierro, L. and Parekh, A. B. (1999) On the characterisation of the mechanism underlying passive activation of the Ca^{2+} release-activated Ca^{2+} current I_{CRAC}. J. Physiol. (Cambridge, U.K.) **520**, 407–416
9 Watras, J., Bezprozvanny, I. and Ehrlich, B. E. (1991) Inositol 1,4,5-trisphosphate-gated channels in cerebellum: presence of multiple conductance states. J. Neurosci. **11**, 3239–3245
10 McLennon, D. H. and Holland, P. C. (1975) Calcium transport in sarcoplasmic reticulum. Annu. Rev. Biophys. Bioeng. **4**, 377–404
11 Glitsch., M. D. and Parekh, A. B. (2000) Ca^{2+} store dynamics determines the pattern of activation of the store-operated Ca^{2+} current I_{CRAC} in response to $InsP_3$ in rat basophilic leukaemia cells. J. Physiol. (Cambridge, Mass.) **523**, 283–290

12 Broad, L. M., Armstrong, D. L. and Putney, J. W. (1999) Role of the inositol 1,4,5-trisphosphate receptor in Ca^{2+} feedback inhibition of calcium release-activated calcium current (I_{CRAC}). J. Biol. Chem. **274**, 32881–32888
13 Bakowski, D. and Parekh, A. B. (2000) Voltage-dependent conductance changes in the store-operated calcium current ICRAC in RBL-1 cells. J. Physiol. (Cambridge, U.K.) **529**, 295–306
14 Marshall, I. C. and Taylor, C. W. (1994) Two calcium-binding sites mediate the interconversion of liver inositol 1,4,5-trisphosphate receptors between three conformational states. Biochem. J. **301**, 591–598
15 Hannaert-Merah, Z., Combettes, L., Coquil, J.-F., Swillens, S., Mauger, J.-P., Claret, M. and Champeil, P. (1995) Characterization of the co-agonist effects of strontium and calcium on myo-inositol trisphosphate-dependent ion fluxes in cerebellar microsomes. Cell Calcium **18**, 390–399
16 Kwan, C. Y. and Putney, J. W. (1990) Uptake and intracellular sequestration of divalent cations in resting and methacholine-stimulated mouse lacrimal acinar cells. Dissociation by Sr^{2+} and Ba^{2+} of agonist-stimulated divalent cation entry from the refilling of the agonist-sensitive intracellular pool. J. Biol. Chem. **265**, 678–684
17 Lupu, V. D., Kaznacheyeva, E., Krishna, U. M., Falck, J. M. and Bezprozvanny, I. (1998) Functional coupling of phosphatidylinositol 4,5-bisphosphate to inositol 1,4,5-trisphosphate receptor. J. Biol. Chem. **273**, 14067–14070
18 Dippel, E., Kalkbrenner, F., Wittig, B. and Schultz, G. (1996) A heterotrimeric G protein complex couples the muscarinic m1 receptor to phospholipase C-β. Proc. Natl. Acad. Sci. U.S.A. **93**, 1391–1396
19 Parekh, A. B. and Penner, R. (1995) Depletion-activated Ca^{2+} current is inhibited by protein kinase in RBL-2H3 cells. Proc. Natl. Acad. Sci. U.S.A. **92**, 7907–7911
20 Roderick, H. L., Lechleiter, J. D. and Camacho, P. (2000) Cytosolic phosphorylation of calnexin controls intracellular Ca^{2+} oscillations via an interaction with SERCA2b. J. Cell Biol. **149**, 1235–1248
21 Liu, J., Farmer, J. D., Lane, W. S., Friedman, J., Weismann, I. and Schreiber, S. L. (1991) Calcineurin is a common target of cyclophilin-cyclosporin A and FKBP-FK506 complexes. Cell (Cambridge, Mass.) **66**, 807–815
22 Zhang, B. X., Zhao, H. and Muallem, S. (1993) Ca^{2+}-dependent kinase and phosphatase control inositol 1,4,5-trisphosphate-mediated Ca^{2+} release. Modification by agonist stimulation. J. Biol. Chem. **268**, 10997–11001
23 Tokumitsu, H., Chijiwa, T., Hagiwara, M., Mizutani, A., Terasawa, M. and Hidaka, H. (1990) KN-62, 1-[N,O-bis(5-isoquinolinesulfonyl)-N-methyl-L-tyrosyl]-4-phenylpiperazine, a specific inhibitor of Ca^{2+}/calmodulin-dependent protein kinase II. J. Biol. Chem. **265**, 4315–4320
24 Kaftan, E. J., Ehrlich, B. E. and Watras, J. (1997) Inositol 1,4,5-trisphosphate ($InsP_3$) and calcium interact to increase the dynamic range of $InsP_3$ receptor-dependent calcium signaling. J. Gen. Physiol. **110**, 529–538
25 Oancea, E. and Meyer, T. (1996) Reversible desensitization of inositol trisphosphate-induced calcium release provides a mechanism for repetitive calcium spikes. J. Biol. Chem. **271**, 17253–17260
26 Brezprovanny, I. and Ehrlich, B. E. (1995) The inositol 1,4,5-trisphosphate ($InsP_3$) receptor. J. Membr. Biol. **145**, 205–216
27 Mogami, H., Gardner, J., Gerasimenko, O. V., Camello, P., Petersen, O. H. and Tepikin, A. V. (1999) Calcium binding capacity of the cytosol and endoplasmic reticulum of mouse pancreatic acinar cells. J. Physiol. (Cambridge, Mass.) **518**, 463–467
28 Mogami, H., Tepikin, A. V. and Petersen, O. H. (1998) Termination of cytosolic Ca^{2+} signals: Ca^{2+} reuptake into intracellular stores is regulated by the free Ca^{2+} concentration in the store lumen. EMBO J. **17**, 435–442
29 Camello, P., Gardner, J., Petersen, O. H. and Tepikin, A. V. (1996) Calcium dependence of calcium extrusion and calcium uptake in mouse pancreatic acinar cells. J. Physiol. (Cambridge, U.K.) **490**, 585–593
30 Gilabert, J.-A. and Parekh, A. B. (2000) Respiring mitochondria determine the pattern of activation and inactivation of the store-operated Ca^{2+} current I_{CRAC}. EMBO J. **19**, 1–7
31 Wuytack, F., Papp, B., Verboomen, H., Raeymakers, L., Dode, L., Bobe, R., Enouf, J., Bokkala, S., Authi, K. S. and Casteels, R. (1994) A sarco/endoplasmic reticulum Ca^{2+}-ATPase 3-type Ca^{2+} pump is expressed in platelets, in lymphoid cells, and in mast cells. J. Biol. Chem. **269**, 1410–1416
32 Huang, Y. and Putney, J. W. (1998) Relationship between intracellular calcium store depletion and calcium release-activated calcium current in a mast cell line (RBL-1). J. Biol. Chem. **273**, 19554–19559
33 Krause, E., Schmid, A., Gonzalez, A. and Schulz, I. (1999) Low cytoplasmic [Ca^{2+}] activates I_{CRAC} independently of global Ca^{2+} store depletion in RBL-1 cells. J. Biol. Chem. **274**, 36957–36962
34 Neher, E. (1995) The use of fura-2 for estimating Ca buffers and Ca fluxes. Neuropharmacology **34**, 1423–1442
35 Hartmann, J. and Verkhratsky, A. (1998) Relations between intracellular Ca^{2+} stores and store-operated Ca^{2+} entry in primary cultured human glioblastoma cells. J. Physiol. (Cambridge, Mass.) **513**, 411–424
36 Liu, K.-Q., Bunnell, S. C., Gurniak, C. B. and Berg, L. J. (1998) T cell receptor-initiated calcium release is uncoupled from capacitative calcium entry in Itk-deficient T cells. J. Exp. Med. **187**, 1721–1727

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