# Rapid Report

## $Ca^{2+}$ store dynamics determines the pattern of activation of the store-operated $Ca^{2+}$ current $I_{CRAC}$ in response to Ins$P_3$ in rat basophilic leukaemia cells

Maike D. Glitsch and Anant B. Parekh

*Department of Physiology, University of Oxford, Parks Road, Oxford OX1 3PT, UK*

(Received 23 November 1999; accepted after revision 17 January 2000)

1. The relationship between the amplitude of the store-operated $Ca^{2+}$ current $I_{CRAC}$ and intracellular inositol 1,4,5-trisphosphate (Ins$P_3$) concentration is complex. In rat basophilic leukaemia (RBL-1) cells dialysed with high intracellular $Ca^{2+}$ buffer, the relationship is supra-linear with a Hill coefficient of 12 and resembles an apparent 'all-or-none' phenomenon. The non-linearity seems to arise from Ins$P_3$ metabolism. However, it is not clear which Ins$P_3$-metabolising pathway engenders the non-linear behaviour nor whether $I_{CRAC}$ is always activated to its maximal extent by Ins$P_3$.

2. Using the whole-cell patch clamp technique, we dialysed RBL-1 cells with different concentrations of the Ins$P_3$ analogue Ins$P_3$-F. Ins$P_3$-F is broken down by Ins(1,4,5)$P_3$ 5-phosphatase but is not a substrate for Ins(1,4,5)$P_3$ 3-kinase. The relationship between Ins$P_3$-F and $I_{CRAC}$ amplitude was supra-linear and very similar to that with Ins$P_3$ but was distinct from the graded relationship seen with the non-metabolisable analogue Ins2,4,5$P_3$.

3. In the presence of high intracellular $Ca^{2+}$ buffer, Ins$P_3$-F activated $I_{CRAC}$ to its maximal extent. With moderate $Ca^{2+}$ buffer, however, sub-maximal $I_{CRAC}$ could be obtained to a maximal Ins$P_3$-F concentration. Nevertheless, the relationship between the amplitude of $I_{CRAC}$ and Ins$P_3$-F concentration was still supra-linear.

4. Submaximal $I_{CRAC}$ in response to Ins$P_3$-F in the presence of moderate $Ca^{2+}$ buffer was due to partial depletion of the stores, because the size of the current could be increased by thapsigargin.

5. The data suggest that first, Ins(1,4,5)$P_3$ 5-phosphatase is an important factor which contributes to the non-linear relationship between Ins$P_3$ concentration and the amplitude of $I_{CRAC}$ and second, Ins$P_3$ does not always activate $I_{CRAC}$ to its maximal extent. At moderate buffer strengths, submaximal $I_{CRAC}$ is evoked by maximal Ins$P_3$. However, the supra-linear relationship between Ins$P_3$ concentration and amplitude of the current still holds.

In a variety of cell types, stimulation of cell-surface receptors that engage the phosphoinositide pathway evokes a biphasic increase in intracellular $Ca^{2+}$: an initial inositol 1,4,5-trisphosphate (Ins$P_3$)-mediated $Ca^{2+}$ release phase is followed by a smaller but sustained $Ca^{2+}$ influx component (Berridge, 1993; Parekh & Penner, 1997). In most non-excitable cells, emptying of the intracellular $Ca^{2+}$ stores activates a selective $Ca^{2+}$ current called $I_{CRAC}$ ($Ca^{2+}$ release-activated $Ca^{2+}$ current; Hoth & Penner, 1992; Parekh & Penner, 1997). $Ca^{2+}$ entry through CRAC channels is necessary for prolonging the $Ca^{2+}$ signal following transient $Ca^{2+}$ release, for refilling the stores as well as for a host of key cellular functions including exocytosis, gene transcription and cell proliferation (Parekh & Penner, 1997).

The relationship between the intracellular Ins$P_3$ concentration and activation of the store-operated $Ca^{2+}$ influx can be graded (Jacob, 1990) or supra-linear (Parekh *et al.* 1997; Hartmann & Verkhratsky, 1998; Liu *et al.* 1998) in different non-excitable cells. In rat basophilic leukaemia (RBL-1) cells, a model system for studying $I_{CRAC}$, the amplitude of the current is steeply related to Ins$P_3$ concentration with a Hill coefficient of 12 (Parekh *et al.* 1997). This highly non-linear behaviour probably reflects metabolism of Ins$P_3$ because the non-metabolisable Ins$P_3$ analogue Ins2,4,5$P_3$ generates a graded response (Hill coefficient of 1). Stimulation of cell-surface receptors that engage the phosphoinositide pathway also generates $I_{CRAC}$ in a non-linear manner, suggesting that this highly supra-

linear behaviour might be of physiological relevance (Parekh *et al.* 1997).

Two questions arise from this. First, which of the Ins$P_3$-metabolising enzymes accounts for the non-linear relationship between Ins$P_3$ concentration and activation of $I_{CRAC}$? Second, is $I_{CRAC}$ invariably activated to its maximal extent by Ins$P_3$ (apparent 'all-or-none' activation) or can the current be activated submaximally under certain conditions? Whilst graded $I_{CRAC}$ has been reported for both a non-metabolisable Ins$P_3$ analogue (Parekh *et al.* 1997) and following passive depletion of stores (Fierro & Parekh, 1999), these conditions are non-physiological. It is not known whether Ins$P_3$ itself activates the current to a submaximal extent. We have designed experiments to address these issues. We have first investigated the biochemical mechanism which engenders supra-linear activation and then examined whether the size of $I_{CRAC}$ is always maximal following a sufficiently high increase in intracellular Ins$P_3$. We find that the relationship between Ins$P_3$ concentration and the size of $I_{CRAC}$ is set largely by cytoplasmic Ins(1,4,5)$P_3$ 5-phosphatase and is complex. $I_{CRAC}$ can be activated maximally or submaximally by a given Ins$P_3$ concentration, and this is determined, at least in part, by $Ca^{2+}$ reuptake into the stores, which operates efficiently at low to moderate concentrations of intracellular $Ca^{2+}$ buffer. Even when $I_{CRAC}$ is submaximal, the steep relationship between Ins$P_3$ concentration and the amplitude of the current still holds.

## METHODS

Rat basophilic leukaemia (RBL-1) cells, which were obtained from the American Type Culture Collection, were cultured as described previously (Parekh *et al.* 1997; Fierro & Parekh, 1999).

Patch clamp experiments were conducted in the tight-seal whole-cell configuration at room temperature (18–25 °C) as described previously (Hamill *et al.* 1981; Parekh *et al.* 1997; Fierro & Parekh, 1999). Sylgard-coated, fire-polished pipettes had DC resistances of 2·5–3·7 MΩ when filled with standard internal solution that contained (mM): caesium glutamate, 145; NaCl, 8; $MgCl_2$, 1; Hepes, 10; Mg-ATP, 2; pH 7·2 with CsOH. The $Ca^{2+}$ chelators EGTA (Sigma) or BAPTA tetracaesium salt (Molecular Probes) were added to this solution at the specified concentrations (described in the text). 3-Deoxy-3-fluoro-*myo*-inositol 4,5-trisphosphate (Ins$P_3$-F; added to the pipette solution) was obtained from Calbiochem. Thapsigargin was from Alomone Laboratories. All other chemicals were purchased from Sigma. 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 (NaOH). $I_{CRAC}$ was measured by applying voltage ramps (−100 to +100 mV in 50 ms) at 0·5 Hz from the holding potential of 0 mV as described previously (Parekh *et al.* 1997). Currents were filtered using an 8-pole Bessel filter at 2·5 kHz and digitised at 100 μs. Currents were normalised by dividing the amplitudes (measured from the voltage ramps at −80 mV) by the cell capacitance. Capacitative currents were compensated before each ramp by using the automatic compensation of the EPC 9-2 amplifier. All leak currents were subtracted by averaging the first two to four 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 Student's unpaired *t* test.

## RESULTS

Ins$P_3$ can be metabolised in mammalian cells through two pathways: an Ins(1,4,5)$P_3$ 3-kinase converts Ins$P_3$ to Ins$P_4$ and Ins(1,4,5)$P_3$ 5-phosphatase degrades Ins$P_3$ to Ins$P_2$ (Shears, 1992). The Ins$P_3$ analogue Ins$P_3$-F is resistant to Ins(1,4,5)$P_3$ 3-kinase activity (Kozikowski *et al.* 1990; Sarrany *et al.* 1992) but is readily susceptible to breakdown by the Ins(1,4,5)$P_3$ 5-phosphatase (Sarrany *et al.* 1992). Ins$P_3$-F is a full agonist on the Ins$P_3$-gated $Ca^{2+}$ release channels, although it has a slightly lower affinity (Sarrany *et al.* 1992). We therefore used Ins$P_3$-F to dissect out the metabolic pathway that underlay the supra-linear activation of $I_{CRAC}$.

Figure 1 summarises the effects of dialysing cells with different concentrations of Ins$P_3$-F. The pipette solution was strongly buffered with BAPTA and $Ca^{2+}$ was clamped close to 120 nM in order to prevent spontaneous depletion of stores and hence activation of $I_{CRAC}$. We constructed a dose–response curve for each coverslip used and Fig. 1*A* summarises a typical experimental protocol in which cells were dialysed with different concentrations of Ins$P_3$-F. The time courses of the currents (obtained from voltage ramps spanning −100 to +100 mV in 50 ms, top right panel of Fig. 1*A*) are shown on the left and the *I–V* relationships on the right. The amplitude of the current to 30 μM Ins$P_3$-F had a peak value of $-3{\cdot}08 \pm 0{\cdot}21$ pA pF$^{-1}$ ($n = 18$, Fig. 1*B*). This was not significantly different to that seen in the presence of 30 μM Ins$P_3$ + 10 mM BAPTA ($-3{\cdot}05 \pm 0{\cdot}38$ pA pF$^{-1}$, $n = 7$) or 30 μM Ins$P_3$-F + 10 mM EGTA + 2 μM thapsigargin ($-2{\cdot}88 \pm 0{\cdot}26$ pA pF$^{-1}$, $n = 5$; we have observed that $I_{CRAC}$ in BAPTA tends to be slightly larger than that in EGTA but this is not significant; Fierro & Parekh, 1999), and indicates that 30 μM Ins$P_3$-F activated $I_{CRAC}$ to its maximal extent. The relationship between Ins$P_3$-F concentration and the amplitude of $I_{CRAC}$ was clearly supra-linear with a Hill coefficient of 17 (Fig. 1*B*, ○). This was strikingly similar to that seen with Ins$P_3$ (Fig. 1*B*, △; Hill coefficient of 13 obtained with EGTA as the chelator; Parekh *et al.* 1997) but markedly different from that seen with the non-metabolisable analogue Ins2,4,5$P_3$ (Fig. 1*B*, ●) (Parekh *et al.* 1997). The lowest concentration of Ins$P_3$-F that activated maximal $I_{CRAC}$ (7·5 μM) did so after a delay that was significantly longer than that seen with higher concentrations ($P < 0{\cdot}05$, Fig. 1*C*). However, once the current was activated, both the time constant of activation and time-to-peak were the same irrespective of the concentration used ($P > 0{\cdot}1$, Fig. 1*C*). The supra-linear relationship between Ins$P_3$ concentration and activation of $I_{CRAC}$ therefore seems to reflect the activity of cytoplasmic Ins(1,4,5)$P_3$ 5-phosphatase. This family of enzymes has a high capacity for Ins$P_3$ and $K_D$

values in the micromolar range (Shears, 1992). Therefore they can effectively suppress modest increases in intracellular $InsP_3$ levels, preventing macroscopic activation of $I_{CRAC}$. We predict that the relationship between the size of $I_{CRAC}$ and the $InsP_3$-F concentration would be less steep in the presence of Ins(1,4,5)$P_3$ 5-phosphatase block. Specific blockers of this class of enzyme are lacking and agents that inhibit the phosphatase are plagued with additional effects (for example 2,3-diphosphoglycerate (DPG), which is often used to inhibit the Ins(1,4,5)$P_3$ 5-phosphatase (Streb *et al.* 1985), can also interfere with the Ins(1,4,5)$P_3$ 3-kinase (Guillemette *et al.* 1990), inhibit the $InsP_3$ receptor directly (Guillemette *et al.* 1990), and affect other signalling pathways like phospholipase D (Kanaho *et al.* 1993). Nevertheless, we tested the effects of DPG and disulfiram (Fowler *et al.* 1993), another putative Ins(1,4,5)$P_3$ 5-phosphatase inhibitor, on the $InsP_3$-F–$I_{CRAC}$ relationship. However, we found that disulfiram (20 $\mu$M, added to the bathing medium) seemed to block CRAC channels directly, because the current could not be activated even in the presence of a maximal concentration

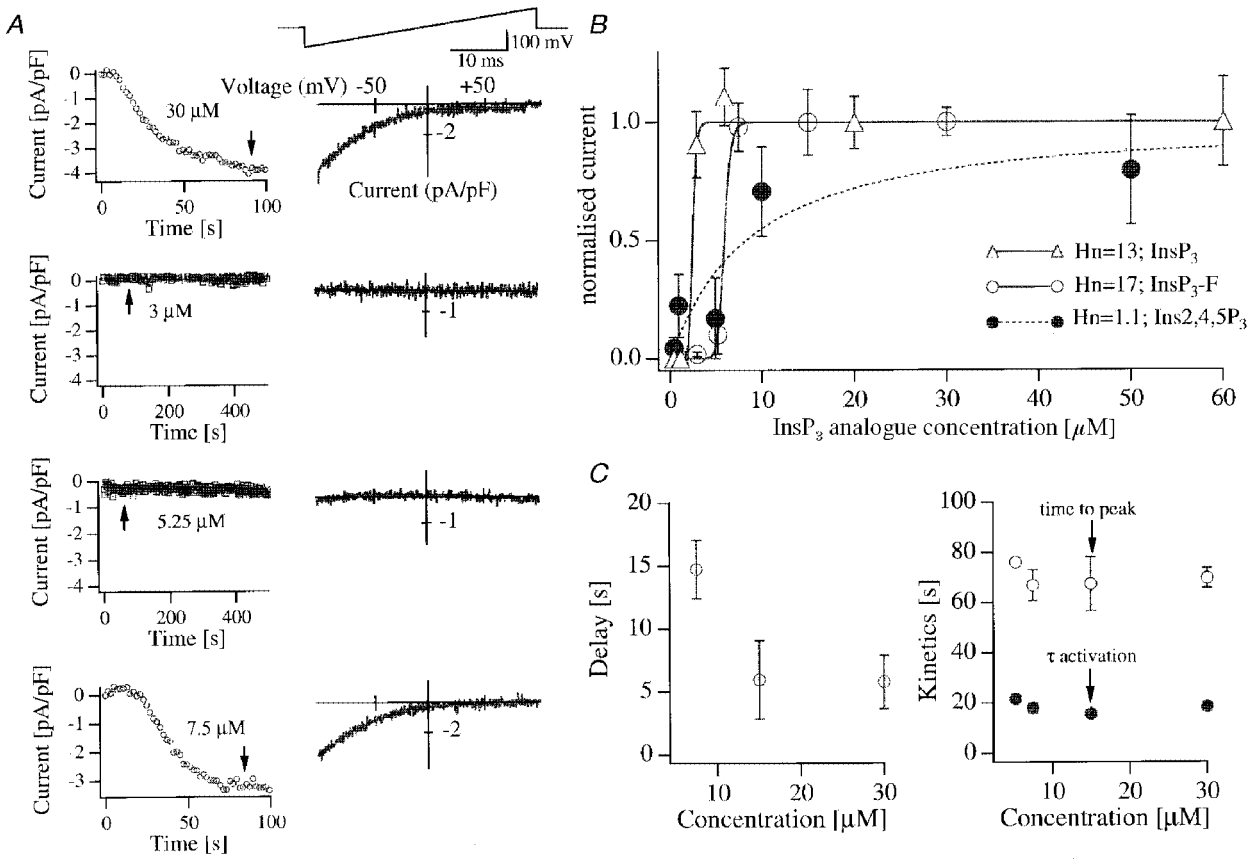


**Figure 1. Highly supra-linear activation of $I_{CRAC}$ by $InsP_3$-F in the presence of high exogenous $Ca^{2+}$ buffer**

*A*, patch clamp recordings investigating the effects of dialysing cells from the same coverslip with different concentrations of $InsP_3$-F are shown exactly as the experiment was carried out. The left-hand graphs depict the time course of $I_{CRAC}$, measured from voltage ramps at −80 mV. The right-hand graphs show the corresponding *I–V* relationships, taken at the time indicated by the arrows. *B* shows the overall $InsP_3$-F concentration–$I_{CRAC}$ amplitude curve. Data were fitted using the analysis program IGOR (Wavemetrics, OR, USA). Included in the graph are the corresponding relationships for $InsP_3$ (the physiological isomer) and Ins2,4,5$P_3$ (non-metabolisable analogue). For the $InsP_3$ curve we have included previously published data (Parekh *et al.* 1997) together with new experiments. The Hill coefficient (Hn) increased from 12 to 13. Our previous Ins2,4,5$P_3$ data have been recently reproduced by Huang & Putney, 1998. Fitting their data yielded a Hill coefficient in the range of 2, reasonably similar to our value of 1. *C* shows the delay (left) and both the time-to-peak and time constant ($\tau$) of the exponential fits (right). Two cells which gave clear currents to 5·25 $\mu$M $InsP_3$-F have been excluded from the left graph (but not the right graph) because the delays (100 and 81 s) would mask the significant difference in delay between 7·5 $\mu$M and the higher concentrations, by increasing the range of the *y*-axis. There were no significant differences between time-to-peak or time constant of activation. For each graph, points represent means ± S.E.M. of 8–18 cells, except for 5·25 $\mu$M $InsP_3$-F in *C* ($n = 2$). Cells were dialysed with standard internal solution supplemented with 12·5 mM BAPTA and 6·0 mM $CaCl_2$. Free $Ca^{2+}$ was calculated to be 122 nM and the free BAPTA concentration 6·25 mM. Dialysis with this solution without $InsP_3$ failed to activate $I_{CRAC}$ over a 400 s period (8 cells).

of Ins$P_3$ (30 $\mu$M in 10 mM EGTA; 4/4 cells). With 1 mM DPG in the pipette, three of seven cells responded to Ins$P_3$ and 10 mM EGTA after a sizeable delay (the other cells behaved normally), consistent with reports that DPG inhibits the Ins$P_3$ receptor (Guillemette *et al.* 1990). We were therefore forced to abandon these drugs.

Taken together, these results indicate that $I_{CRAC}$ activates in a supra-linear manner to Ins$P_3$ in the presence of high intracellular $Ca^{2+}$ buffer. Furthermore, the range of Ins$P_3$ concentrations over which $I_{CRAC}$ activates is so narrow (2-fold) under these conditions that it approximates to an apparent 'all-or-none' process.

We then examined whether first, Ins$P_3$ supra-linearly activated $I_{CRAC}$ under more physiological conditions (i.e. weak intracellular $Ca^{2+}$ buffering) and second, if this was always to its maximal extent. Unfortunately, the current cannot be measured when cells are dialysed with Ins$P_3$ in low $Ca^{2+}$ buffer (Fig. 2*A*, Ins$P_3$-F + 0·1 mM EGTA; see also Huang *et al.* 1998; Fierro & Parekh, 2000). We therefore determined the lowest concentrations of buffer that would still enable us

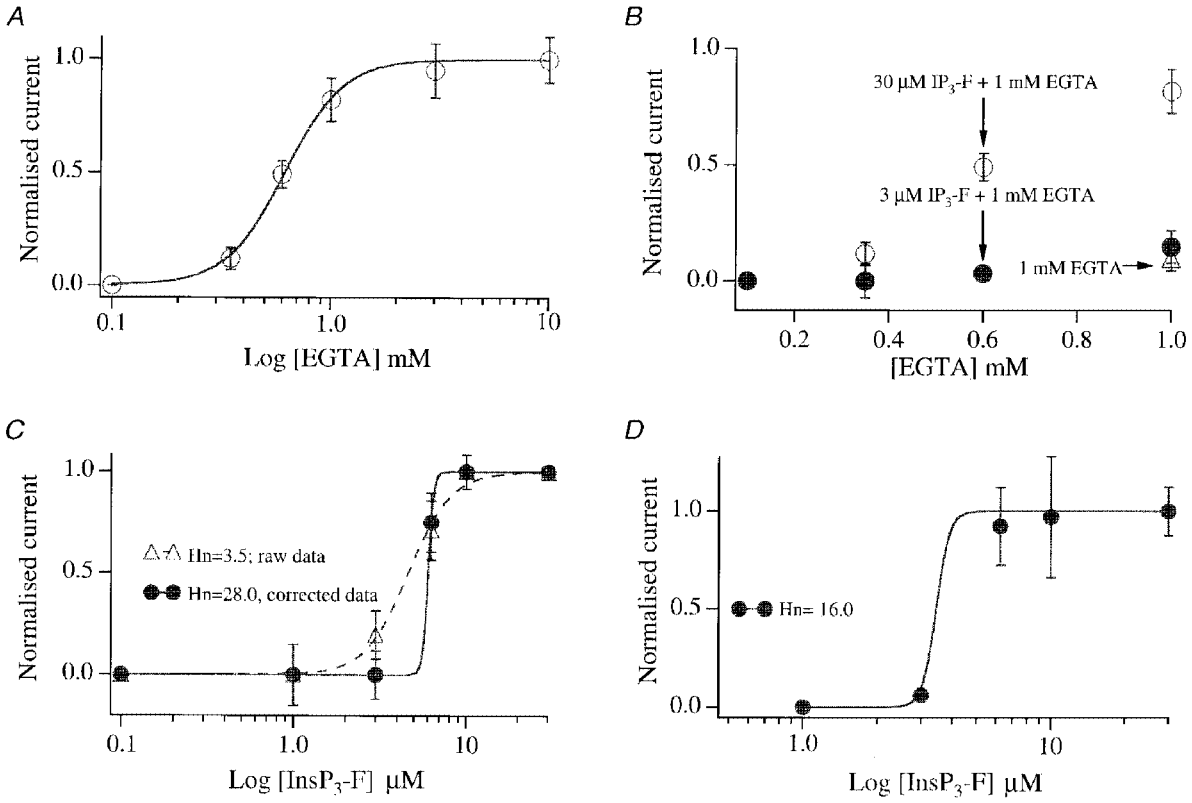


**Figure 2. Supra-linear activation of $I_{CRAC}$ in the presence of moderate $Ca^{2+}$ buffering**

*A*, relationship between intra-pipette EGTA concentration and the amplitude of $I_{CRAC}$ (evoked by dialysis with 30 $\mu$M Ins$P_3$-F). Note that $I_{CRAC}$ was clearly submaximal in the presence of 0·6 mM EGTA. Each point is the mean ± S.E.M. of at least 5 cells. *B*, graph showing the relationship between EGTA concentration and size of $I_{CRAC}$ when the threshold dose of 3 $\mu$M Ins$P_3$-F ($IP_3$-F) was used. Data for 30 $\mu$M have been included for comparison. The EGTA concentration ranged from 0·1 to 1·0 mM (each point is the mean ± S.E.M. of at least 5 cells and error bars are within the circles). The open triangle that overlaps with the filled circle at 1 mM is the size of $I_{CRAC}$ seen following dialysis with 1 mM EGTA and no Ins$P_3$-F. *C*, summary of pooled results when cells were dialysed with different concentrations of Ins$P_3$-F in the presence of 1 mM EGTA. Each point is the mean ± S.E.M. of at least 5 cells. Depending on how we treated the response to 3 $\mu$M Ins$P_3$-F, two different fits were obtained. If we included the size of $I_{CRAC}$ with 1 mM EGTA alone (−0·3 ± 0·11 pA $pF^{-1}$), and then subtracted this value from the amplitudes seen when different concentrations of Ins$P_3$-F were included, we obtained the dashed fit (raw data). However, if we arbitrarily set the amplitude of $I_{CRAC}$ to zero for 3 $\mu$M Ins$P_3$-F (because there was no significant difference between 1 mM EGTA alone and 3 $\mu$M Ins$P_3$-F + 1 mM EGTA), and subtracted the value for 3 $\mu$M Ins$P_3$-F + 1 mM EGTA from the currents seen with higher concentrations of Ins$P_3$-F, then we obtained the continuous fit (corrected data). The dashed fit is an underestimate, whereas the continuous one is an overestimate, of the true Hill coefficient (Hn). *D*, graph summarising the Ins$P_3$-F concentration–$I_{CRAC}$ relationship in the presence of 0·6 mM EGTA. Whereas the majority of cells failed to generate a current to 3 $\mu$M Ins$P_3$-F + 0·6 mM EGTA or 0·6 mM EGTA alone, two cells from one preparation did generate a clear $I_{CRAC}$ to 3 $\mu$M Ins$P_3$-F (−1·5 and −1·2 pA $pF^{-1}$). In this same preparation, 0·6 mM EGTA also produced a current rapidly (−1·0 pA $pF^{-1}$). Because this was so uncharacteristic, we did not include any cells from this preparation.

to reliably measure macroscopic $I_{CRAC}$ and, having found this, we then examined the relationship between $InsP_3$-F concentration and the size of $I_{CRAC}$. Cells were dialysed with 30 $\mu$M $InsP_3$-F and different concentrations of EGTA (range, 0·1–10 mM). For 30 $\mu$M $InsP_3$-F, concentrations of EGTA of less than 0·35 mM generally failed to support any macroscopic activation of $I_{CRAC}$ (Fig. 2*A*). Close to maximum current was seen with around 1 mM EGTA ($-2·33 \pm 0·19$ compared with $-2·64 \pm 0·31$ pA pF$^{-1}$ for 10 mM EGTA). However, in the presence of 0·6 mM EGTA, we obtained $I_{CRAC}$ that clearly had a submaximal amplitude ($-1·39 \pm 0·21$ pA pF$^{-1}$, $n = 8$; Fig. 2*A*). The relationship between exogenous buffer concentration and $I_{CRAC}$ amplitude in Fig. 2*A* was supra-linear with a Hill coefficient of 3·5. Figure 2*B* shows an experiment with a threshold concentration of 3 $\mu$M $InsP_3$-F instead of 30 $\mu$M (30 cells; 0·1–1 mM EGTA). For comparison, the corresponding data with 30 $\mu$M $InsP_3$-F were also included. For EGTA concentrations of 0·6 mM or less, no macroscopic $I_{CRAC}$ was detected. In the presence of 1 mM EGTA and 3 $\mu$M $InsP_3$-F, a small current was occasionally seen (6 of 13 cells) but its properties were no different to those of the current evoked by 1 mM EGTA alone (50% of cells responded to 1 mM EGTA alone compared with 46% for 1 mM EGTA + 3 $\mu$M $InsP_3$-F; their time-to-peak values were $120·1 \pm 20·4$ and $122·67 \pm 44·52$ s, respectively, and the overall extent of $I_{CRAC}$ was similar; $P > 0·1$; Fig. 2*B*; see also Fierro & Parekh, 1999).

We then determined the relationship between $InsP_3$-F concentration and activation of $I_{CRAC}$ in the presence of moderate intracellular $Ca^{2+}$ buffering (1 and 0·6 mM EGTA). Results are summarised in Fig. 2*C* and *D*. The relationship between $InsP_3$-F concentration and the amplitude of $I_{CRAC}$ was clearly supra-linear for both cases (Hill coefficient in the range 3·5–28 and apparent $K_D$ of 4·1 $\mu$M for 1 mM EGTA (see legend to Fig. 2); corresponding values for 0·6 mM EGTA were 16 and 3·4 $\mu$M, respectively). Therefore even in the presence of quite moderate amounts of $Ca^{2+}$ buffer and when the current is submaximal (as in 0·6 mM EGTA), $I_{CRAC}$ is still supra-linearly related to the cytoplasmic $InsP_3$ concentration.

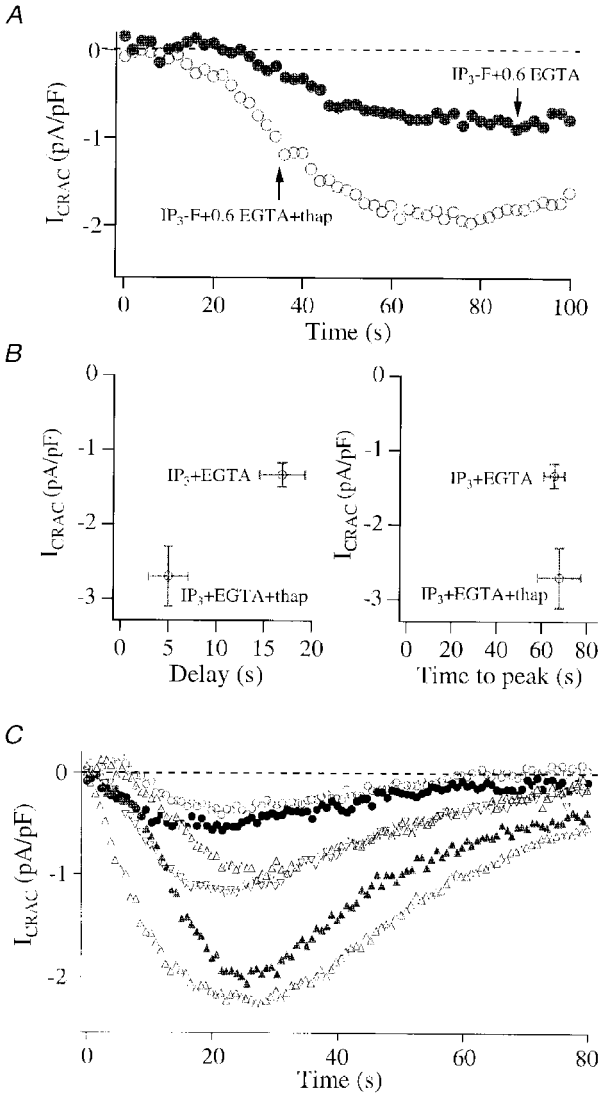


**Figure 3. Graded $I_{CRAC}$ can be obtained in the presence of moderate $Ca^{2+}$ buffer because of SERCA uptake and inhibition of the $InsP_3$ receptor**

*A* shows recordings for a cell dialysed with 30 $\mu$M $InsP_3$-F and 0·6 mM EGTA (●) and for a cell dialysed with this solution but supplemented with 2 $\mu$M thapsigargin (thap), a SERCA blocker. Note the dramatic increase in the size of $I_{CRAC}$ following inhibition of the pumps. *B*, summary of amplitudes, delays and times to peak for experiments as in *A*. *C*, cells dialysed with $InsP_3$ and 1 mg ml$^{-1}$ heparin (an inhibitor of the $InsP_3$ receptor) can produce a wide range of different $I_{CRAC}$ amplitudes, in spite of the presence of a maximal $InsP_3$ concentration. This experiment will depend on the relative speed of entry of $InsP_3$ and heparin into the cytosol (proportional to series resistance), subsequent diffusion in the cytosol and access to the $InsP_3$ receptors. Cells were dialysed with a solution in which $Ca^{2+}$ was buffered at 225 nM (3 mM Ca-EGTA and 2 mM EGTA). The decay in the current reflects store refilling because it could be reduced by inclusion of thapsigargin in the pipette (5 cells; data not shown). Dialysis with heparin fails to alter the activation of $I_{CRAC}$ evoked by ionomycin (Parekh & Penner, 1995) or passive depletion (Fierro & Parekh, 1999), indicating that it is not interfering directly with the activation mechanism itself nor with the CRAC channels.

We designed experiments to understand why $I_{CRAC}$ was submaximal in the presence of 0·6 mM EGTA, despite dialysis with a maximal concentration of Ins$P_3$-F. We reasoned that stores might refill partially in the presence of such a concentration of $Ca^{2+}$ buffer and that this would reduce the overall extent of $I_{CRAC}$ activation. If this was the case, then one would predict that the size of $I_{CRAC}$ should increase in the presence of the sarco–endoplasmic reticulum $Ca^{2+}$-ATPase (SERCA) blocker thapsigargin (Thastrup *et al.* 1989), since the latter should prevent any $Ca^{2+}$ reuptake and hence refilling of the stores. The results of Fig. 3*A* and *B* show that this was indeed the case. Inclusion of thapsigargin in the pipette solution (with 30 $\mu$M Ins$P_3$-F and 0·6 mM EGTA) had quite dramatic effects on the properties of $I_{CRAC}$. The delay before the current activated was substantially reduced (3-fold, $P < 0{\cdot}01$; Fig. 3*A* and *B*) and, strikingly, the size of the current increased significantly ($P < 0{\cdot}01$, Fig. 3*A* and *B*). Hence SERCA-mediated reuptake can slow the onset of $I_{CRAC}$ as well as reduce the overall extent of activation. However, the rate of development of $I_{CRAC}$ was the same for both conditions (Fig. 3*B*). The size of $I_{CRAC}$ in 30 $\mu$M Ins$P_3$-F + 0·6 mM EGTA + thapsigargin was similar to that seen in the presence of either Ins$P_3$ + 10 mM EGTA or Ins$P_3$ + 10 mM EGTA + thapsigargin and is therefore the maximal amplitude that can be recorded. Inhibition of SERCA pumps can therefore increase the size of $I_{CRAC}$ evoked by a maximal concentration of Ins$P_3$-F in moderate (0·6 mM) but not high (10 mM) EGTA, consistent with enhanced $Ca^{2+}$-dependent SERCA reuptake in the presence of moderate $Ca^{2+}$ buffer (Mogami *et al.* 1998). An alternative explanation for the actions of thapsigargin is that the subsequent elevation of intracellular $Ca^{2+}$ inhibits the activity of the Ins(1,4,5)$P_3$ 5-phosphatase so that Ins$P_3$ concentrations in the cytosol are increased and hence $I_{CRAC}$ becomes larger. However, the Ins(1,4,5)$P_3$ 5-phosphatase is only weakly sensitive to $Ca^{2+}$ changes and the most parsimonious explanation of the thapsigargin results is that it reflects reduced $Ca^{2+}$ reuptake.

Because inhibition of SERCA-mediated $Ca^{2+}$ uptake into the stores increases the size of $I_{CRAC}$ in moderate $Ca^{2+}$ buffer, we examined whether the converse was also true: namely, if inhibition of $Ca^{2+}$ efflux from the stores, as $I_{CRAC}$ developed, could turn the current off. We reasoned that, in the absence of further Ins$P_3$-mediated $Ca^{2+}$ release, SERCA pumps should rapidly refill the stores, thereby deactivating the current. The experiment of Fig. 3*C* was designed to test this. We dialysed cells with 30 $\mu$M Ins$P_3$ and heparin (1 mg ml$^{-1}$), a competitive antagonist of the Ins$P_3$ receptor. Because of its large size, heparin diffuses into the cytosol relatively slowly and hence $I_{CRAC}$ would initially start to activate before appreciable amounts of intracellular heparin would have built up. However, as intracellular heparin levels rise, it should displace Ins$P_3$ from its receptor, thereby causing $I_{CRAC}$ to switch off as SERCA pumps refill the stores. Results are summarised in Fig. 3*C*. We obtained a wide dispersion of $I_{CRAC}$ amplitudes, despite dialysis with a maximally effective Ins$P_3$ concentration. Some cells generated a large $I_{CRAC}$ whereas others produced rather small currents. Such pronounced variation was not seen in the absence of heparin. Hence a range of $I_{CRAC}$ amplitudes can be obtained to a fixed Ins$P_3$ concentration when $Ca^{2+}$ release is compromised. The results also indicate that both activation and maintainance of $I_{CRAC}$ in moderate $Ca^{2+}$ buffering requires the Ins$P_3$ receptor to be in an open conducting state in order to compensate SERCA reuptake. Although our results suggest that the Ins$P_3$ receptor indirectly controls $I_{CRAC}$ through the $Ca^{2+}$ content of the stores, it is also conceivable that a more direct regulation involving a physical interaction between the CRAC channels and Ins$P_3$ receptors also contributes (Kiselyov *et al.* 1998). The results of Fig. 3 demonstrate that activation of $I_{CRAC}$ by Ins$P_3$ is not a true 'all-or-none' phenomenon because, once the current was activated, it did not invariably reach its maximal extent.

## DISCUSSION

Our results provide new insights into the activation of $I_{CRAC}$. The second messenger Ins$P_3$ does not invariably activate $I_{CRAC}$ to its maximal extent, because submaximal currents can be observed at moderate levels of intracellular $Ca^{2+}$ buffering. This seems to reflect a dynamic interplay between $Ca^{2+}$ release and thapsigargin-sensitive $Ca^{2+}$ reuptake at the level of the Ins$P_3$-sensitive $Ca^{2+}$ stores. It is interesting that, in moderate buffer, SERCA-mediated reuptake prevents Ins$P_3$ from depleting the stores to the extent that $I_{CRAC}$ activates maximally. It seems unlikely that primary active transport ($Ca^{2+}$ uptake) can match the efflux of $Ca^{2+}$ through the large conductance Ins$P_3$-gated channels. As we have recently suggested (Fierro & Parekh, 2000), the Ins$P_3$ receptors on the stores may inactivate, at least partially, hence reducing the extent of $Ca^{2+}$ movement out of the stores. Furthermore, the fall in intraluminal as well as the increase in cytosolic $Ca^{2+}$ may both further enhance SERCA-mediated reuptake (Mogami *et al.* 1998).

The finding that $I_{CRAC}$ activates only partially in response to Ins$P_3$ in moderate buffer and that this is due to SERCA activity indicates that regulation of $Ca^{2+}$ uptake may represent a powerful means for grading the extent of activation of $I_{CRAC}$ in the presence of the weak to moderate levels of intracellular $Ca^{2+}$ buffering seen in intact cells.

Although $I_{CRAC}$ can be submaximal in the presence of moderate intracellular $Ca^{2+}$ buffer, the highly supra-linear relationship between Ins$P_3$ concentration and activation of the current still holds. However, we cannot be sure that the supra-linear behaviour is valid under conditions of weak buffering, since we and others consistently fail to record activation of the current by Ins$P_3$ under these conditions.

The supra-linear relationship between Ins$P_3$ concentration and activation of $I_{CRAC}$ seen in moderate to high intracellular $Ca^{2+}$ buffer probably arises from the cytoplasmic activity of

Ins(1,4,5)$P_3$ 5-phosphatase. Although this conclusion is drawn from experiments with a phosphatase-resistant Ins$P_3$ analogue and the standard pharmacological tools we have employed are not refined enough to selectively interfere with the enzyme, nevertheless this opens up the possibility of new experiments using molecular genetics to test this hypothesis in the future. Although the human Ins(1,4,5)$P_3$ 5-phosphatase has been cloned, the corresponding enzyme in the rat has not. Nevertheless, we have tried to reduce expression of the rat inositol 5-phosphatase using antisense oligonucleotides based on the human sequence (supplied by Biognostik, Goettingen, Germany), but without success. Cloning of the rat enzyme should enable us to design more rational antisense oligonucleotides. Interestingly, in certain human malignant haemopoetic cells, the activity of this enzyme is significantly reduced compared with that in normal cells (Mengubas *et al.* 1994). Because $Ca^{2+}$ influx through CRAC channels has been linked to cell growth and proliferation (Berridge, 1995), it is tempting to speculate that the abnormal cell growth in these forms of human leukaemia reflects, at least in part, the non-linear relationship between Ins$P_3$ concentration and the size of $I_{CRAC}$.


Berridge, M. J. (1993). Inositol trisphosphate and calcium signalling. *Nature* **361**, 315–325.

Berridge, M. J. (1995). Calcium signalling and cell proliferation. *Bioessays* **17**, 491–500.

Fierro, L. & Parekh, A. B. (1999). On the characterisation of the mechanism underlying passive activation of the $Ca^{2+}$ release-activated $Ca^{2+}$ current $I_{CRAC}$ in rat basophilic leukaemia cells. *Journal of Physiology* **520**, 407–416.

Fierro, L. & Parekh, A. B. (2000). 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. *Journal of Physiology* **522**, 247–257.

Fowler, C. J., Brannstrom, G., Ahlgren, P. C., Florvall, L. & Akerman, K. E. (1993). Inhibition of inositol 1,4,5-trisphosphate 5-phosphatase by micromolar concentrations of disulfiram and its analogues. *Biochemical Journal* **289**, 853–859.

Guillemette, G., Favreau, I., Lamontagne, S. & Boulay, G. (1990). 2,3-Diphosphoglycerate is a nonselective inhibitor of inositol 1,4,5-trisphosphate action and metabolism. *European Journal of Pharmacology* **188**, 251–260.

Hamill, O. P., Marty, A., Neher, E., Sakmann, B. & Sigworth, F. J. (1981). Improved patch-clamp techniques for high-resolution current recording from cells and cell-free membrane patches. *Pflügers Archiv* **391**, 85–100.

Hartmann, J. & Verkhratsky, A. (1998). Relations between intracellular $Ca^{2+}$ stores and store-operated $Ca^{2+}$ entry in primary cultured human glioblastoma cells. *Journal of Physiology* **513**, 411–424.

Hoth, M. & Penner, R. (1992). Depletion of intracellular calcium stores activates a calcium current in mast cells. *Nature* **355**, 353–356.

Huang, Y. & Putney, J. W. Jr (1998). Relationship between intracellular calcium store depletion and calcium release-activated calcium current in a mast cell-line (RBL-1). *Journal of Biological Chemistry* **273**, 19554–19559.

Huang, Y., Takahashi, M., Tanzawa, K. & Putney, J. W. Jr (1998). Effect of adenophostin A on $Ca^{2+}$ entry and calcium release-activated calcium current (Icrac) in rat basophilic leukemia cells *Journal of Biological Chemistry* **273**, 31815–31821.

Jacob, R. (1990). Agonist-stimulated divalent cation entry into single cultured human umbilical vein endothelial cells. *Journal of Physiology* **421**, 55–77.

Kanaho, Y., Nakai, Y., Katoh, M. & Nozawa, Y. (1993). The phosphatase inhibitor 2,3-diphosphoglycerate interferes with phospholipase D activation in rabbit peritoneal neutrophils. *Journal of Biological Chemistry* **268**, 12492–12497.

Kiselyov, K., Xu, X., Mozhayeva, G., Kuo, T., Pessah, I., Mignery, G., Zhu, X., Birnbaumer, L. & Muallem, S. (1998). Functional interaction between $InsP_3$ receptors and store-operated Htrp3 channels. *Nature* **396**, 478–482.

Kozikowski, A. P., Faruq, A. H., Aksoy, I. A., Seewald, M. J. & Powis, G. (1990). Synthesis of the first optically pure, fluorinated inositol 1,4,5-trisphosphate of myo-inositol stereochemistry and its effects on $Ca^{2+}$ release in Swiss 3T3 cells. *Journal of the American Chemical Society* **112**, 7403–7404.

Liu, K.-Q., Bunnell, S. C., Gurniak, C. B. & Berg, L. J. (1998). T cell receptor-initiated calcium release is uncoupled from capacitative calcium entry in Itk-deficient T cells. *Journal of Experimental Medicine* **187**, 1721–1727.

Mengubas, K., Jabbar, S. A. B., Nye, K. E., Wilkes, S., Hoffbrand, A. V. & Wickremasinghe, R. G. (1994). Inactivation of calcium ion-regulating inositol polyphosphate second messengers is impaired in subpopulations of human leukemia cells. *Leukemia* **8**, 1718–1725.

Mogami, H., Tepikin, A. V. & 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 Journal* **17**, 435–442.

Parekh, A. B., Fleig, A. & Penner, R. (1997). The store-operated calcium current $I_{CRAC}$: Nonlinear activation by $InsP_3$ and dissociation from calcium release. *Cell* **89**, 973–980.

Parekh, A. B. & Penner, R. (1995). Activation of store-operated calcium influx at resting Ins$P_3$ levels by sensitization of the Ins$P_3$ receptor in rat basophilic leukaemia cells. *Journal of Physiology* **489**, 377–382.

Parekh, A. B. & Penner, R. (1997). Store depletion and calcium influx. *Physiological Reviews* **77**, 901–930.

Sarrany, S. T., Wilcox, R. A., Liu, C., Potter, B. V. L. & Nahorski, S. R. (1992). 3-Position modification of myo-inositol 1,4,5-trisphosphate: Consequences for intracellular calcium mobilisation and enzyme recognition. *European Journal of Pharmacology* **8**, 265–272.

Shears, S. B. (1992). Metabolism of inositol phosphates. *Advances in Second Messenger and Phosphoprotein Research* **26**, 63–92.

Streb, H., Heslop, J. P., Irvine, R. F., Schulz, I. & Berridge, M. J. (1985). Relationship between secretagogue-induced $Ca^{2+}$ release and inositol polyphosphate production in permeabilized pancreatic acinar cells. *Journal of Biological Chemistry* **260**, 7309–7315.

Thastrup, O., Cullen, P. J., Drobak, B. K., Hanley, M. R. & Dawson, A. P. (1989). Thapsigargin, a tumor promoter, discharges intracellular $Ca^{2+}$ stores by specific inhibition of the endoplasmic reticulum $Ca^{2+}$-ATPase. *Proceedings of the National Academy of Sciences of the USA* **87**, 2466–2470.

**Acknowledgements**

We are grateful to Professors Alison F. Brading and H. Criss Hartzell and Dr Leonardo Fierro for discussion/critical comments on various stages of the manuscript. This work was supported by a grant from the Wellcome Trust to A.B.P. (no. 049236/Z/96/Z). A.B.P. is the Amersham Fellow in Medical Cell Biology, Keble College, Oxford. M.D.G. is a recipient of an international Human Frontiers of Science Programme long-term Fellowship.

**Corresponding author**

A. B. Parekh: Department of Physiology, University of Oxford, Parks Road, Oxford OX1 3PT, UK.

Email: anant.parekh@physiol.ox.ac.uk