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The Store-Operated Calcium Current I_{CRAC}: Nonlinear Activation by $InsP_3$ and Dissociation from Calcium Release

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Summary

Patch-clamp experiments aimed at determining the relationship between intracellular Ca^{2+} release and activation of store-operated calcium current I_{CRAC} reveal that both agonist and $InsP_3$-mediated activation of I_{CRAC} are highly nonlinear, occurring over a narrow concentration range. Ca^{2+} release and Ca^{2+} influx can be dissociated, as they possess differential sensitivities to $InsP_3$: low concentrations induce substantial Ca^{2+} release without any activation of I_{CRAC}, whereas micromolar concentrations of $InsP_3$ are required to activate Ca^{2+} influx. This suggests functionally distinct stores controlling Ca^{2+} release and influx and enables cells to switch between sources of Ca^{2+} to fit best their current needs.

Introduction

In electrically nonexcitable cells, activation of cell-surface receptors that stimulate inositol 1,4,5-trisphosphate ($InsP_3$) production evokes a biphasic increase in cytosolic free Ca^{2+}. The rapid phase reflects Ca^{2+} release from internal stores, whereas the sustained phase is due to Ca^{2+} influx into the cell (Putney, 1986; Tsien and Tsien, 1990; Berridge, 1993). The Ca^{2+} influx pathway is linked to the Ca^{2+} content of the stores and has been termed store-operated Ca^{2+} influx (formerly capacitative Ca^{2+} influx). Depletion of stores activates a Ca^{2+} current, called I_{CRAC}, in several nonexcitable cells (Hoth and Penner, 1992). We have examined the relationship between Ca^{2+} release and activation of I_{CRAC} using a variety of different ways to deplete $InsP_3$ stores in rat basophilic leukemia cells (RBL-1). We find I_{CRAC} activates fully over a very narrow range of stimulus intensity, suggesting that activation of the current is nonlinearly related to store release. Furthermore, despite maximal activation of I_{CRAC}, we report that Ca^{2+} influx can be graded, and this arises both from graded changes in membrane potential and from additional inhibitory signals like protein kinase C, which are activated after receptor stimulation (Parekh and Penner, 1995b). It appears that activation of I_{CRAC} is an essentially all-or-none process, which is then fine tuned by additional regulatory mechanisms.

By directly measuring cytosolic Ca^{2+}, we find substantial Ca^{2+} release at $InsP_3$ concentrations that do not activate I_{CRAC} at all. Ca^{2+} release and Ca^{2+} influx can therefore be dissociated. We suggest that there are at least two types of functional $InsP_3$ stores: one involved in Ca^{2+} release and another that is primarily responsible for activation of Ca^{2+} influx. Given the importance of Ca^{2+} influx for a variety of cellular processes, such as Ca^{2+} oscillations (Tsien and Tsien, 1990; Berridge, 1993), secretion (Parekh and Penner, 1995b), and enzymatic regulation (Chiono et al., 1995), our results are likely to be of widespread importance to a plethora of physiological processes.

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Results and Discussion

Highly Nonlinear Activation of I_{CRAC}

To probe the relationship between Ca^{2+} release from $InsP_3$ stores and the corresponding activation of I_{CRAC}, we dialyzed RBL cells with different concentrations of $InsP_3$, the physiologically relevant isomer, via the patch pipette. Figure 1 summarizes the relationship between $InsP_3$ concentration and amplitude of I_{CRAC} in 40 individual cells. When 60 μM $InsP_3$ was included in the patch pipette, I_{CRAC} activated with a latency of 2 $\pm$ 1 s after obtaining the whole-cell configuration (Figures 1A and 1C; n = 8). The activation time constant (τ) was 18 $\pm$ 1.7 s, and the current reached a peak amplitude of -2.35 ± 0.45 pA/pF at -80 mV (Figure 1B). This is the maximal rate and level of activation of I_{CRAC}, because first, external application of 14 μM ionomycin activates the current with the same kinetics and size (Parekh and Penner, 1995a), and second, application of ionomycin once I_{CRAC} has peaked in response to 60 μM $InsP_3$ fails to evoke any further current (4/4 cells; data not shown). With 6 μM $InsP_3$ in the patch pipette, I_{CRAC} activated after a longer latency (73 $\pm$ 19 s) and slower τ (49 $\pm$ 8.1 s) but nevertheless evoked the maximal activation of I_{CRAC} (-2.6 ± 0.25 pA/pF, 10 cells) (Figures 1A and 1B). A small decrease in $InsP_3$ concentration to 3 μM still maximally activated I_{CRAC} (-2.13 ± 0.31 pA/pF) in 6 of 8 cells (after a latency of 147.2 $\pm$ 35.4 s and τ of 46.7 $\pm$ 10.6 s). However, two cells failed to show I_{CRAC} at all. Under identical recording conditions, a further small reduction of $InsP_3$ concentration to 1.2 μM resulted in complete failure of I_{CRAC} activation in 8 out of 8 cells. Lower doses of 60 and 600 nM $InsP_3$ also failed to activate I_{CRAC} (4 and 10 cells, respectively).

Activation of I_{CRAC} therefore has a highly nonlinear dependence on the $InsP_3$ concentration. The dose-response curve of Figure 1B yielded a Hill coefficient of 12, indicating tremendous cooperativity. Although we were using an immortalized cell line, we unexpectedly observed that different batches of cells (frozen stocks) occasionally had different sensitivities to $InsP_3$. One extreme batch gave maximal I_{CRAC} to 600 nM $InsP_3$, but 60 nM gave no response at all. In these cells, a 5-fold increase in $InsP_3$ evoked maximal I_{CRAC}. Although different preparations had different sensitivities to $InsP_3$, the nonlinear relationship was always observed.

Dissociation between Ca^{2+} Release and Ca^{2+} Influx

Superimposed in Figure 1B is the relationship between $InsP_3$ concentration and Ca^{2+} release in permeabilized

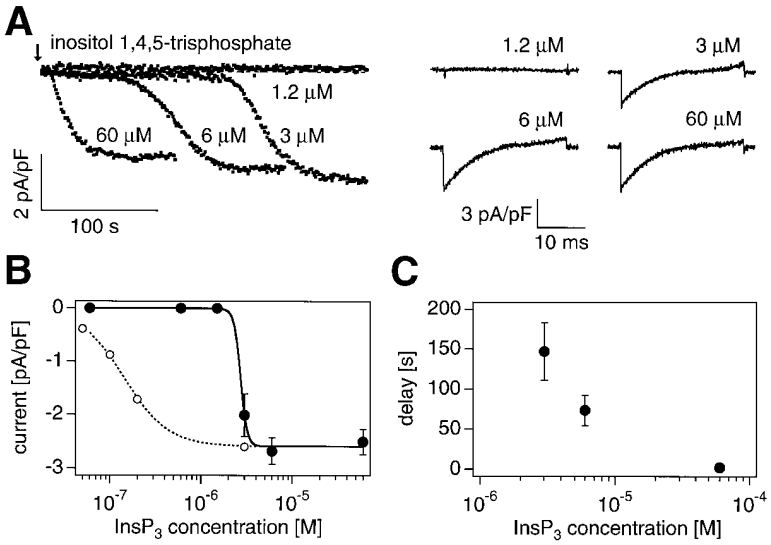

Figure 1. Nonlinear Dependence of I_{CRAC} on $InsP_3$ Concentration

(A) Typical examples of currents evoked by perfusion of cells with different concentrations of $InsP_3$. The Ca^{2+} current was monitored by voltage ramps (right panels) spanning −100 to +100 mV in 50 ms at intervals of 1 or 2 s. From these ramps, amplitudes of currents measured at −80 mV and normalized for cell capacitance are plotted versus time (left panel). Normally, $InsP_3$ at 0.6 and 1.2 μM does not evoke I_{CRAC}, whereas at 3 and 6 μM it does. All 4 cells were from the same coverslip.

(B) Dose-response curve relating pipette $InsP_3$ concentration to normalized I_{CRAC} amplitude (closed circles). The apparent K_D was 2.7 μM, and the Hill coefficient was 12. In these experiments, $InsP_3$ was tested over the entire concentration range of the dose-response on each experimental session, and then all the data from one preparation were pooled. Data are means ± SEM (n = 4–11). Open circles and the dashed dose-response curve plot $InsP_3$ versus Ca^{2+} release in permeabilized RBL cells. These data were scanned from Figure 5 of Meyer and Streyer (1990), digitized, and subsequently analyzed. For better comparison, the data points were scaled such that maximal release matches the maximal amplitude of I_{CRAC}. This analysis yielded an apparent K_D of 140 nM and a Hill coefficient of 1.7.

(C) Dose-response curve relating pipette $InsP_3$ concentration to the delay in I_{CRAC} activation. Data are means ± SEM (n = 6–11).

RBL cells (open circles and dotted line, taken from Meyer and Stryer, 1990). This relationship has a Hill coefficient of 1.7. A recent study arrives at a Hill coefficient of 2 for $InsP_3$-induced Ca^{2+} release in HL-60 granulocytes (Schrenzel et al., 1995). Hence, the very large Hill coefficient for the activation of I_{CRAC} cannot be completely accounted for by cooperativity in $InsP_3$-mediated Ca^{2+} release. The graphs in Figure 1B also suggest a large discrepancy between $InsP_3$-evoked Ca^{2+} release and activation of I_{CRAC}. Low $InsP_3$ causes substantial Ca^{2+} release in permeabilized RBL cells but no activation of the current. To examine the relationship between Ca^{2+} release and activation of I_{CRAC} further, we attempted to measure the amount of Ca^{2+} released by different $InsP_3$ concentrations in the patch pipette. The results, which were obtained in nominally Ca^{2+}-free external medium, are shown in Figure 2. Breaking into the cell with a very low concentration of $InsP_3$ (10 nM) failed to give any Ca^{2+} transient (Figure 2A, left panel; 5/5 cells). Note that the 360 nm (Ca^{2+}-independent wavelength of Fura 2) and the 390 nm (Ca^{2+}-dependent) both increase rapidly and monoexponentially upon breaking into the cell. Raising the $InsP_3$ concentration slightly resulted in large Ca^{2+} release. Concentrations of $InsP_3$ that failed to activate I_{CRAC} at all (60–100 nM; cf. Figure 1B) evoked substantial Ca^{2+} release (Figure 2A, middle and right panels). This can also be seen in the delay before the rise of the Fura 2 fluorescence signal at 390 nm excitation as compared with the 360 nm signal, which increased rapidly and monoexponentially. The key point is that we could observe substantial Ca^{2+} release with $InsP_3$ concentrations that failed to evoke any I_{CRAC}, which suggests a dissociation between the extent of Ca^{2+} release and the subsequent activation of I_{CRAC}.

The next set of experiments was carried out in the presence of external Ca^{2+}. To demonstrate that substantial Ca^{2+} release was not associated with any Ca^{2+} influx in the same cell, we applied hyperpolarizing pulses to increase the electrical gradient for Ca^{2+} entry. Although 600 nM $InsP_3$ caused very large and rapid Ca^{2+} release (Figure 2B), this was not associated with Ca^{2+} entry because hyperpolarizing pulses to either −20 or −70 mV (from a holding potential of +20 mV) did not cause any Ca^{2+} influx (Figure 2B). When 60 μM $InsP_3$ was added to the pipette solution, similar hyperolarizing steps evoked substantial Ca^{2+} entry (Figure 2C). The Ca^{2+} release was so rapid that we were unable to measure the rise phase because an insufficient time had elapsed for adequate amounts of Fura 2 to dialyze into the cell. We were able to catch only the trailing edge of the transient, and this was in excess of 1 μM, as has also been observed in mast cells (Neher, 1988). We interpret these results as evidence that release of a substantial amount of stored Ca^{2+} from the $InsP_3$-sensitive stores does not activate I_{CRAC} at all. An alternative explanation is that the stores refill in the presence of $InsP_3$ concentrations <1 μM and, hence, are depleted only transiently. To distinguish between these possibilities, we measured the Ca^{2+} that remained in the stores after dialyzing individual RBL cells with different concentrations of $InsP_3$. In the first series of experiments, we assessed the store contents in RBL cells by first activating release with different doses of $InsP_3$ and subsequently challenging the cell with ionomycin. Unfortunately, this approach did not yield consistent results, as we obtained highly variable Ca^{2+} signals for identical $InsP_3$ concentrations. There was even some release by ionomycin when large concentrations of $InsP_3$ (60 μM) were present, suggesting the presence of $InsP_3$-insensitive Ca^{2+} compartments in these cells.

In a second approach, we used RBL cells transfected with a muscarinic receptor that coupled to the phosphoinositidase pathway (Choi et al., 1993). When cells were dialyzed with solutions lacking $InsP_3$, a hyperpolarization to −40 mV from +20 mV produced a very small Ca^{2+} signal, which reflects Ca^{2+} influx through the leak

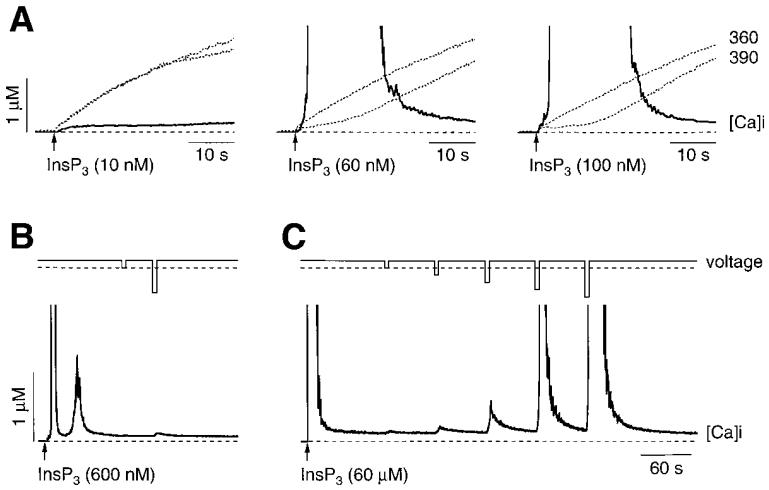

Figure 2. Dissociation of Ca^{2+} Release and Ca^{2+} Influx

(A) Typical examples of changes in $[Ca^{2+}]_i$ as monitored by Fura 2 fluorescence. Cells were dialyzed with the standard internal solution supplemented with 100 μM Fura 2 and the indicated concentrations of $InsP_3$. For the length of the experiment, the cells were perfused with nominally Ca^{2+}-free external solution locally applied from a puffer pipette. The arrow indicates the time at which the whole-cell configuration was established. Dotted lines represent the fluorescence measured at the two excitation wavelengths, and the solid trace is the free $[Ca^{2+}]_i$ calculated from the fluorescence ratio of the two excitation wavelengths. Calcium signals above 2 μM are truncated, as Fura 2 does not reliably report $[Ca^{2+}]_i$ above several micromolar.

(B) The cell was dialyzed with the standard internal solution supplemented with 600 nM $InsP_3$ in the presence of 2 mM external Ca^{2+}. The holding potential was set to +20 mV and hyperpolarized for 5 s to 0 and −70 mV as indicated in the voltage protocol. Note the prominent Ca^{2+} release but the absence of significant Ca^{2+} influx even at strong hyperpolarization.

(C) Similar experiment as in (B), except that a saturating concentration of $InsP_3$ was employed. Now even mild hyperpolarizations cause significant Ca^{2+} influx graded with the hyperpolarization strength.

pathway (Figure 3, control; averaged responses from 10 cells). Subsequent application of carbachol evoked a large Ca^{2+} release transient (Figure 3), which depeleted internal stores and activated Ca^{2+} entry, as evidenced by the increase in intracellular Ca^{2+} concentration by the second hyperpolarizing pulse. If transfected cells were dialyzed with 1 μM $InsP_3$, Ca^{2+} release occurred shortly after breaking into the cell, and a step hyperpolarization applied after 120 s (when pipette $InsP_3$ had equilibrated with the cytosol) failed to stimulate Ca^{2+} entry above that induced by the leak pathway (Figure 3; averaged responses of 7 cells), as observed in nontransfected cells (Figure 2). Subsequent application of carbachol resulted in a much smaller Ca^{2+} release transient (<20% of control), demonstrating that the stores were severely depleted and had not refilled in the continuous presence of 1 μM $InsP_3$. Despite the small Ca^{2+} release by carbachol, a second hyperpolarization now resulted in prominent Ca^{2+} influx (Figure 3). Dialysis with 10 μM $InsP_3$ produced a large Ca^{2+} transient and Ca^{2+} influx upon hyperpolarization (Figure 3; averaged responses of 5 cells). Subsequent exposure to carbachol failed to generate any further Ca^{2+} release, demonstrating that the $InsP_3$-sensitive and the carbachol-sensitive Ca^{2+} stores overlapped completely. These results clearly demonstrate that low concentrations of $InsP_3$ can significantly reduce the Ca^{2+} content of the stores without activating Ca^{2+} entry at all.

There is considerable evidence that the $InsP_3$ concentration–Ca^{2+} release relationship is nonlinear. First, $InsP_3$-induced Ca^{2+} release exhibits cooperativity with Hill coefficients of about 2 (Meyer and Stryer, 1990; Schrenzel et al., 1995). Second, there is probably additional cooperativity owing to positive feedback by Ca^{2+} on the $InsP_3$ receptor (Schrenzel et al., 1995), and this effect will enhance the release in the experiments of

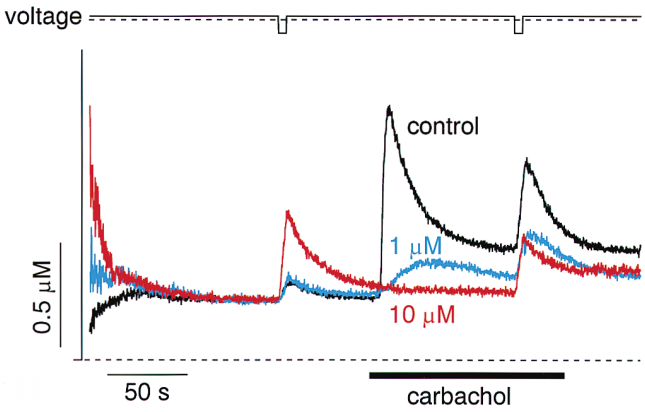

Figure 3. Relation between IP_3-Induced Calcium Release, Receptor-Stimulated Store Depletion, and Activation of I_{CRAC}

Averaged data traces showing changes in $[Ca^{2+}]_i$ as monitored by Fura 2 fluorescence in RBL-2H3 cells stably transfected with the muscarinic receptor M1. The cells were dialyzed with the standard internal solution supplemented with 200 μM Fura 2 and either 10 μM (n = 5), 1 μM (n = 7), or no $InsP_3$ (n = 10). The bar indicates the time during which external solution containing 100 μM carbachol was applied locally from a puffer pipette. The holding potential was set to +20 mV and hyperpolarized for 5 s to −40 mV before and after carbachol application as indicated in the voltage protocol. For display purposes, the traces corresponding to 10 and 1 μM $InsP_3$ were shifted by −42 and −14 nM relative to the control trace, respectively, to adjust for the slightly different baseline $[Ca^{2+}]_i$ between the different data sets.

Figure 2, where Ca^{2+} buffering capacity was low (unlike the situation for the current measurements). Even if we assumed the minimal cooperativity of 2 (neglecting the Ca^{2+} feedback), 600 nM $InsP_3$ is expected to deplete the stores significantly, since 60 nM $InsP_3$ already caused some release (Figure 2A), and a dose-response curve with a Hill coefficient of 2 predicts about 90% release for a 10-fold concentration change. Despite significant release, this was not associated with any Ca^{2+} entry (Figures 1B and 2B).

These results demonstrate that Ca^{2+} release has a higher sensitivity to $InsP_3$ than Ca^{2+} influx. This could arise if there are two populations of $InsP_3$ stores having different sensitivities to $InsP_3$, as has been discussed (Berridge, 1992). In this scheme, one set of stores would be essentially involved in Ca^{2+} release, and the other lower affinity store would be mainly involved in Ca^{2+} entry. Alternatively, partial emptying of one homogenous store by low $InsP_3$ is insufficient to activate Ca^{2+} influx. Higher $InsP_3$ further depletes the store, and only now can I_{CRAC} be activated.

Nonlinear Activation of I_{CRAC} Following Receptor Stimulation

Physiologically, $InsP_3$ levels increase after receptor stimulation, so we constructed a dose-response curve to an agonist that increases $InsP_3$ levels. Activation of adenosine receptors with the adenosine analog NECA routinely evokes I_{CRAC} in RBL cells, provided that protein kinase C activity is blocked, because the kinase inactivates CRAC channels (Parekh and Penner, 1995b). In Figure 4A, the effects of different concentrations of NECA on I_{CRAC} are shown. Two observations are striking. First, if a cell responded, then the peak amplitude of I_{CRAC} was the same, independent of the agonist concentration. Over a 10,000-fold concentration range, the amplitude of I_{CRAC} was similar (Figures 4A and 4B). I_{CRAC} activated by 3 nM NECA was as large as that generated in response to 30 μM, despite 3 nM being at least 100× lower than a maximal concentration (Ali et al., 1990). A plot of the dose-response curve yielded a Hill coefficient of 4 (Figure 4B). Each open circle depicts a single cell, and the closed circles reflect the averaged response. At the critical concentration of 0.3 nM, either a cell responded or it did not. If it responded, it gave the maximal I_{CRAC}. Increasing the NECA concentration increases the probability that a cell will respond but not the size of the response. Therefore, the averaged response is somewhat misleading, because it provides a mean that does not reflect the all-or-none behavior. The second striking observation was that I_{CRAC} did not gradually develop during the application of NECA (Figure 4A). If we had a graded linear process relating store emptying to CRAC activation, we would expect a gradual increase in the current with time, as $InsP_3$ levels increase and empty more stores. Instead, a long latency of several hundred seconds in some cases preceded rapid activation of the current (Figure 4A). This would be indicative of a threshold level below which no current is activated, but above which activation is rapid.

Small Ca^{2+} Influx Despite Maximal I_{CRAC}

Protein kinase C is a strong feedback inhibitor of I_{CRAC} (Parekh and Penner, 1995b). To measure routinely I_{CRAC}

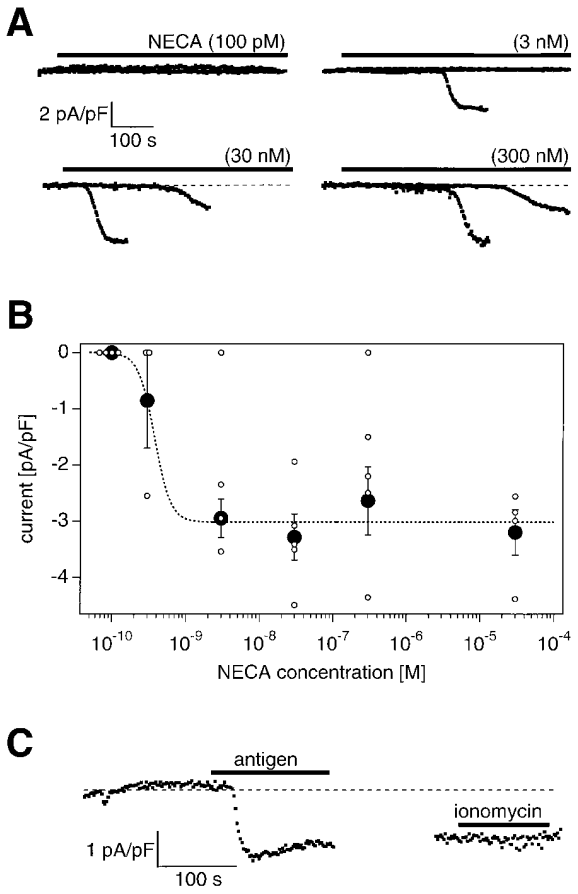

Figure 4. Nonlinear Activation of I_{CRAC} by Receptor Stimulation

(A) Development of I_{CRAC} at −80 mV to different concentrations of NECA (two examples for each concentration). Increasing NECA concentration tended to reduce the latency before the onset of the current, although this was somewhat variable between individual cells. In these experiments, the bath solution was supplemented with 500 nM of the protein kinase C inhibitor bisindolylmaleimide to prevent feedback inhibition of I_{CRAC}.

(B) Normalized I_{CRAC} amplitude as a function of NECA concentration. Each dot represents a single cell, and closed circles represent the means ± SEM (the two failures at 3 and 300 nM were excluded from the means). The fit to the mean data yielded an apparent K_D of 0.4 nM and a Hill coefficient of 4.

(C) Antigen stimulation in the absence of bisindolylmaleimide activates I_{CRAC} with smaller average amplitudes. Extracellular application of ionomycin (20 μM) cannot further increase I_{CRAC}. The recording was suspended between 310 and 470 s to change the application pipettes.

after NECA stimulation requires the presence of protein kinase C inhibitors like bisindolylmaleimide. Activation of antigen receptors in RBL cells reliably activates I_{CRAC}, suggesting less protein kinase C feedback after antigen stimulation (Parekh and Penner, 1995b). However, some inhibition is present because antigen-activated I_{CRAC} is around 50% smaller compared with that evoked by $InsP_3$, ionomycin, or NECA and bisindolylmaleimide (Parekh and Penner, 1995b). Because antigen maximally depletes the $InsP_3$ stores in RBL cells (Ali et al., 1995; data not shown), some inhibitory signal arising from activated receptors (like protein kinase C) is probably reducing the number of maximally active CRAC channels rather than antigen only partially depleting the $InsP_3$

stores and causing smaller I_{CRAC}. We therefore tried to increase further I_{CRAC} amplitude after stimulation of antigen receptors. Once I_{CRAC} had been activated with supramaximal antigen stimulation, we applied ionomycin to see whether we could increase I_{CRAC} to its maximal level. However, ionomycin failed to evoke any further I_{CRAC} (Figure 4C). This clearly demonstrates that receptor stimulation can evoke smaller I_{CRAC} despite maximal depletion of stores, and this arises because of receptor-evoked partial inhibition of CRAC channels. Submaximal Ca^{2+} influx is therefore not diagnostic of submaximal activation of I_{CRAC} because additional signals can reduce Ca^{2+} entry through maximally activated CRAC channels.

Graded influx has been observed in cell-population experiments (Demaurex et al., 1992; Hu et al., 1994), but interpretation of these experiments runs into several complications. First, many cell types respond in an all-or-none manner to receptor stimulation. Ca^{2+} release in rat hepatocytes (Chiavaroli et al., 1994), smooth muscles (Iino et al., 1993), and NIH-3T3 cells (Giovannardi et al., 1992) occurs in an all-or-none way to receptor stimulation. Crucially, different cells have different sensitivities to agonist (Giovannardi et al., 1992; Iino et al., 1993; Chiavaroli et al., 1994), so increasing agonist concentration may simply recruit more cells in an all-or-none manner, rather than all cells responding in a graded way. This vital information would be lost in population studies. Second, unless the membrane potential is clamped, graded influx can arise from graded changes in the electrical driving force for Ca^{2+} entry, as shown in Figure 2C. Third, protein kinase C inactivation of I_{CRAC} (Parekh and Penner, 1995b), as well as additional signals from an activated receptor, can grade the extent of Ca^{2+} entry. Finally, Ca^{2+} feedback inhibition on I_{CRAC} can also reduce the size of Ca^{2+} entry (Zweifach and Lewis, 1995).

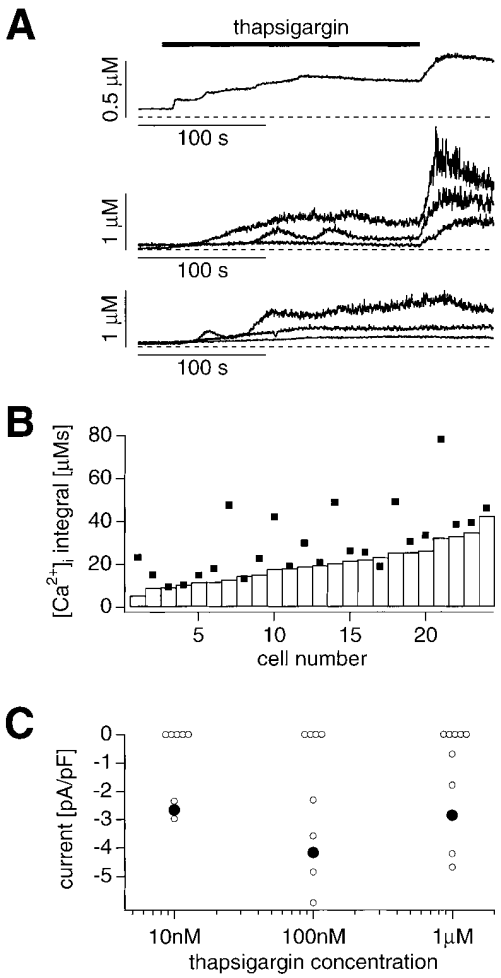

Figure 5. Relation between Ca^{2+} Release and Subsequent Ca^{2+} Influx by Thapsigargin Stimulation

(A) Examples of $[Ca^{2+}]_i$ signals evoked by thapsigargin in intact Fura 2 ester–loaded cells (three examples in each panel). Thapsigargin (1 μM) was applied from a puffer pipette in nominally Ca^{2+}-free external solution for the time indicated. Stopping the application reexposed the cells to the normal bath solution, which contained 2 mM Ca^{2+}. The middle panel illustrates examples of cells in which readmission of Ca^{2+} produced $[Ca^{2+}]_i$ elevations (off-response), whereas the bottom panel shows examples in which $[Ca^{2+}]_i$ did not rise or even decreased.

(B) Analysis of $[Ca^{2+}]_i$ changes experienced by individual cells following the protocol described in (A). The histogram bars reflect the integral of $[Ca^{2+}]_i$ over 60 s just prior to the readmission of external Ca^{2+}, whereas the closed squares reflect the integral over the first 60 s after readmission. Note the variability in the responses and the lack of correlation in the size of the thapsigargin effect and its ability to promote the off-response.

(C) Normalized I_{CRAC} current amplitudes as a function of thapsigargin concentration. Amplitudes of I_{CRAC} were determined by current recordings as described in Figures 1 and 2. The standard pipette solution had $[Ca^{2+}]_i$ buffered to about 90 nM. Data of individual cells are represented by open circles, and the average amplitudes of cells that produced an inward current are shown as closed circles.

Further Dissociation between Ca^{2+} Release and Influx: Thapsigargin

The ER Ca^{2+}-ATPase inhibitor thapsigargin has been reported to deplete stores and thereby activate capacitative Ca^{2+} influx independent of an elevation of $InsP_3$ (Thastrup et al., 1990). We therefore investigated the relationship between thapsigargin concentration and activaion of I_{CRAC}. The results are summarized in Figure 5. Including 1 μM thapsigargin in the patch pipette activated I_{CRAC} in 5 out of 9 cells (Figure 5C). The latency before onset of I_{CRAC} was 108.6 ± 48 s (range 8–280). When 100 nM thapsigargin was included in the pipette solution, 4 out of 8 cells responded, and this occurred after a latency of 160 ± 62 s (Figure 5C). With 10 nM thapsigargin, only 2 out of 7 cells reponded, and these 2 cells had latencies of 46 and 7 s, respectively. Again, just like with $InsP_3$, if a cell clearly responded, then it gave around the maximum I_{CRAC}. Interestingly, application of ionomycin to cells that had not responded to thapsigargin generated large I_{CRAC} (3/3 cells). These cells therefore did possess both the activation mechanism of I_{CRAC} and CRAC channels themselves. The inability of thapsigargin to activate the current presumably reflects its inability to deplete the stores in these cells despite the high concentrations used, although we cannot rule out that thapsigargin concentrations achieved by intracellular application might be lower than anticipated if thapsigargin diffused quickly out of the cell, owing to its lipophilicity.

We were surprised that these high concentrations of thapsigargin (1 μM) failed to activate routinely any I_{CRAC}, since application of 1 μM to single Fura ester–loaded

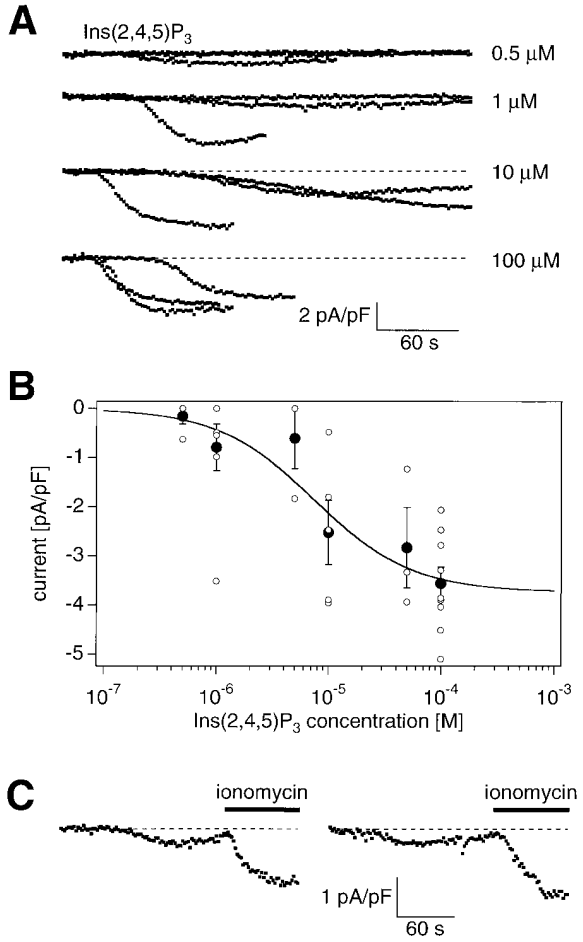

Figure 6. Graded Activation of I_{CRAC} by $Ins(2,4,5)P_3$

(A) Development of I_{CRAC} at −80 mV at different concentrations of intracellularly perfused $Ins(2,4,5)P_3$ (three examples for each concentration).

(B) Normalized I_{CRAC} amplitude as a function of $Ins(2,4,5)P_3$ concentration. Each dot represents a single cell, and closed circles represent the means ± SEM. The fit to the mean data yielded an apparent K_D of 7.5 μM and a Hill coefficient of 1.

(C) Two examples of cells in which low doses of $Ins(2,4,5)P_3$ caused graded submaximal activation of I_{CRAC}. Extracellular application of ionomycin (20 μM) caused a further increase in I_{CRAC}.

cells evoked substantial Ca^{2+} signals (peaks in the range 150 nM to 2 μM) in 109 out of 109 cells. Because substantial Ca^{2+} release to $InsP_3$ fails to activate I_{CRAC} (see above), we examined the relationship between Ca^{2+} release and Ca^{2+} influx to thapsigargin. By applying thapsigargin in Ca^{2+}-free solution and then readmitting Ca^{2+}, Ca^{2+} entry occurs because capacitative Ca^{2+} influx has been activated by thapsigargin and induces the off-response. Although the off-response does not simply reflect the degree of activation of the influx pathway, since membrane potential, Ca^{2+} buffering, and kinase activation can all affect the Ca^{2+} signal, it does provide a rough indication of the magnitude of Ca^{2+} influx.

The top panel of Figure 5A shows the averaged response of a total of 24 cells. A prominent off-response is observed. However, the behavior at the single-cell level was rather diverse. The middle panel of Figure 5A presents three examples of cells where a clear influx component was observed, while the bottom panel shows three other cells from the same coverslip, where Ca^{2+} levels did not increase after readmission of external Ca^{2+}, despite substantial Ca^{2+} release. In fact, a strong off-response was observed in only 7/24 cells, whereas all other cells from the same coverslips gave only minor or no off-responses at all. The averaged response is therefore quite misleading, since it does not reveal what is happening at the single-cell level. These results highlight the caution needed in interpreting Ca^{2+} signals from cell populations. Figure 5B quantifies the relationship between Ca^{2+} release and capacitative Ca^{2+} influx in all of the 24 cells. The bars depict the integral of the Ca^{2+} release phase, and the dots the size of the corresponding Ca^{2+} influx for each cell. Despite substantial Ca^{2+} release in some cells, there appears to be no influx, whereas in other cells a similar amount of Ca^{2+} release gives rise to large influx. The lack of influx was not due to kinase C block, because the kinase C inhibitor bisindolylmaleimide did not convert nonresponding cells into responding ones. However, we cannot rule out possible effects of thapsigargin on the membrane potential, although the Ca^{2+} measurements in intact cells and our patch-clamp recordings are complementary.

It would appear that thapsigargin is less effective than $InsP_3$ in activating I_{CRAC}, which indicates that CRAC stores are either less leaky or relatively thapsigargin insensitive. These results confirm and extend the results with $InsP_3$ and establish a dissociation between the amount of Ca^{2+} released and subsequent activation of Ca^{2+} influx. Few studies have examined the relationship between Ca^{2+} release and subsequent activation of store-operated Ca^{2+} influx. In endothelial cells, Jacob has observed a linear relationship between histamine-stimulated Ca^{2+} release and Ca^{2+} entry (Jacob, 1990). In his discussion, however, Jacob was careful to point out that: "No conclusions can be drawn from the fortuitous linear relationship between the two parameters" (namely Ca^{2+} release and Mn^{2+} entry) (Jacob, 1990, p. 73). Reasons for this difference between RBL and endothelial cells may be based on several facts. First, the patch-clamp technique allowed us to measure calcium entry directly by recording I_{CRAC}, whereas Jacob monitored entry indirectly using Mn^{2+} quenching of Fura 2. Furthermore, Ca^{2+} entry pathways, in addition to the store-operated one, coexist in endothelial cells ($InsP_4$-modulated Mn^{2+}-permeable channels and nonselective channels permeable to Ca^{2+} [Lückhoff and Clapham, 1992; Nilius et al., 1993]), resulting in a mixed Ca^{2+} entry signal. Also, changes in electrical driving force for Ca^{2+} could occur in the endothelial cells, since the cells were not voltage clamped, and this would therefore change the amount of Ca^{2+} or Mn^{2+} that enters the cell. Finally, the size of the functionally distinct Ca^{2+} pools might differ in different cell types: in RBL cells, the pools are clearly distinct, whereas in endothelial cells they may overlap appreciably.

The Nonlinear Activation Arises from $InsP_3$ Metabolism

The highly nonlinear activation of I_{CRAC} could reside either in a step prior to the binding of $InsP_3$ to its receptor

($InsP_3$ metabolism) or in the activation mechanism itself. To distinguish between these two possibilities, we took advantage of the nonphysiological $InsP_3$ isomer inositol 2,4,5-trisphosphate (Ins[2,4,5]P_3), which activates $InsP_3$ receptors but is relatively resistant to metabolic degradation (Bird et al., 1991). Figure 6A shows current records from individual cells that were dialyzed with different concentrations of Ins(2,4,5)P_3. Graded I_{CRAC} can easily be discerned. Figure 6B plots the relationship between Ins(2,4,5)P_3 concentration and the peak amplitude of I_{CRAC} measured at −80 mV in 30 cells. Each point represents a single cell. With this $InsP_3$ analog, we were able to record small I_{CRAC} with low doses, which was not the case with $InsP_3$. The resulting curve is not as steep as that for $InsP_3$. In fact, the dose-response curve had a Hill coefficient of 1, although this was somewhat complicated by the inclusion of all nonresponding cells. The small I_{CRAC} to low concentrations of Ins(2,4,5)P_3 could be increased by subsequent application of ionomycin (Figure 6C), ruling out possible partial agonist effects of Ins(2,4,5)P_3 or that low doses were fully depleting stores but somehow additionally reducing I_{CRAC} amplitude.

The major site of cooperativity therefore likely arises from metabolism of $InsP_3$. Both the enzymes that metabolize $InsP_3$ have high affinity for the substrate, which is required to account for the observed steep relationship. The 5-phosphatase has a K_m of 100 nM, and the 3-kinase one of 1 μM (Shears, 1992), and these values are in the range of the present study's K_d for activation of I_{CRAC} with $InsP_3$. Our results suggest that, following production of $InsP_3$, activation of I_{CRAC} will not occur until the $InsP_3$ has saturated the metabolizing enzymes. Once this has occurred, even a small increase in $InsP_3$ concentration will result in a substantial increase in the free $InsP_3$ and, therefore, maximal activation of I_{CRAC}.

Conclusions

Our results clearly demonstrate that, first, activation of I_{CRAC} is a highly nonlinear function of the $InsP_3$ concentration and, second, Ca^{2+} release from $InsP_3$ stores is not tightly coupled to activation of I_{CRAC}. We suggest that partial depletion of a homogenous population of $InsP_3$ stores or full depletion of one set of multiple $InsP_3$ stores does not activate I_{CRAC}. Instead, a functionally distinct $InsP_3$ store might be involved in activation of store-operated Ca^{2+} influx. This store is characterized by an apparently lower sensitivity to $InsP_3$, presumably owing to stronger metabolism, which effectively reduces the free $InsP_3$ concentration seen by the $InsP_3$ receptors in that store. It seems reasonable to assume that this store is close to the plasma membrane (Parekh and Penner, 1995a). In rat hepatocytes (Chiavaroli et al., 1994), smooth muscle (Iino et al., 1993), and NIH-3T3 cells (Giovannardi et al., 1992), receptor-induced Ca^{2+} release is an all-or-none process, as is $InsP_3$-mediated release in mast cells (Neher, 1989). Strikingly, all these cell types have prominent capacitative Ca^{2+} influx. It is therefore very likely that Ca^{2+} influx in these cells will also be an all-or-none phenomenon. The highly nonlinear activation of I_{CRAC} in response to $InsP_3$ we have observed may therefore be found in a variety of nonexcitable cells. Our results can also explain certain puzzling aspects of Ca^{2+} oscillations. During baseline spiking to cholecystokinin in acinar cells (Yule et al., 1991), increasing agonist concentration increases the frequency of the oscillations but not the amplitude. These oscillations are large but are completely independent of extracellular Ca^{2+}. However, a substantial fraction of released Ca^{2+} is extruded from the cell during each Ca^{2+} oscillation, so the stores should lose some Ca^{2+} after each Ca^{2+} spike (Tepikin et al., 1993). Hence, partial depletion of the stores evokes no Ca^{2+} influx at all. A small further increase in agonist concentration suddenly evokes large Ca^{2+} influx. The nonlinear relationship between Ca^{2+} release and subsequent activation of I_{CRAC} that we have observed provides an attractive explanation for these results, since partial emptying of the stores with each spike is insufficient to activate I_{CRAC}.

Although activation of I_{CRAC} under physiological conditions appears to be an all-or-none process, this does not mean that I_{CRAC} or Ca^{2+} influx is not graded. Our results with antigen-receptor stimulation clearly show reduced I_{CRAC} despite maximal depletion of stores. But this arises because of inhibitory signals (like protein kinase C) that reduce maximal CRAC channel activity. This also means that stimulation of different receptors (or different concentrations of one agonist) can evoke different sizes of I_{CRAC} owing to different levels of activation of the inhibitory signal. A further way to achieve graded influx despite maximal depletion of stores and full activation of I_{CRAC} is by changing the membrane potential. Simply altering the electrical gradient for Ca^{2+} entry can evoke graded Ca^{2+} influx. Since many nonexcitable cells have Ca^{2+}- and/or second messenger–activated conductances, there is ample room for modulation of Ca^{2+} entry to produce graded $[Ca^{2+}]_i$ signals. Since I_{CRAC} itself is a small and Ca^{2+}-selective current, it can raise cytosolic Ca^{2+} substantially, but it will not depolarize the cell much itself. This makes it an ideal Ca^{2+} influx pathway, as opposed to a nonselective cation pathway, which would itself depolarize the cell substantially and limit its Ca^{2+} transport capacity.

Experimental Procedures

Rat basophilic leukemia cells (RBL-1) were cultured on glass coverslips with Dulbecco modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum, 45 mM $NaHCO_3$, 5 mM glucose, 0.12 mg/ml streptomycin, and 0.60 mg/ml penicillin. For experiments, coverslips were transferred to the recording chamber and kept in a Ringer's solution of the following composition: 145 mM NaCl, 2.8 mM KCl, 10 mM CsCl, 10 mM $CaCl_2$, 2 mM $MgCl_2$, 10 mM glucose, 10 mM HEPES–NaOH (pH 7.2). Sylgard-coated patch pipettes had resistances between 2–4 MΩ after filling with the standard intracellular solution that contained the following: 145 mM Cs–glutamate, 8 mM NaCl, 1 mM $MgCl_2$, 2 mM Mg–ATP, 10 mM EGTA ($[Ca^{2+}]_i$ clamped to about 90 nM by a Ca–EGTA/EGTA mixture of 3.3/6.7 mM) (pH 7.2 adjusted with CsOH). Inositol 1,4,5-trisphosphate (Amersham) or Inositol 2,4,5-trisphosphate (Calbiochem) were added to the pipette solution at the indicated concentrations. When receptors were stimulated by either NECA or antigen, 200 μM GTP was added to the standard pipette solution. NECA was purchased from Sigma, and antigen stimulation was performed in cells sensitized by overnight preincubation with 2 μg/ml IgE and stimulation with 1 μg/ml DNP–BSA as described (Parekh and Penner, 1995b). Bisindolylmaleimide (Calbiochem) was added to the bath solution at 500 nM, and ionomycin (Calbiochem) was applied at 20 μM.

Patch-clamp experiments were performed in the tight-seal whole-cell configuration at 21°C–25°C. High resolution current recordings

were acquired by a computer-based patch-clamp amplifier system (EPC-9; HEKA, Lambrecht, Germany). Holding potential was usually 0 mV unless otherwise indicated. The development of I_{CRAC} over time was assessed by measuring the current amplitudes at a potential of −80 mV, taken from high resolution currents in response to voltage ramps spanning the voltage range between −100 to +100 mV over a period of 50 ms, and delivered at a rate of 0.5–1 Hz. All voltages were corrected for a liquid-junction potential of 10 mV between external and internal solutions. Currents were filtered at 2.3 kHz and digitized at 100 μs intervals. Capacitive currents and series resistance were determined and corrected before each voltage ramp using the automatic capacitance compensation of the EPC-9. For analysis, the very first ramps before activation of I_{CRAC} were digitally filtered at 1 kHz, pooled, and used for leak subtraction of the subsequent current records.

The concentration of $[Ca^{2+}]_i$ was monitored at a rate of 5 Hz with a photomultiplier-based system as described (Neher, 1989). Cells were loaded with 100–200 μM Fura 2 by diffusion from the patch pipette, and $[Ca^{2+}]_i$ was calculated from the fluorescence ratio at 2 excitation wavelengths (360/390 nm). Ester loading of intact cells was performed by incubation of cells in normal Ringer's solution (2 mM Ca^{2+}) supplemented with 2 μM Fura 2–AM for 30 min.

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