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The Wellcome Prize Lecture

Store-operated Ca^{2+} entry: dynamic interplay between endoplasmic reticulum, mitochondria and plasma membrane

Anant B. Parekh

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

In eukaryotic cells, hormones and neurotransmitters that engage the phosphoinositide pathway evoke a biphasic increase in intracellular free Ca^{2+} concentration: an initial transient release of Ca^{2+} from intracellular stores is followed by a sustained phase of Ca^{2+} influx. This influx is generally store-dependent and is required for controlling a host of Ca^{2+}-dependent processes ranging from exocytosis to cell growth and proliferation. In many cell types, store-operated Ca^{2+} entry is manifest as a non-voltage-gated Ca^{2+} current called I_{CRAC} (Ca^{2+} release-activated Ca^{2+} current). Just how store emptying activates CRAC channels remains unclear, and some of our recent experiments that address this issue will be described. No less important from a physiological perspective is the weak Ca^{2+} buffer paradox: whereas macroscopic (whole cell) I_{CRAC} can be measured routinely in the presence of strong intracellular Ca^{2+} buffer, the current is generally not detectable under physiological conditions of weak buffering following store emptying with the second messenger InsP_3. In this review, I describe some of our experiments aimed at understanding just why InsP_3 is ineffective under these conditions and which lead us to conclude that respiring mitochondria are essential for the activation of I_{CRAC} in weak intracellular Ca^{2+} buffer. Mitochondrial Ca^{2+} uptake also increases the dynamic range over which InsP_3 functions as the second messenger that controls Ca^{2+} influx. Finally, we find that Ca^{2+}-dependent slow inactivation of Ca^{2+} influx, a widespread but poorly understood phenomenon that helps shape the profile of an intracellular Ca^{2+} signal, is regulated by mitochondrial Ca^{2+} buffering. Thus, by enabling macroscopic store-operated Ca^{2+} current to activate and then by controlling its extent and duration, mitochondria play a crucial role in all stages of store-operated Ca^{2+} influx. Store-operated Ca^{2+} entry reflects therefore a dynamic interplay between endoplasmic reticulum, mitochondria and plasma membrane.

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Correspondence A. B. Parekh: Department of Physiology, University of Oxford, Parks Road, Oxford OX1 3PT, UK.
Email: anant.parekh@physiol.ox.ac.uk

An increase in intracellular free Ca^{2+} concentration is a ubiquitous signalling mechanism that regulates a broad spectrum of kinetically disparate processes ranging from exocytosis to cell growth and proliferation (Carafoli, 2002). Two major ways whereby eukaryotic cells can increase their cytosolic Ca^{2+} concentration are release of the ions from intracellular stores (mainly the endoplasmic/sarcoplasmic reticulum) or entry of Ca^{2+} into the cell from the external solution (Berridge, 1993; Pozzan *et al.* 1994). Because the intracellular stores have a limited capacity, Ca^{2+} entry into the cell down an enormous electrochemical potential gradient is important for supporting the activities of numerous Ca^{2+}-dependent processes. In excitable tissues, like nerve and muscle, Ca^{2+} entry is accomplished by the opening of Ca^{2+}-permeable ion channels (voltage- and ligand-gated; Burnashev, 1998; Catterall, 2000). Much is known about the biophysical properties of these proteins, their stoichiometry, and even the identity of amino acids that are crucial for key channel functions like selectivity and gating (Burnashev, 1998; Catterall, 2000; Hille, 2001).

However, until relatively recently, our understanding of how Ca^{2+} enters non-excitable cells has been weak. Non-excitable cells are cells that do not fire action potentials and include the cells of the immune system, endothelia lining the blood vessels, epithelia that line the respiratory and digestive tracts, and glial cells in the brain. In these cell types, Ca^{2+} entry is essential for maintaining normal cell function but voltage-operated Ca^{2+} channels tend not to be

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expressed and ligand-gated Ca^{2+}-permeable channels are sparse. Instead, a major route for Ca^{2+} influx in these cells is via store-operated Ca^{2+} channels (Parekh & Penner, 1997).

Store-operated Ca^{2+} influx

In an erudite paper published in 1986, James Putney proposed a model for Ca^{2+} influx in non-excitable cells, which he called capacitative Ca^{2+} entry (Putney, 1986). The fundamental tenet of this model was that the Ca^{2+} entry pathway in the plasma membrane was controlled by the Ca^{2+} content of the intracellular stores. As the store Ca^{2+} content fell, a signal was somehow sent from the stores which caused the Ca^{2+} entry pathway to open (now referred to as store-operated Ca^{2+} entry). Although indirect evidence began to accrete in support of Putney's model, it was not until 1992 that Reinhold Penner directly demonstrated, in an elegant series of experiments, that Ca^{2+} store depletion resulted in the activation of a Ca^{2+}-selective current which was called I_{CRAC} (Hoth & Penner, 1992). Ca^{2+} entry through store-operated Ca^{2+} channels is important for regulating a host of temporally diverse processes from exocytosis and enzymatic activity to gene transcription and cell proliferation (Parekh & Penner, 1997; Putney & McKay, 1999). Aberrant store-operated entry has been reported to underlie debilitating human diseases like certain primary immunodeficiencies (Partiseti *et al.* 1994) and acute pancreatitis (Raraty *et al.* 2000; Parekh, 2000). It now seems clear that store-operated Ca^{2+} channels are not a homogeneous class but instead reflect a family with different biophysical properties. The best characterised store-operated current is I_{CRAC}, which is found in several types of cell including mast cells, lymphocytes, macrophages, megakaryocytes and hepatocytes (Parekh & Penner, 1997; Lewis, 1999).

Although store-operated entry is a widespread Ca^{2+} influx pathway, it is by no means the only route for Ca^{2+} entry in non-excitable cells. In addition to a range of second-messenger operated Ca^{2+}-permeable channels (activated by Ca^{2+}, InsP_3/InsP_4, cyclic nucleotides; reviewed in Clapham, 1995), growing evidence indicates that a Ca^{2+} influx pathway controlled by arachidonic acid might be prominent under certain conditions (reviewed in Taylor, 2002).

In this Wellcome Prize Lecture review, I shall focus on some of our work over the past several years that has been aimed at understanding the mechanisms that control I_{CRAC}. It is, as befits a prize lecture, a subjective account and is not a general review of the literature. Because of space

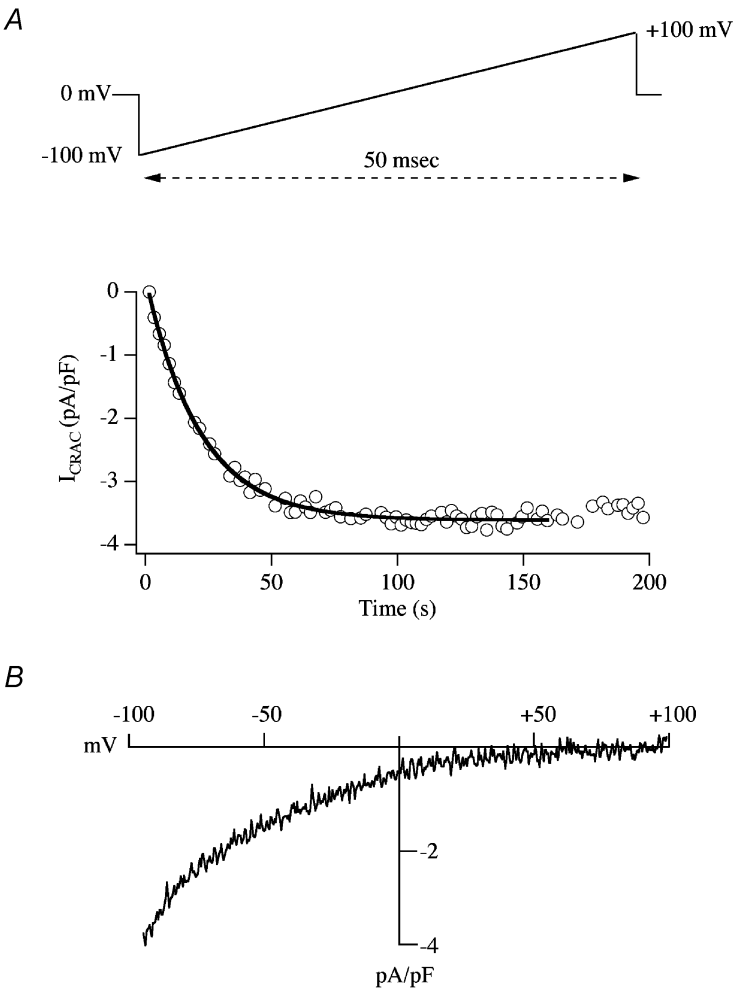

Figure 1. Development of I_{CRAC} following store depletion

A, the upper panel shows the typical voltage ramp protocol used to monitor I_{CRAC} in mast cells, the related rat basophilic leukaemia (RBL-1) cell line and jurkat T-lymphocytes. The ramp spans –100 to +100 mV in 50 ms, and is applied from a holding potential of 0 mV once every 2 s. The lower panel depicts the time course of activation of I_{CRAC}. The current amplitude, measured at –80 mV from each ramp, has been normalised for cell size by dividing the current by cell capacitance. The thick line shows a mono-exponential fit to the activation time course. *B* shows the current–voltage relationship, once the current had reached steady state. Note the inward rectification and very positive reversal potential, characteristic of I_{CRAC}. The pipette solution is caesium glutamate based, supplemented with InsP_3 and 10 mM EGTA.

constraints, certain papers have not been cited and I apologise to those authors for this.

Basic features of I_{CRAC}

CRAC channels are activated by the process of emptying the intracellular Ca^{2+} stores. It is of little consequence how the stores are actually emptied, the net result is the same (Hoth & Penner, 1992; Parekh & Penner, 1997). Physiologically, stores are emptied following cell-surface receptor stimulation which increases the levels of the Ca^{2+} releasing messenger InsP_3. Experimentally, stores can be emptied by increasing net Ca^{2+} flux out of the stores following application of receptor agonists, increasing intracellular InsP_3 by dialysis with a patch pipette or by exposure to Ca^{2+} ionophores. Alternatively, suppressing Ca^{2+} re-uptake into the stores allows the endogenous Ca^{2+} leak pathway to deplete the stores. Inhibition of store refilling can occur by exposure to thapsigargin, a potent inhibitor of the sarcoplasmic/endoplasmic reticular Ca^{2+} ATPase (SERCA) pumps on the stores.

The Ca^{2+} flux through CRAC channels gives rise to the small Ca^{2+} current I_{CRAC}, which can be measured directly and unambiguously using the whole cell patch clamp technique. With Ca^{2+} as the charge carrier, I_{CRAC} activates mono-exponentially (time constant of around 20 s; Fig. 1*A*) and is a non-voltage-gated, inwardly rectifying current with a very positive reversal potential (> +60 mV; Fig. 1*B*), the latter indicating high selectivity for Ca^{2+}. Although Na^+ ions outnumber Ca^{2+} by 70 : 1 in the external solution, neither the extent of I_{CRAC} nor the positive reversal potential (still > +60 mV) appear to be altered by substituting external Na^+ for organic cations like $NMDG^+$ in the presence of physiological levels of external Ca^{2+} (1–2 mM) (Fierro & Parekh, 2000). Moreover, removal of external Ca^{2+} in the continuous presence of external Na^+ and Mg^{2+} abolishes the current completely (Hoth & Penner, 1992; Zweifach & Lewis, 1993; Fierro & Parekh, 2000). Because of the lack of a clear reversal potential for I_{CRAC} with Ca^{2+} as the charge carrier, the permeability ratio of Ca^{2+} to Na^+ (P_{Ca}/P_{Na}) has been hard to establish. Using the 'added buffer method', where cells are loaded with sufficient Fura-2 to capture all the incoming Ca^{2+} through CRAC channels, it has been estimated that CRAC channels are as selective for Ca^{2+} as their voltage-operated counterparts (Hoth, 1995), the latter exhibiting a P_{Ca}/P_{Na} of 1000. However, CRAC channels discriminate between various divalent cations, with Ba^{2+} and Sr^{2+} supporting less current than Ca^{2+} (Hoth & Penner, 1992; Zweifach & Lewis, 1993; Fierro & Parekh, 1999*a*). The single CRAC channel conductance is low. Stationary noise analysis has estimated it to be well below 1 pS, beyond the current level of detection (Hoth & Penner, 1992; Zweifach & Lewis, 1993). A consequence of this is that there are estimated to be at least 5000 functional CRAC channels in the plasma membrane following stimulation.

Activation mechanism

The unique gating of I_{CRAC} has made it an attractive target of investigation for the identification of new signal transduction pathways. CRAC channels are activated by the emptying of intracellular Ca^{2+} stores. But just how a reduction in store content is translated into opening of these channels remains unclear. A sensor within the stores presumably detects the fall in Ca^{2+} content and this then initiates a process resulting in a signal being sent from the stores to the channels in the plasma membrane. Virtually nothing is known about the identity of the sensor, other than it is probably not calreticulin (Fasolato *et al.* 1998), the major intraluminal Ca^{2+} binding protein of non-muscle cells. As for the activation signal, three models have been put forward (see Fig. 2; reviewed in Parekh & Penner, 1997; Putney & McKay, 1999): (1) the vesicular fusion hypothesis (in which CRAC channels are inserted into the plasma membrane upon store depletion via an exocytotic mechanism; Yao *et al.* 1999; Alderton *et al.* 2000); (2) conformational coupling (where InsP_3 receptors on the stores are physically attached to CRAC channels in the plasma membrane; Berridge, 1995; Kiselyov *et al.* 1999), including the related secretion-like coupling model (where peripheral endoplasmic reticulum (ER) moves to the plasma membrane upon store depletion so that coupling can take place and this movement is regulated by the peripheral cytoskeleton; Patterson *et al.* 1999; Rosado *et al.* 2000), and (3) the diffusible messenger model (in which a small mobile factor is released from the stores upon their emptying and which then directly opens CRAC channels; Ramdriamampita & Tsien, 1993; Csutora *et al.* 1999).

The vesicular fusion model does not provide any insight into the activation signal itself. It postulates instead that this signal results in the insertion of CRAC channels into the plasma membrane. The fusion model therefore could be accommodated within either of the other two contemporary models. However, we found that dialysing the cytosol with small GTP-binding proteins known to be involved in vesicular trafficking (Parekh & Penner, 1996), interfering with GTP-binding protein function (Fierro & Parekh, 1999*b*) or exposing cells to either clostridial neurotoxins or to recombinant proteins that suppress exocytosis (in collaboration with Professor Burgoyne, Liverpool) all fail to affect the activation of I_{CRAC}, findings that are not readily reconcilable with a fusion mechanism. The secretion-like conformational coupling model predicts that, first, intimate interactions between the ER and plasma membrane are essential for I_{CRAC} to activate, second, InsP_3 receptors are required for all stages of store-operated Ca^{2+} entry, and third, that interfering with the peripheral cytoskeleton should impair the ability of I_{CRAC} to activate. We have tested these predictions in rat basophilic leukaemia (RBL-1) cells (Bakowski *et al.* 2001). Inflating the cell by applying positive pressure to the patch

pipette (cell ballooning) did not interefere with the ability of CRAC channels to activate, in spite of some rather marked changes in the cellular architecture (Bakowski *et al.* 2001). A variety of InsP_3 receptor inhibitors did not affect I_{CRAC} development, in spite of occupying InsP_3 receptors, and pharmacological tools directed towards re-shaping the cytoskeleton consistently failed to interfere with the activation of I_{CRAC} (Bakowski *et al.* 2001). Hence, a secretion-like coupling model is probably not a viable candidate for the activation of CRAC channels in RBL-1 cells. As for the diffusible messenger hypothesis, no convincing candidate has yet been identified. Recently, we have found that inhibition of the lipoxygenase family of enzymes impairs the activation of I_{CRAC} (Glitsch *et al.* 2002*a*). The inhibitors are much less effective if administered after CRAC channels have been activated. Such findings raise the intriguing possibility that a lipoxygenase might be involved in the activation mechanism, although, as discussed in Glitsch *et al.* (2002), such a conclusion is not unequivocal as it is based on a pharmacological approach.

Clearly, the ability to study single CRAC channel currents in an inside-out patch would greatly facilitate identification of key regulatory molecules involved in channel gating, as would the identification of the CRAC channel gene(s). It was recently suggested that the protein TRPV6 comprises all or part of the CRAC channel pore (Yue *et al.* 2001). But work by us and others suggests that this is probably not the case (Bakowski & Parekh, 2002*a*,*b*; Hermosura *et al.* 2002; Prakriya & Lewis, 2002). Understanding the molecular identity of the CRAC channel as well as the activation signal remain outstanding questions in the field.

The weak buffer paradox

Another major problem concerning I_{CRAC} is the weak Ca^{2+} buffer paradox. Whereas I_{CRAC} can be activated maximally following whole cell dialysis with a patch pipette containing InsP_3 and strong buffer (10 mM EGTA), the current generally fails to develop in the presence of weak Ca^{2+} buffer (0.1 mM EGTA; Fig. 3, modified from Bakowski & Parekh, 2001; see also Broad *et al.* 1999; Fierro & Parekh, 2000). Such low concentrations of EGTA are only slightly larger than the levels of endogenous mobile Ca^{2+} buffers (Zhou & Neher, 1993). InsP_3 is able to release Ca^{2+} from the stores under these conditions (Parekh *et al.* 1997). Hence the inability of InsP_3 to activate I_{CRAC} in weak Ca^{2+} buffer does not reflect a failure to mobilise Ca^{2+} from the stores. Clearly, resolution of why InsP_3 is so effete in weak buffer is essential if we are to place I_{CRAC} in any kind of physiological context.

The first explanation that we considered was that the inability of InsP_3 to activate I_{CRAC} in weak Ca^{2+} buffer was an idiosyncrasy of our recording conditions. Like most electrophysiologists, we routinely work at room temperature. To see whether InsP_3 was effective at physiological temperature, we compared the effects of InsP_3 in weak *versus* strong buffer at 36°C. However, InsP_3 was still ineffective in weak buffer (Fig. 4*A* and *B*, modified from

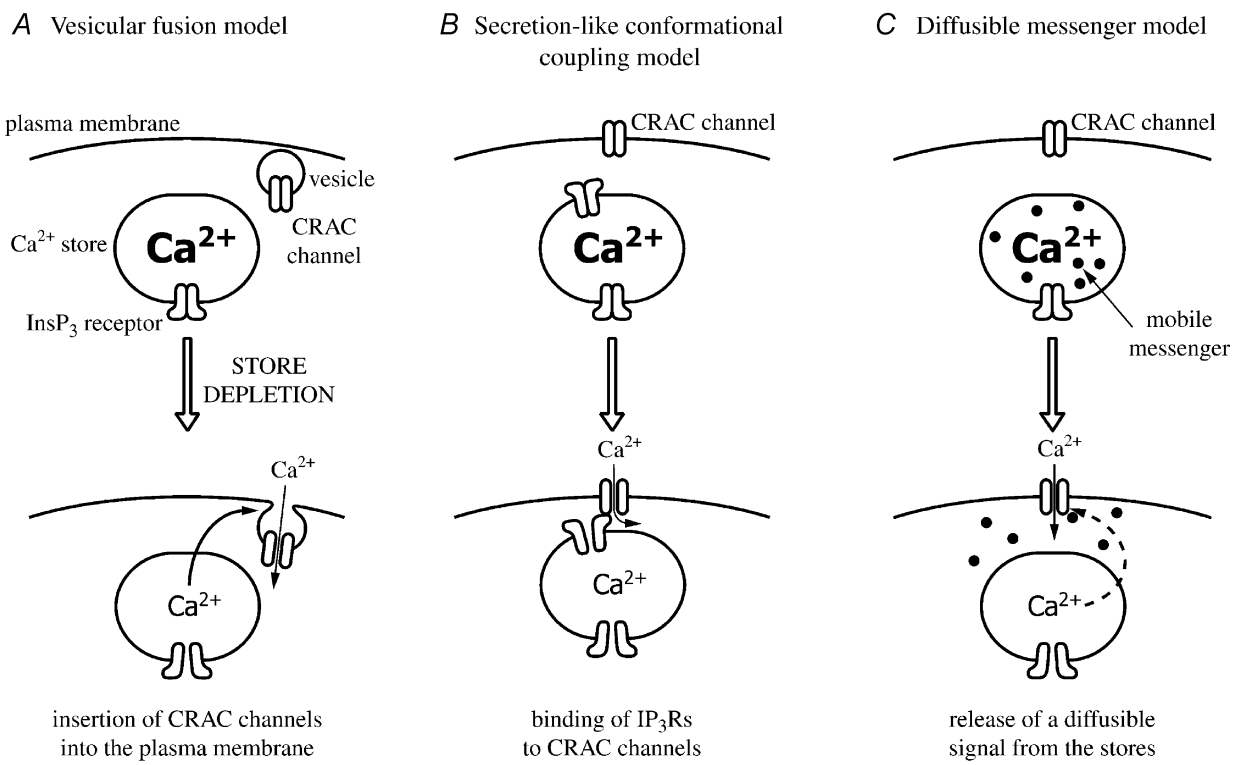

Figure 2. The three contemporary models proposed to account for the activation mechanism of I_{CRAC}

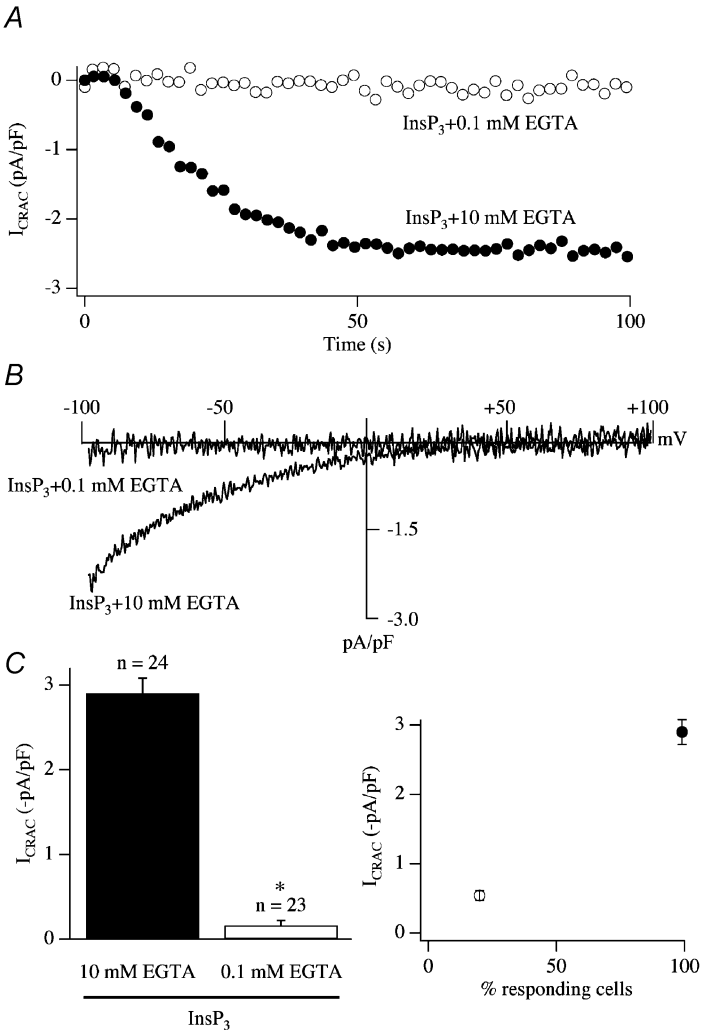

Figure 3. The weak buffer paradox

A, InsP_3 generally fails to activate I_{CRAC} in weak intracellular Ca^{2+} buffer (0.1 mM EGTA) whereas it is effective in strong buffer (10 mM EGTA). *B*, current–voltage relationships for the two recordings from *A*, taken at steady state. *C*, the mean amplitude of I_{CRAC} (left-hand panel) and the fraction of cells responding (right-hand panel) for each condition is shown. In this and all subsequent figures, data from RBL-1 cells are shown. $^*P < 0.01$.

Fierro *et al.* 2000). We also systematically altered the composition of the pipette solution to see whether we could render InsP_3 more effective. However, InsP_3 was still largely ineffective in weak buffer in either a Cs^+- or K^+-based pipette solution, when Cl^- was the dominant anion or when EGTA was replaced by BAPTA or dimethyl BAPTA (Fierro & Parekh, 2000; Gilabert & Parekh, 2000). Non-metabolisable analogues of InsP_3 were also incapable

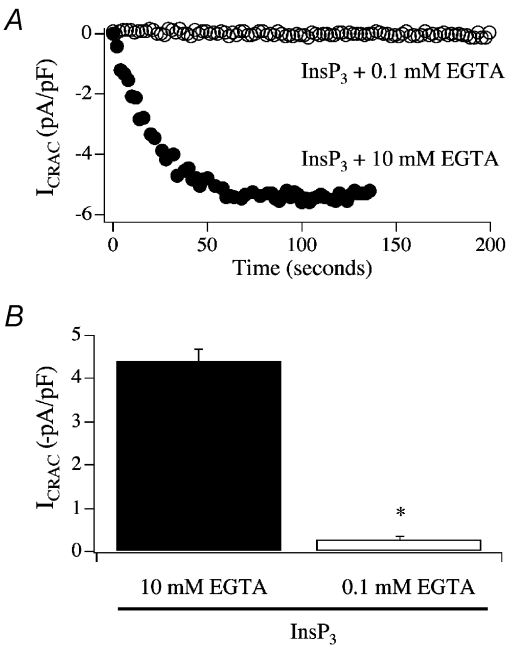

Figure 4. InsP_3 still fails to activate I_{CRAC} in weak Ca^{2+} buffer at physiological temperature

Experiments were carried out at 36 °C. *A*, whereas InsP_3 evoked a large I_{CRAC} at 36 °C in 10 mM EGTA, no current was detectable in 0.1 mM EGTA. *B*, aggregate data are summarised. $^*P < 0.01$ (Student's *t* test).

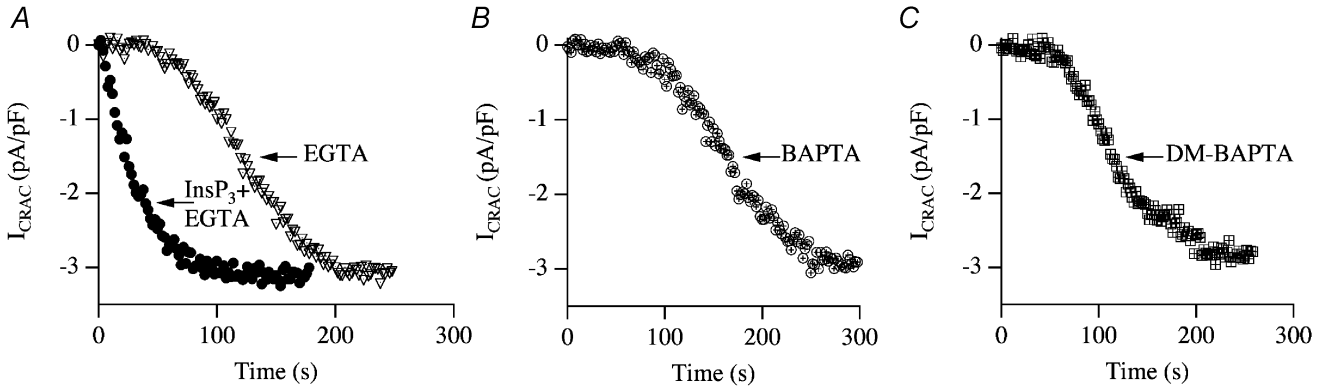

Figure 5. Passive activation of I_{CRAC}

A, comparison of the time course of development of I_{CRAC} following dialysis with either 10 mM EGTA alone or 30 μM InsP_3 + EGTA. Note the initial slow development of I_{CRAC} followed by a second, faster phase in response to EGTA alone. *B*, development of I_{CRAC} following dialysis with 10 mM BAPTA or dimethyl BAPTA (panel *C*).

of routinely generating the current, as was receptor stimulation, the latter being used as a physiological route to generate InsP_3.

Passive activation of I_{CRAC}

An important clue came from experiments designed to try to understand the mechanism underlying activation of I_{CRAC} following passive depletion of stores (Fierro & Parekh, 1999*c*). Dialysis with a high concentration of Ca^{2+} chelator alone (10 mM EGTA or BAPTA) is sufficient to maximally activate I_{CRAC}, a process referred to as passive depletion of stores (Hoth & Penner, 1992). Passive depletion is thought to rely only on the endogenous leak of Ca^{2+} from the stores. In strong buffer (several millimolar chelator), cytoplasmic Ca^{2+} is strongly clamped at very low levels, so as Ca^{2+} leaks out of the stores it is captured by the chelator and cannot therefore be taken back up. Stores will be drained gradually of Ca^{2+} and hence empty sufficiently for I_{CRAC} to activate (passive activation). Figure 5*A–C* shows the pattern of development of I_{CRAC} following dialysis with 10 mM of EGTA, BAPTA or dimethyl BAPTA. Superimposed on the EGTA trace in Fig. 5*A* is a recording from a cell in which InsP_3 was also included in the pipette. With InsP_3, I_{CRAC} activated rapidly and mono-exponentially. In marked contrast, the pattern of development of the current to passive depletion was much slower than that seen with InsP_3. With EGTA (Fig. 5*A*), BAPTA (Fig. 5*B*) or dimethyl BAPTA (Fig. 5*C*), the development of the current was kinetically complex: after a sizeable delay of around 60 s, I_{CRAC} initially activated slowly (first phase of development), followed by a faster developing phase (second phase of

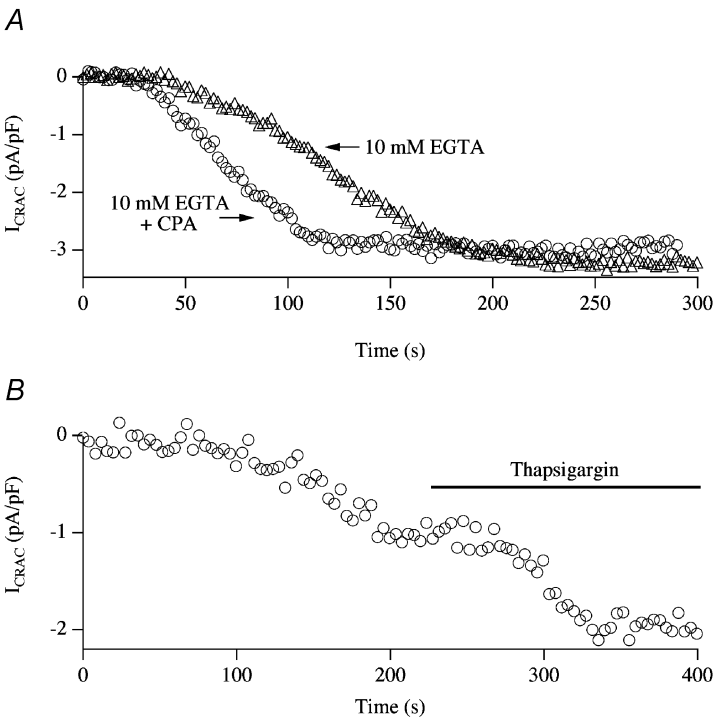

Figure 6. SERCA pumps shape the pattern of passive activation of I_{CRAC}

A, inclusion of cyclopiazonic acid (CPA) eliminates the initial slow phase of current development, leaving the second component intact. *B*, following dialysis with 2.5 mM EGTA, I_{CRAC} activated partially in this cell. Subsequent application of thapsigargin increased the extent of the current, as well as its rate of development.

development). We analysed the various features of the current (delay, rate of development of the two phases as well as their durations, their relative contributions to total current amplitude) and these were not significantly different between the chelators (Fierro & Parekh, 1999*b*). These chelators have apparent equilibrium dissociation constants of 150, 225 and 150 nM, respectively, and on-rates of 1.5×10^6 M^{-1} s^{-1} for EGTA and 5×10^8 M^{-1} s^{-1} for BAPTA and dimethyl BAPTA at pH 7.2. Hence neither the speed of binding Ca^{2+} nor the equilibrium affinity of the chelators accounts for the complex kinetic profile.

Importance of SERCA pumps

We found that SERCA pumps were extremely influential in shaping the pattern of development of I_{CRAC} following passive store depletion (Fierro & Parekh, 1999*c*). When SERCA pumps were inhibited with either cyclopiazonic acid or thapsigargin, the kinetics of I_{CRAC} changed dramatically compared with the current activated in 10 mM EGTA alone (Fig. 6*A*). The biphasic nature of the current (initial slow phase followed by a second faster phase), which was always seen with passive depletion using 10 mM EGTA, was not present when SERCA pumps were blocked. Now, the intial slow component was lost and instead the rapid phase developed in isolation. The slow initial activation therefore reflects a dynamic interplay between Ca^{2+} leakage from the stores and SERCA pump-mediated Ca^{2+} reuptake. The Ca^{2+} pumps help shape the profile of I_{CRAC} development, even in 10 mM EGTA.

For lower concentrations of EGTA (1–2.5 mM), the effects of SERCA pumps were even more striking. Following dialysis with EGTA < 2.5 mM, I_{CRAC} activated slowly and mono-phasically to reach a submaximal steady-state amplitude (Fig. 6*B*). Once the small current had reached steady-state, application of the SERCA pump blocker thapsigargin increased the size of the current further. This demonstrates that the submaximal I_{CRAC} reflected a steady state between Ca^{2+} efflux from the stores and reuptake by SERCA pump activity (Fierro & Parekh, 1999*b*). Moreover, even in the presence of a relatively high concentration of EGTA (2.5 mM), SERCA pumps were still active and were able to resequester Ca^{2+} into the stores.

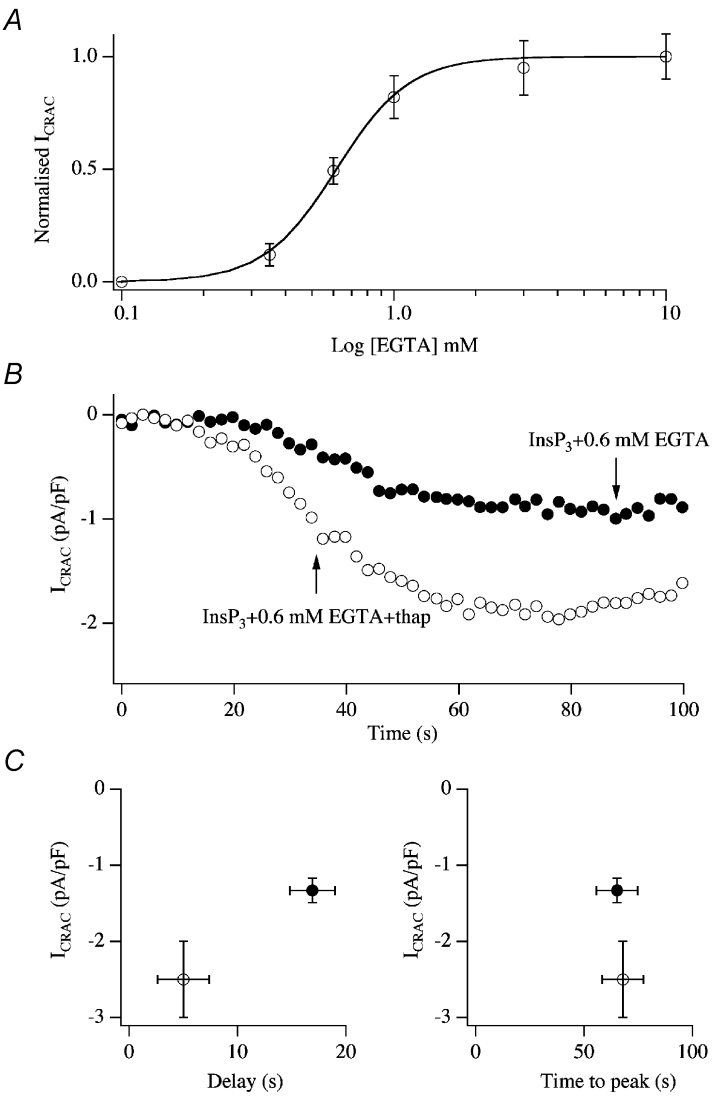

Figure 7. SERCA pumps function in the presence of InsP_3

A, the relationship between intrapipette EGTA concentration and amplitude of I_{CRAC} (evoked by dialysis with 30 μM InsP_3) is plotted. 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*, recordings are shown for a cell dialysed with 30 μM InsP_3 and 0.6 mM EGTA (●) and for one dialysed with this solution but supplemented with 2 μM thapsigargin, a SERCA blocker (○). Note the increase in the size of I_{CRAC} following inhibition of the pumps. *C*, the amplitudes, delays and times to peak are summarised for experiments as in *B*. Filled circles denote InsP_3 + 0.6 mM EGTA whereas open cirlces represent InsP_3 + 0.6 mM EGTA + thapsigargin.

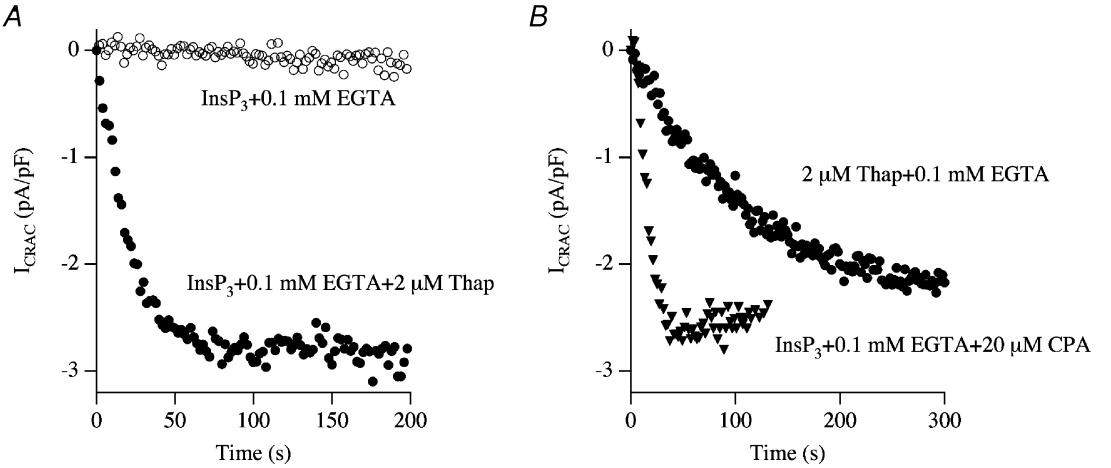

Figure 8. InsP_3 activates I_{CRAC} in weak buffer provided SERCA pump activity is compromised

A, InsP_3 together with thapsigargin activates I_{CRAC} in weak buffer whereas InsP_3 alone is ineffective. *B*, InsP_3 with cyclopiazonic acid, a structurally distinct SERCA pump blocker, is also effective. SERCA pump block alone (2 μM Thap + 0.1 mM EGTA) can also activate I_{CRAC}, albeit at a much slower rate.

SERCA and InsP_3

Although the preceding results demonstrate that SERCA pumps are very powerful and their activity needs to be overcome in order for I_{CRAC} to activate fully, they do not address the key question as to whether the pumps can sculpt the ability of the natural trigger, namely InsP_3, to activate I_{CRAC} under physiological conditions of weak Ca^{2+} buffering. Because the current cannot be measured when cells are dialysed with InsP_3 in weak Ca^{2+} buffer (Fig. 3), we therefore determined the lowest concentration of EGTA that would still enable us to reliably measure macroscopic I_{CRAC} and, having found this, we examined the effects of SERCA pump block on the features of the current (Glitsch & Parekh, 2000). Figure 7*A* summarises results from experiments in which cells were dialysed with a maximally effective concentration of InsP_3 and different concentrations of EGTA. Concentrations of EGTA less than 0.35 mM generally failed to support any macroscopic activation of I_{CRAC} whereas close to maximal activation was seen with 1 mM EGTA. However, with 0.6 mM EGTA, I_{CRAC} clearly had a sub-maximal amplitude in spite of the cells being continuously exposed to a high concentration of InsP_3 via the patch pipette. We reasoned that the stores might nevertheless refill partially in the presence of such a moderate Ca^{2+} chelator concentration and this would therefore reduce the overall extent of I_{CRAC} activation. If true, then one might expect that the size of I_{CRAC} would increase under these conditions if SERCA pumps were to be blocked since this would suppress store refilling. The results of Fig. 7*B* show that this was indeed the case. Inclusion of thapsigargin in the recording pipette together with InsP_3 and 0.6 mM EGTA significantly increased the size of the current compared with InsP_3 and 0.6 mM EGTA alone. Moreover, the delay before the current activated was also reduced by blocking SERCA pumps, although the time to peak was unaffected (Fig. 7*C*).

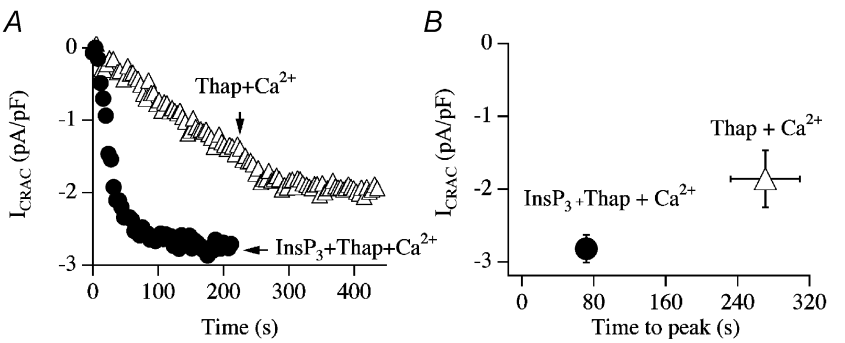

Figure 9. SERCA pump block is sufficient to activate I_{CRAC} even in high intracellular Ca^{2+}

A, InsP_3 and thapsigargin can activate I_{CRAC} in weak buffer even in the presence of high intracellular Ca^{2+} (100 μM total $CaCl_2$ in the pipette solution, free Ca^{2+} concentration estimated to be in the micromolar range). Thapsigargin alone was also able to evoke I_{CRAC} under these conditions, but after a delay and at a slower rate. *B*, aggregate data are summarised.

Collectively, these results demonstrate that SERCA pumps can refill stores, even in the continuous presence of InsP_3, and to the extent that I_{CRAC} activates only partially.

InsP_3 activates I_{CRAC} in physiological buffer provided SERCA pumps are blocked

The preceding results provide an explanation as to why InsP_3 is largely ineffective in activating I_{CRAC} under physiological conditions of weak Ca^{2+} buffering. The SERCA pumps are so effective that they can prevent InsP_3 from depleting the stores sufficiently or long enough for I_{CRAC} to activate. If true, then one would expect InsP_3 together with a SERCA pump blocker to activate I_{CRAC} in weak buffer. Figure 8 shows experiments designed to test this (Fierro & Parekh, 2000). Whereas InsP_3 was ineffective in weak Ca^{2+} buffer, the combination of InsP_3 and thapsigargin evoked a large current (Fig. 8*A*). The effects of thapsigargin were mimicked by the different SERCA pump blockers cyclopiazonic acid (Fig. 8*B*) and thapsigargicin (which is almost as potent as thapsigargin but less hydrophobic). Inhibition of SERCA pumps alone, in the absence of InsP_3, also activated I_{CRAC} (Fig. 8*B*; Fierro & Parekh, 2000). This occurred at a much slower rate than that seen in the presence of InsP_3 because store depletion to thapsigargin alone occurs via the background leak pathway.

In fact, provided SERCA pumps were blocked, I_{CRAC} could activate even in the absence of any exogenous Ca^{2+} chelator (Fierro & Parekh, 2000). Figure 9 shows the development of I_{CRAC} following dialysis with InsP_3, thapsigargin, 100 μM Ca^{2+} and no added Ca^{2+} chelator. Ca^{2+} in the pipette solution was weakly buffered by ATP (2 mM) and glutamate (main anion in the patch pipette). The current activated at the normal rate (trace marked InsP_3 + Thap + Ca^{2+} in Fig. 9*A*). Dialysis with thapsigargin and 100 μM Ca^{2+} (in the absence of InsP_3) also activated I_{CRAC}, with the typical slow kinetics expected for thapsigargin alone (trace marked Thap + Ca^{2+}). Aggregate data are summarised in Fig. 9*B*. Although the free Ca^{2+} concentration in the cells is not known precisely under these conditions (due to exogenous buffering/removal), in experiments with thapsigargin alone the slow development of I_{CRAC} would ensure that reasonable dialysis with micromolar Ca^{2+} concentrations would have occurred as I_{CRAC} activated. I_{CRAC} can therefore activate when cytosolic Ca^{2+} is in the micromolar range. Moreover, exogenous Ca^{2+} chelators are not required for I_{CRAC} to be activated.

SERCA and secretion

Upon stimulation, mast cells and basophils degranulate, thereby releasing a host of factors into the extracellular medium which are important in shaping a local inflammatory response. We measured secretion from individual RBL-1 cells using the capacitance method to track vesicular fusion (Artalejo *et al.* 1998). We found that exocytosis required both an increase in Ca^{2+} and a GTP-dependent step, at least in the whole cell configuration of the patch clamp technique. Strikingly, dialysis with InsP_3 in weak Ca^{2+} buffer failed to evoke any clear secretory response unless thapsigargin was present (Fig. 10). No doubt, part of the effects of thapsigargin are likely to involve reduced Ca^{2+} removal by SERCA pumps. But it seems likely that Ca^{2+} entry following the development of I_{CRAC}, as a consequence of exposure to the combination of InsP_3 and thapsigargin, will also promote a larger secretory response.

Central role for mitochondria in development of I_{CRAC}

SERCA pump activity therefore appears to be one of the main determinants of whether I_{CRAC} activates or not under physiological conditions. Only when SERCA pump activity is reduced does I_{CRAC} develop. How might SERCA pump activity be reduced under physiological conditions of weak Ca^{2+} buffering? We reasoned that an increase in the rate of Ca^{2+} removal from the cytosol by another clearance mechanism might be particularly effective because this would compete effectively with SERCA pumps and hence reduce the rate and extent of store refilling. Stores would therefore empty more and I_{CRAC} should activate. Moreover, enhanced Ca^{2+} clearance away from the endoplasmic reticulum might also reduce Ca^{2+}-dependent inactivation of InsP_3 receptors (Taylor & Traynor, 1995). Combined, this would enable InsP_3 to deplete stores sufficiently for I_{CRAC} to activate. We focused on mitochondria for three reasons. First, much recent evidence has established that they can and do take up significant amounts of Ca^{2+} following cytosolic Ca^{2+} increases under physiological conditions (Rizzuto *et al.* 1993, 1998; Jouavaille *et al.* 1995;

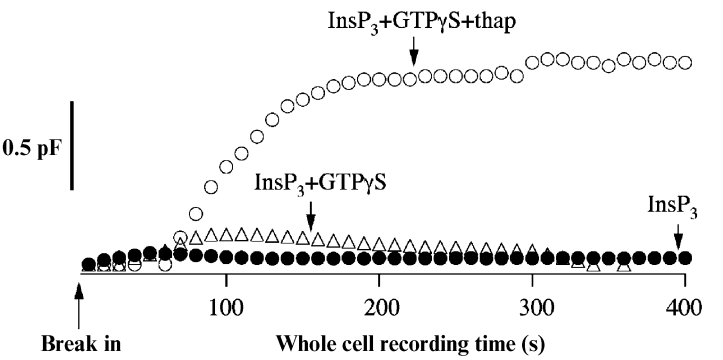

Figure 10. The combination of InsP_3 and thapsigargin evokes a robust secretory response in weak buffer, whereas InsP_3 alone is largely ineffective

InsP_3 alone (●) or in combination with GTPγS (△) did not trigger a detectable increase in membrane capacitance, whereas InsP_3 together with thapsigargin and GTPγS produced a prominent secretory response (○). In these experiments, cells were clamped at –60 mV.

Babcock *et al.* 1997; Hajnoczky *et al.* 1999; Tinel *et al.* 1999; Montero *et al.* 1999; Park *et al.* 2001; Arnaudeau *et al.* 2001) and this Ca^{2+} uptake, which occurs via a relatively low affinity ruthenium red-sensitive uniporter, is driven by the large inner mitochondrial membrane potential (Duchen, 2000). Second, mitochondria are often physically very close to the endoplasmic reticulum (Rizzuto *et al.* 1998; Park *et al.* 2001; Csordas & Hajnoczky, 2001), an arrangement that would greatly facilitate interaction between the two Ca^{2+}-handling organelles. Third, mitochondria can take up Ca^{2+} that has entered via the store-operated pathway (Lawrie *et al.* 1996; Hoth *et al.* 1997; Hartmann & Verkhratsky, 1999).

To examine the effects of mitochondria on the ability of InsP_3 to activate I_{CRAC} in weak Ca^{2+} buffer, we dialysed cells with a pipette solution containing weak buffer and no thapsigargin but supplemented with a cocktail (malate, pyruvate, NaH_2PO_4, cAMP and GTP) known to be important for sustaining respiring mitochondria in the whole cell recording configuration (Gunter & Pfeiffer, 1990; Herrington *et al.* 1996). The results were dramatic (Fig. 11). InsP_3 in the absence of cocktail usually failed to activate any detectable I_{CRAC} (Fig. 11*A*–*C*) and, in the small fraction of cells that responded (Fig. 11*D*), the current was very small. Inclusion of the cocktail had striking effects (Gilabert & Parekh, 2000). A robust I_{CRAC} could now be activated (Fig. 11*A*–*C*) and the vast majority of cells responded (Fig. 11*D*), although the size of the current was smaller than that seen in strong buffer. The effects of the cocktail were prevented by pre-treatment with the combination of antimycin A and oligomycin or by including ruthenium red in the pipette together with InsP_3 and cocktail (Fig. 11*C*). Oligomycin alone was without effect (Glitsch *et al.* 2002*b*). Hence mitochondrial Ca^{2+} uptake is required for InsP_3 to activate I_{CRAC} under physiological conditions.

The effects of the mitochondrial cocktail solution were Ca^{2+}-dependent, because no enhancing effect was seen in strong Ca^{2+} buffer (Fig. 12*A*). Strong Ca^{2+} buffer prevents a rise in intracellular Ca^{2+} by chelating Ca^{2+} both released from the stores and entering the cell, and hence a requirement for mitochondrial Ca^{2+} uptake would be obviated. Importantly, neither antimycin A and oligomycin nor ruthenium red impaired the ability of I_{CRAC} to activate in strong buffer, arguing against a direct channel blocking action of these drugs (Glitsch *et al.* 2002*b*).

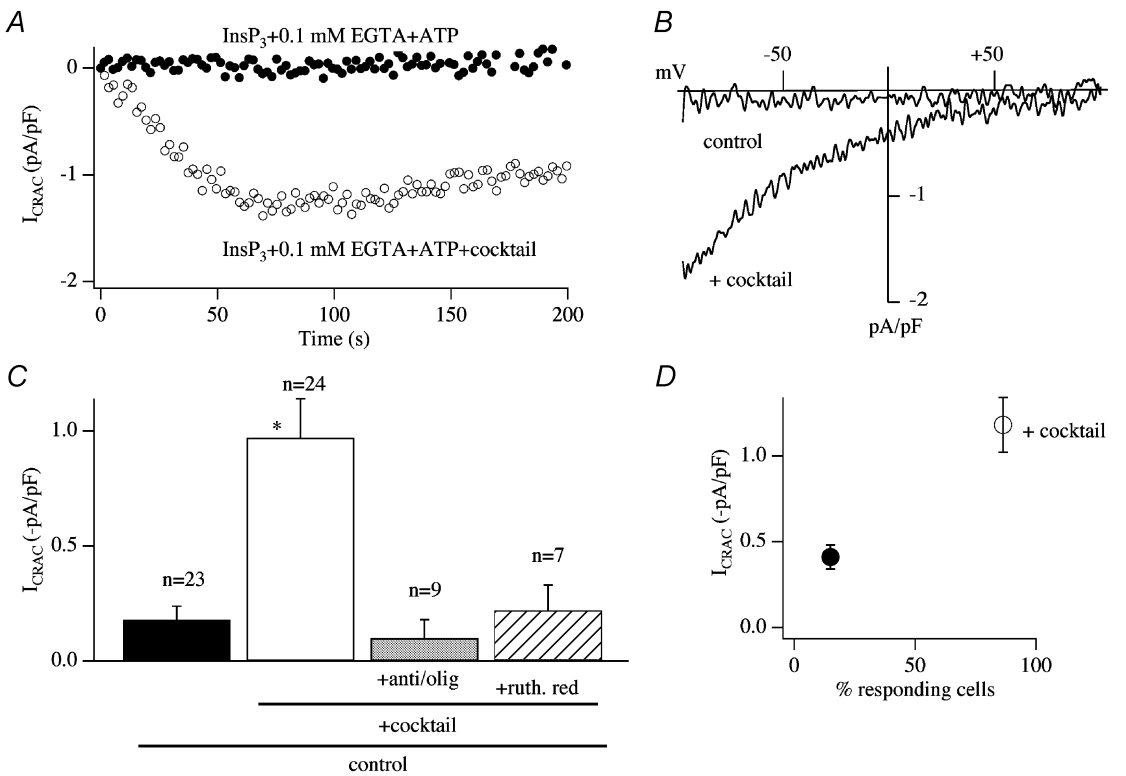

Figure 11. InsP_3 activates I_{CRAC} in weak buffer provided mitochondria are energised

A, time course of activation of I_{CRAC} in the presence of the mitochondrial cocktail (○). I_{CRAC} does not activate in weak Ca^{2+} buffer in response to InsP_3 alone (●). *B*, steady-state *I–V* relationships are shown for the cells in *A*. *C*, the effects of cocktail are suppressed by preventing mitochondrial Ca^{2+} uptake by either depolarising mitochondria (pre-treatment with antimycin A (5 μg ml^{-1}) and oligomycin (0.5 μg ml^{-1})) or inhibiting the uniporter with ruthenium red. *D*, the presence of cocktail dramatically increases the fraction of cells that respond to InsP_3 in weak Ca^{2+} buffer. * $P < 0.01$.

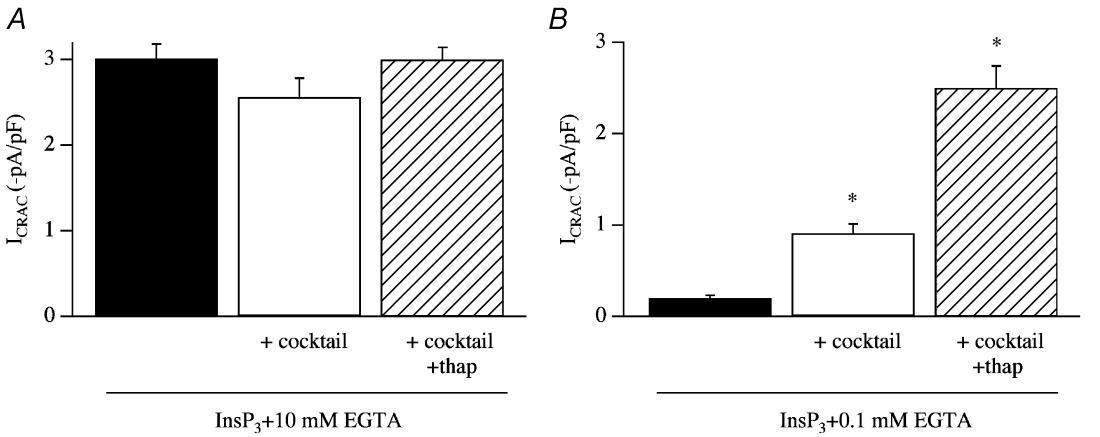

Figure 12. Physiological antagonism between SERCA pumps and mitochondria

A, the potentiation of I_{CRAC} by cocktail is suppressed by strong intracellular Ca^{2+} buffer (10 mM EGTA), indicating that the effect is Ca^{2+}-dependent. *B*, the extent of I_{CRAC} to InsP_3 in weak buffer alone, in mitochondrial cocktail and cocktail plus thapsigargin is compared. * $P < 0.01$.

SERCA pumps compete with mitochondria for removal of cytosolic Ca^{2+}

In the presence of energised mitochondria, the extent of I_{CRAC} following dialysis with InsP_3 is only around 35 % the size of that seen in strong buffer. This smaller current in weak buffer could reflect some Ca^{2+}-dependent store refilling, which would imply that not all the Ca^{2+} that has been released from the stores has been taken up by mitochondria but instead that some has been resequestrated into the stores by the SERCA pumps. To examine this, we measured the size of I_{CRAC} evoked by dialysis with InsP_3 in weak buffer in the absence and

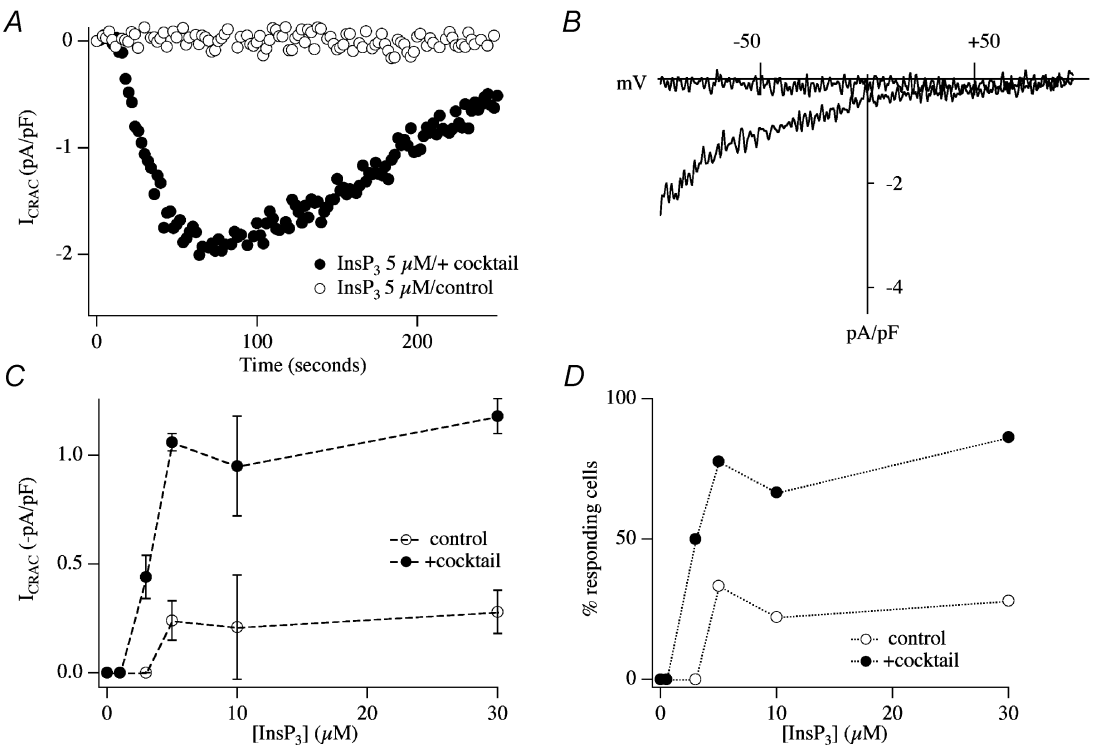

Figure 13. Energised mitochondria increase the ability of InsP_3 to activate I_{CRAC} in weak Ca^{2+} buffer

A, whereas 5 μM InsP_3 is ineffective in weak buffer (○), it evokes a prominent I_{CRAC} in the presence of energised mitochondria (●). *B*, *I–V* relationships for the cells shown in *A*, taken at steady state. *C*, concentration–response curve to InsP_3 in the absence (○) and presence (●) of cocktail. *D*, the fraction of cells responding *versus* concentration of InsP_3 is plotted in the absence (○) and presence (●) of energised mitochondria.

presence of thapsigargin in the cocktail (Gilabert *et al.* 2001). Results are summarised in Fig. 12*B*. Inclusion of thapsigargin resulted in an almost threefold increase in the size of the current, and the amplitude now was similar to that seen in strong buffer. No such potentiating effects were seen when these experiments were repeated in strong buffer (Fig. 12*A*). The results are consistent with the notion that mitochondria and SERCA pumps compete for removing Ca^{2+} and this form of physiological antagonism between two major Ca^{2+} clearance mechanisms, which is particularly acute under physiological conditions of weak intracellular Ca^{2+} buffering, impacts upon the extent of store depletion and subsequent activation of I_{CRAC}. In RBL cells, there is good morphological evidence documenting a very close apposition between mitochondria and SERCA pumps on the endoplasmic reticulum (Csordas & Hajnoczky, 2001), which would greatly promote competition for Ca^{2+} removal between these two organelles.

Respiring mitochondria reduce the threshold for activation of I_{CRAC} by InsP_3

The previous experiments have all involved supramaximal concentrations of InsP_3. To investigate whether mitochondrial Ca^{2+} uptake potentiates I_{CRAC} to more moderate levels of InsP_3, we compared the size of I_{CRAC} over a range of InsP_3 concentrations in the presence and absence of mitochondrial cocktail (Gilabert *et al.* 2001). The results are summarised in Fig. 13. Whereas 5 μM InsP_3 generally failed to activate I_{CRAC} in weak Ca^{2+} buffer (Fig. 13*A* and *B*), it evoked a sizeable current in the presence of the cocktail (Fig. 13*A* and *B*). Aggregate data are depicted in Fig. 13*C*. Comparing the results in the absence and presence of mitochondrial cocktail revealed several striking differences. First, the size of I_{CRAC} was potentiated in cells exposed to mitochondrial cocktail over a range of concentrations of InsP_3 (5–30 μM; Fig. 13*C*). Second, the fraction of cells that responded over this concentration range increased substantially in the presence of energised mitochondria (Fig. 13*D*). Third, whereas 3 μM InsP_3 consistently failed to evoke any current and is therefore a subthreshold concentration in the absence of the cocktail, around half the cells responded to this dose when mitochondria were energised (Fig. 13*C* and *D*). Mitochondrial Ca^{2+} uptake therefore increases the sensitivity of store-operated Ca^{2+} influx to InsP_3, and lowers the threshold concentration of InsP_3 that is required to activate I_{CRAC}. Mitochondrial Ca^{2+} buffering increases the dynamic range of concentrations over which InsP_3 is able to function as

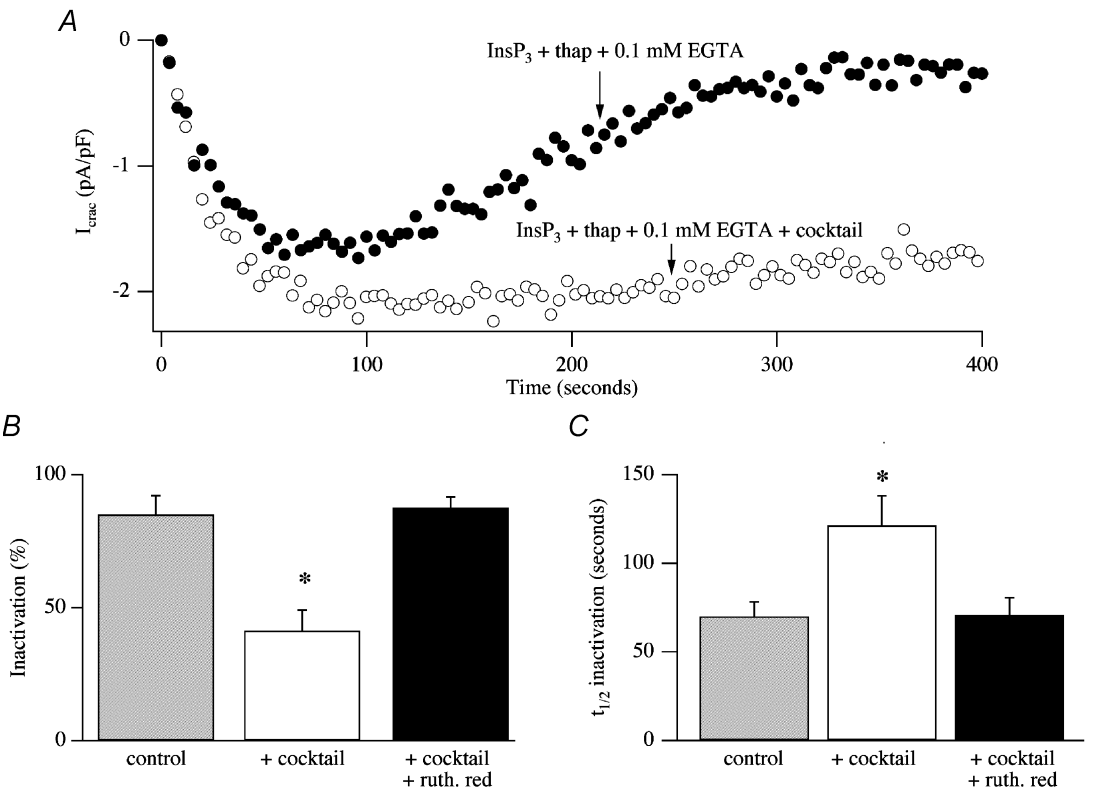

Figure 14. Mitochondrial Ca^{2+} uptake reduces the rate and extent of Ca^{2+}-dependent slow inactivation

A, dialysis with InsP_3 and thapsigargin in weak buffer (control) activates I_{CRAC} but then the current inactivates slowly (●). The extent of inactivation is reduced if mitochondria are energised (○). *B* and *C* summarise aggregate data comparing the extent (*B*) and rate (*C*) of inactivation of I_{CRAC} in control cells, in those in which cocktail was added to the solution and in cells exposed to cocktail and ruthenium red, to inhibit mitochondrial Ca^{2+} uptake. * $P < 0.01$. $t_{½}$, time at which I_{CRAC} had inactivated by 50 %.

the physiological messenger that triggers activation of store-operated Ca^{2+} influx (Gilabert *et al.* 2001).

Ca^{2+}-dependent inactivation of CRAC channels is reduced by mitochondria

We have identified three mechanisms whereby a rise in cytosolic Ca^{2+} can inhibit CRAC channel activity. Ca^{2+}-dependent fast inactivation occurs when permeating Ca^{2+} ions feed back to partially inactivate the channel through which they permeated (Fierro & Parekh, 1999*a*). Inactivation occurs with time constants of 10 and 100 ms, is largely independent of the macroscopic current (consistent with local feedback) and is more effectively reduced by BAPTA than EGTA, suggesting that the intracellular Ca^{2+} binding site is within a few nanometres of the pore (Fierro & Parekh, 1999*a*). This is very similar to fast inactivation first described in mast cells and jurkat T-cells (Hoth & Penner, 1993; Zweifach & Lewis, 1995*a*). Importantly, fast inactivation is unaffected by either energising mitochondria (Gilabert & Parekh, 2000) or depolarising them (Glitsch *et al.* 2002*b*), indicating that mitochondrial Ca^{2+} uptake does not seem to interfere with this form of Ca^{2+}-dependent inactivation. Two slower Ca^{2+}-dependent regulatory pathways also exist, operating over a time frame of several tens of seconds: Ca^{2+}-dependent store refilling (Bakowski *et al.* 2001) and Ca^{2+} entry-dependent but store-independent inactivation (Parekh, 1998). These pathways can be separated by the use of thapsigargin, as was originally described in jurkat T cells (Zweifach & Lewis, 1995*b*). By inhibiting store refilling, thapsigargin eliminates the store-dependent component and enables the slow Ca^{2+}-dependent inactivation mechanism to be studied in relative isolation, although the latter's contribution is probably exaggerated somewhat by thapsigargin because a major Ca^{2+} removal mechanism has been inhibited. Figure 14*A* shows an experiment in which a cell was dialysed with a pipette solution containing InsP_3 and thaspigargin in weak Ca^{2+} buffer. I_{CRAC} activated but then gradually decayed with a half-time of around 100 s, as Ca^{2+}-dependent slow inactivation developed (● in Fig. 14*A*). Strikingly, if mitochondria were maintained in an energised state by including the cocktail, the extent of this slow inactivation was reduced significantly and the rate at which it developed was slowed (○ in Fig. 14*A*). Pooled data are summarised in Fig. 14*B* and *C*. Blocking mitochondrial Ca^{2+} uptake with ruthenium red prevented the cocktail from reducing

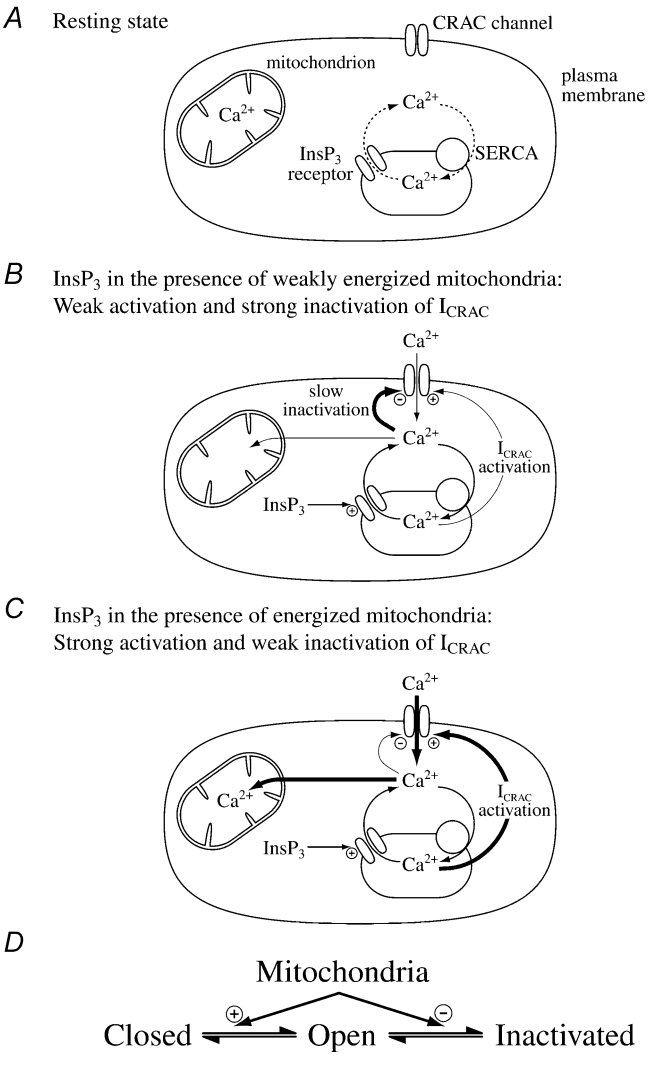

Figure 15. A diagrammatic scheme for the role of mitochondria in controlling I_{CRAC} under physiological conditions of weak intracellular Ca^{2+} buffering

A, the resting state, where I_{CRAC} is not functioning, is shown. Stores are largely full and any Ca^{2+} that leaks from the stores is taken back up by the SERCA pumps. *B*, increasing InsP_3 levels in the absence of active mitochondrial Ca^{2+} uptake, releases Ca^{2+} from the stores. However, the SERCA pumps are able to resequester sufficient Ca^{2+} so only a very small fraction of CRAC channels are activated (undetectable in whole cell mode). Furthermore, the rise in cytosolic Ca^{2+} results in Ca^{2+}-dependent slow inactivation of CRAC channels, and possibly InsP_3 receptors as well. *C*, in the presence of respiring mitochondria, InsP_3 activates macroscopic I_{CRAC}. Ca^{2+} released from the stores by InsP_3 is taken up by mitochondria. This reduces the amount of Ca^{2+} available to the SERCA pumps and in the vicinity of open InsP_3 receptors such that the stores are depleted sufficiently for macroscopic I_{CRAC} to activate (less refilling by SERCA pumps and less inactivation of InsP_3 receptors). Some refilling does occur because inclusion of thapsigargin enhances the size of the current. Furthermore, mitochondrial Ca^{2+} buffering reduces the rate and extent of Ca^{2+}-dependent slow inactivation, thereby increasing the size and duration of the current. *D*, a simplified gating scheme for CRAC channels summarising the role of mitochondrial Ca^{2+} buffering. Mitochondria facilitate opening (Closed to Open transition) whilst, simultaneously, reducing inactivation (Open to Inactivated transition). In this way, mitochondria have a much larger impact on I_{CRAC} than through either transition alone.

the extent of slow inactivation (Fig. 14*B* and *C*). Hence mitochondrial Ca^{2+} uptake can prolong the time course of store-operated Ca^{2+} influx by reducing Ca^{2+}-dependent slow inactivation (Gilabert & Parekh, 2000). A similar process has recently been reported in jurkat T cells (Hoth *et al.* 2000).

Dual actions of mitochondria on I_{CRAC}

It is particularly striking that the same mechanism, Ca^{2+} uptake by mitochondria, should have such pronounced effects on both the activation and inactivation of I_{CRAC}. By facilitating the ability of InsP_3 to activate I_{CRAC} (by promoting more extensive store emptying) and, at the same time, reducing Ca^{2+}-dependent slow inactivation, mitochondria will both increase the extent of I_{CRAC} as well as its duration to a much greater extent than through an action on activation or inactivation alone. A diagram summary depicting this dual action is shown in Fig. 15.

Under physiological conditions therefore, mitochondria play a pivotal role in all stages of store-operated Ca^{2+} entry, determining whether the whole cell current develops or not, to what extent and for how long it stays operational. We are now trying to understand how mitochondrial Ca^{2+} buffering impacts upon physiologically relevant responses like exocytosis.

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