Energized mitochondria increase the dynamic range over which inositol 1,4,5-trisphosphate activates store-operated calcium influx

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In eukaryotic cells, activation of cell surface receptors that couple to the phosphoinositide pathway evokes a biphasic increase in intracellular free Ca\(^{2+}\) concentration: an initial transient phase reflecting Ca\(^{2+}\) release from intracellular stores, followed by a plateau phase due to Ca\(^{2+}\) influx. A major component of this Ca\(^{2+}\) influx is store-dependent and often can be measured directly as the Ca\(^{2+}\) release-activated Ca\(^{2+}\) current (I\(_{\text{CRAC}}\)). Under physiological conditions of weak intracellular Ca\(^{2+}\) buffering, respiring mitochondria play a central role in store-operated Ca\(^{2+}\) influx. They determine whether macroscopic I\(_{\text{CRAC}}\) activates or not, to what extent and for how long. Here we describe an additional role for energized mitochondria: they reduce the amount of inositol 1,4,5-trisphosphate (InsP\(_3\)) that is required to activate I\(_{\text{CRAC}}\). By increasing the sensitivity of store-operated influx to InsP\(_3\), respiring mitochondria will determine whether modest levels of stimulation are capable of evoking Ca\(^{2+}\) entry or not. Mitochondrial Ca\(^{2+}\) buffering therefore increases the dynamic range of concentrations over which the InsP\(_3\) is able to function as the physiological messenger that triggers the activation of store-operated Ca\(^{2+}\) influx.

**Keywords:** calcium influx/inositol 1,4,5-trisphosphate/ mitochondria

**Introduction**

In non-excitable cells, the second messenger inositol 1,4,5-trisphosphate (InsP\(_3\)) evokes Ca\(^{2+}\) release from intracellular stores followed by Ca\(^{2+}\) influx across the plasma membrane (Putney, 1986; Berridge, 1993). One major route for this Ca\(^{2+}\) entry is through store-operated Ca\(^{2+}\) channels (SOCs), which are activated by the process of emptying the intracellular Ca\(^{2+}\) stores (Parekh and Penner, 1997). Ca\(^{2+}\) influx through SOCs is required not only for refilling the intracellular stores but also for regulating a host of physiological processes including secretion, gene transcription and cell proliferation (Parekh and Penner, 1997).

Although several types of SOC have been described, the best characterized to date are the Ca\(^{2+}\) release-activated Ca\(^{2+}\) (CRAC) SOCs, which give rise to a highly selective whole-cell Ca\(^{2+}\) current called I\(_{\text{CRAC}}\) (Hoth and Penner, 1992; Parekh and Penner, 1997). I\(_{\text{CRAC}}\) can be measured directly using the whole-cell patch-clamp technique. Until recently, I\(_{\text{CRAC}}\) was studied routinely in the presence of very high concentrations of Ca\(^{2+}\) chelators (mM concentrations of EGTA/BAPTA) in the recording pipette. Such strong Ca\(^{2+}\) buffering was used because the current could not be detected with weaker, more physiological levels of Ca\(^{2+}\) buffer (0.1 mM EGTA/BAPTA), and this inability to record I\(_{\text{CRAC}}\) was attributed to Ca\(^{2+}\)-dependent inactivation of the underlying CRAC channels. However, we and others have shown that this explanation cannot account for the inability to record I\(_{\text{CRAC}}\) in weak Ca\(^{2+}\) buffer (Broad et al., 1999; Fierro and Parekh, 2000). We have found that SERCA pumps, which refill the stores, are very powerful in rat basophilic leukaemia-1 (RBL-1) cells (Fierro and Parekh, 1999) and that InsP\(_3\) is unable to deplete stores sufficiently for whole-cell (macroscopic) I\(_{\text{CRAC}}\) to develop in weak Ca\(^{2+}\) buffer unless the SERCA pumps are inhibited (Fierro and Parekh, 2000; Bakowski and Parekh, 2001). Recently, we have demonstrated that I\(_{\text{CRAC}}\) can be activated in weak buffer, even when SERCA pumps are active, provided Ca\(^{2+}\) uptake into mitochondria is functional (i.e. when mitochondria are in an energized state in the whole-cell configuration; Gilabert and Parekh, 2000). Furthermore, Ca\(^{2+}\)-dependent slow inactivation of CRAC channels was reduced when mitochondrial Ca\(^{2+}\) uptake was operational. Hence mitochondria are important determinants of store-operated Ca\(^{2+}\) influx, determining whether macroscopic I\(_{\text{CRAC}}\) activates in weak buffer, to what extent and for how long (Gilabert and Parekh, 2000).

Mitochondrial Ca\(^{2+}\) buffering has quite marked effects on Ca\(^{2+}\) release to submaximal concentrations of InsP\(_3\). In permeabilized hepatocytes, mitochondrial Ca\(^{2+}\) uptake suppresses the positive feedback actions of cytosolic Ca\(^{2+}\) on adjacent InsP\(_3\) receptors and this results in less Ca\(^{2+}\) release to a submaximal InsP\(_3\) concentration (Hajnoczy et al., 1999). A similar conclusion was reached by Boitier et al. (1999), who found that mitochondrial Ca\(^{2+}\) uptake reduced the rate of propagation of Ca\(^{2+}\) waves in astrocytes. In Xenopus oocytes, on the other hand, mitochondrial Ca\(^{2+}\) uptake increased the frequency of Ca\(^{2+}\) waves and this was thought to reflect a reduction in the extent of Ca\(^{2+}\)-dependent inactivation of InsP\(_3\) receptors (Jouyville et al., 1995).

Since many Ca\(^{2+}\)-dependent processes are activated by modest increases in intracellular InsP\(_3\) levels, we have now investigated the effects of mitochondrial Ca\(^{2+}\) uptake on the ability of submaximal concentrations of InsP\(_3\) to activate I\(_{\text{CRAC}}\) in weak Ca\(^{2+}\) buffer. We find that respiring mitochondria enhance the extent of I\(_{\text{CRAC}}\), thus enabling moderate levels of InsP\(_3\) to be more effective in triggering Ca\(^{2+}\) influx. In addition, the threshold concentration of InsP\(_3\) that triggers I\(_{\text{CRAC}}\) is reduced in the presence of energized mitochondria. Mitochondrial Ca\(^{2+}\) uptake
Therefore increases the dynamic range over which InsP₃ is an effective stimulus for Ca²⁺ entry and may determine the efficacy of relatively weak stimuli in promoting store-dependent Ca²⁺ influx.

Results

A range of InsP₃ concentrations fail to activate I_{CRAC} consistently in weak Ca²⁺ buffer

When RBL-1 cells are dialysed with a patch pipette containing strong Ca²⁺ buffer (10 mM EGTA) and a maximally effective concentration of InsP₃ (30 μM), virtually all cells respond by generating a relatively large store-operated Ca²⁺ current (Fierro and Parekh, 2000; Gilabert and Parekh, 2000; Bakowski and Parekh, 2001). However, if this experiment is repeated in the presence of more physiological levels of intracellular Ca²⁺ buffering (0.1 mM EGTA), the same concentration of InsP₃ is largely ineffective. The majority of cells (>75%) fail to develop any detectable I_{CRAC} and, for those that do, the current is substantially smaller than in strong buffer. To examine whether lower, and presumably more physiological, concentrations of InsP₃ were similarly ineffective in weak Ca²⁺ buffer, we dialysed RBL-1 cells with different concentrations of InsP₃ (0.1 mM EGTA). In Figure 1A1, the time course of I_{CRAC} in response to 5 μM InsP₃, a concentration that is just maximal in strong buffer (Parekh et al., 1997), is depicted (open circles in Figure 1A1). Figure 1A2 shows the current–voltage relationship, taken at 100 s. InsP₃ at 5 μM failed to evoke any detectable I_{CRAC} in this cell. Averaged data from several cells, exposed to different concentrations of InsP₃, are shown in Figure 1B, and the fraction of responding cells is depicted in Figure 1C. In Figure 1B, cells that responded have been pooled together with those that did not. Concentrations of InsP₃ ≤3 μM consistently failed to evoke any discernible current. These concentrations nevertheless do release Ca²⁺ from the stores (Parekh et al., 1997). For higher InsP₃ concentrations, I_{CRAC} was very small and only ~25% of the cells responded. For those cells that did respond, I_{CRAC} was still several fold smaller than the amplitude in strong buffer (~0.51 pA/pF compared with ~2.85 pA/pF in strong buffer for 30 μM InsP₃). Hence, over a range of concentrations, InsP₃ is a very weak activator of I_{CRAC} in the presence of weak intracellular Ca²⁺ buffer.

InsP₃ still fails to activate I_{CRAC} consistently when K⁺ is the major intracellular cation

In the preceding experiments, we used Cs⁺ as the dominant cation in our pipette solution. For example, RBL cells express a GTP-dependent K⁺ conductance (McCloskey and Cahalan, 1990), and GTP is a component of the intracellular cocktail used to maintain mitochondria in an energized state (Gilabert and Parekh, 2000; see below). The GTP-dependent K⁺ current is not permeable to Cs⁺, and, therefore, in the presence of Cs⁺, it would not contaminate our recordings. However, under physiological...
conditions, $K^+$ is the major intracellular cation. It is possible that counter-movement of $K^+$ is required in order to sustain $Ca^{2+}$ release from the stores, and $Cs^+$ might be unable to substitute for $K^+$ in this action.

Both InsP$_3$-gated channels and ryanodine receptors are non-selective cation channels permeable to monovalent and divalent cations, with a higher permeability for divalents (Bezprozvanny and Ehrlich, 1995). The selectivity profile is believed to be very similar for these two $Ca^{2+}$ release channels, which is not unexpected as there is significant homology between the genes encoding these proteins. Although there is little information on the relative permeabilities of $Cs^+$ and $K^+$ for the InsP$_3$ receptors, $Cs^+$ is quite permeable through ryanodine-sensitive channels. It is more permeable than $K^+$ in the channels from skeletal muscle (Smith et al., 1988) and is 0.61 times that of $K^+$ in cardiac muscle (Lindsay et al., 1991). Nevertheless, we compared the ability of 30 $\mu$M InsP$_3$ to activate $I_{CRAC}$ in weak buffer in the presence of either $Cs^+$ or $K^+$. With $Cs^+$, the mean $I_{CRAC}$ was $-0.34 \pm 0.04$ pA/PF and in $K^+$ it was $-0.41 \pm 0.05$ pA/PF (six cells for each condition), and the amplitudes were not significantly different ($p > 0.3$). These results are entirely consistent both with our previous findings in which carbachol, acting via an increase in InsP$_3$, evoked robust $Ca^{2+}$ release after cells had been dialysed with $Cs^+$ (see figure 3 of Parekh et al., 1997), and with a recent report by Hermosura et al. (2000). These authors showed that the size of the carbachol-evoked transient in RBL cells was very similar in intact cells (where $K^+$ is the major intracellular cation) and after extensive dialysis of the cytosol with $Cs^+$ (figure 4 of Hermosura et al., 2000).

Respiring mitochondria potentiate $I_{CRAC}$ and reduce the threshold for activation by InsP$_3$

We have found that mitochondrial $Ca^{2+}$ uptake is essential in order for 30 $\mu$M InsP$_3$ to activate $I_{CRAC}$ in weak $Ca^{2+}$ buffer (Gilabert and Parekh, 2000). Whereas most cells fail to respond to InsP$_3$ in weak buffer, and for those that do the current is very small (see above), dialysis with a solution designed to maintain mitochondria in an energized state (mitochondrial cocktail solution, see Materials and methods) results in virtually all cells responding to produce a robust $I_{CRAC}$. The effects of the cocktail are due entirely to an action on mitochondria because manoeuvres that suppress mitochondrial $Ca^{2+}$ uptake abolish the enhancing effects of the cocktail (Gilabert and Parekh, 2000). To investigate whether mitochondrial $Ca^{2+}$ uptake similarly potentiates $I_{CRAC}$ to more moderate levels of InsP$_3$, we dialysed cells with different concentrations of InsP$_3$ in weak $Ca^{2+}$ buffer but now also in the presence of the mitochondrial cocktail. The results were dramatic. Whereas 5 $\mu$M InsP$_3$ generally failed to activate $I_{CRAC}$ in weak $Ca^{2+}$ buffer (Figure 1A1, open circles), it evoked a sizeable current in the presence of the cocktail (Figure 1A2, filled circles). The I–V relationship is shown in Figure 1A2. The current is inwardly rectifying, voltage-independent (over 50 ms) and reverses at potentials $>50$ mV, which are the hallmarks of $I_{CRAC}$ (Parekh and Penner, 1997). The extent of $I_{CRAC}$ to different InsP$_3$ concentrations in the absence (open circles) and presence (filled circles) of the mitochondrial cocktail solution is compared in Figure 1B, where data from responders and non-responders have been pooled together. The percentage of responding cells for each condition is shown in Figure 1C. Kinetic features of the current for the two different conditions are summarized in Figure 2A1 and A2. Several striking differences are apparent in the presence of energized mitochondria. (i) The size of $I_{CRAC}$ is potentiated over the range of concentrations of InsP$_3$ that evoke a response (5–30 $\mu$M; Figure 1B). (ii) The fraction of cells that respond over this concentration range increases substantially in the presence of respiring mitochondria (Figure 1C). (iii) Whereas 3 $\mu$M InsP$_3$ consistently fails to evoke $I_{CRAC}$ and is hence a subthreshold concentration in the absence of mitochondrial cocktail, around half of the cells respond to this dose of InsP$_3$ when mitochondria are energized (Figure 1B and C). (iv) The only kinetic parameter that changes with InsP$_3$ concentration is the delay before $I_{CRAC}$ starts to activate (Figure 2A1). However, once the current is initiated, then it develops at the same rate (Figure 2A2). (v) If we consider the amplitude of $I_{CRAC}$ in the presence of cocktail for responding cells only, then this was quite similar for all InsP$_3$ concentrations that evoked a response $[-0.44 \pm 0.08$ ($n = 5$), $-1.06 \pm 0.25$ ($n = 7$), $-0.81 \pm 0.16$ ($n = 6$) and $-1.18 \pm 0.16$ ($n = 21$)] pA/PF for 3, 5, 10 and 30 $\mu$M InsP$_3$, respectively; the only significant difference was between 3 and 30 $\mu$M InsP$_3$. Collectively, these results indicate that the threshold concentration of InsP$_3$ required to evoke $I_{CRAC}$ is reduced in the presence of energized mitochondria. Mitochondrial $Ca^{2+}$ uptake therefore increases the sensitivity of store-operated $Ca^{2+}$ influx to InsP$_3$. However, lowering the InsP$_3$ concentration further (0.5 $\mu$M) failed to activate $I_{CRAC}$ in weak buffer, even in the presence of the mitochondrial cocktail.

The relationship between InsP$_3$ concentration and extent of $I_{CRAC}$ is highly non-linear in weak $Ca^{2+}$ buffer

We had reported previously that the relationship between InsP$_3$ concentration and the amplitude of $I_{CRAC}$ was highly non-linear in strong $Ca^{2+}$ buffer, with a Hill coefficient of 12 (Parekh et al., 1997; Glitsch and Parekh, 2000). This supralinear relationship still held in the presence of moderate $Ca^{2+}$ buffering (Glitsch and Parekh, 2000). However, it was not clear whether the steep relationship was also valid in the presence of weak $Ca^{2+}$ buffer because the current could not be measured consistently under those conditions. Since we can record robust $I_{CRAC}$ in weak $Ca^{2+}$ buffer provided mitochondria are energized, we have been able to address this important issue directly. As shown in Figure 1B, concentrations of InsP$_3$ < 3 $\mu$M fail to evoke any detectable $I_{CRAC}$, whereas 5 $\mu$M InsP$_3$ generates maximal current. Fitting this dose–response curve with a modified Hill equation yielded a Hill coefficient of 17. Because the relationship is so steep, the Hill coefficient (derived from the fit) is only an approximation. However, the key point is that $I_{CRAC}$ is related supralinearly to InsP$_3$ concentration in the presence of physiological levels of intracellular $Ca^{2+}$ buffering and energized mitochondria. Once the threshold concentration of InsP$_3$ is exceeded, then only small further increases in InsP$_3$ will result in maximal activation of $Ca^{2+}$ influx.
**Kinetics of decay of I_{CRAC} in the presence of energized mitochondria**

Mitochondrial Ca^{2+} uptake reduces both the rate and extent of Ca^{2+}-dependent slow inactivation of CRAC channels in RBL cells (Gilabert and Parekh, 2000) and Jurkat T lymphocytes (Hoth et al., 2000). In order to isolate this inactivation mechanism, Ca^{2+}-dependent store refilling by SERCA pumps had to be suppressed. Because SERCA pumps are very powerful in RBL cells, we wanted to see whether mitochondria could prolong the duration of I_{CRAC} even when these pumps were active. To this end, we compared the time course of I_{CRAC} following activation by InsP_{3} in weak Ca^{2+} buffer in the absence and presence of the mitochondrial cocktail. Most cells failed to generate I_{CRAC} to InsP_{3} in the absence of cocktail, whereas for the minority that did so the current was transient. Figure 2B1 shows a typical response to InsP_{3} in the absence of cocktail (open circles, control) and the extent of decline of the current is summarized in the histogram of Figure 2B2. I_{CRAC} declined almost completely within 200 s. However, in the presence of cocktail, the current decayed much more slowly such that the amplitude of I_{CRAC} at times >100 s was significantly larger than the case when mitochondria were not maintained in an energized state (Figure 2B1 and B2). Therefore, under conditions where SERCA pump activity is maintained, mitochondrial Ca^{2+} buffering is still an important factor that prolongs the time course of I_{CRAC}. There was some variability in the extent of decay for I_{CRAC} in the presence of energized mitochondria between different cells (see also Gilabert and Parekh, 2000), but overall the decay was not significantly different between the various InsP_{3} concentrations (Figure 2B3).

**The facilitatory effects of mitochondria are suppressed by moderate concentrations of Ca^{2+} chelator**

Thin-section electron microscopic studies on RBL-2H3 cells have revealed that a small fraction of the mitochondrial surface is closely apposed to the endoplasmic reticulum (ER) membrane (Hajnoczky et al., 2000). This suggests that Ca^{2+} released by InsP_{3} receptors on the ER might need to diffuse, at least over short distances, in the cytosol before they are taken up by mitochondria. To see whether a slow Ca^{2+} chelator could intercept the diffusing Ca^{2+} ions and what concentration of this chelator suppressed the facilitatory effects of mitochondrial Ca^{2+} uptake, we constructed dose–response curves to EGTA in the absence and presence of the mitochondrial cocktail solution. Results are summarized in Figure 3A. For control (non-cocktail-treated) cells, 30 μM InsP_{3} failed to evoke any I_{CRAC} in 0.1 mM EGTA. EGTA at 0.35 mM was at around the threshold concentration for detection (3/9 cells failed to respond). In the presence of 0.6 mM EGTA, all cells responded and I_{CRAC} was ~50% of the maximum, the maximum being obtained in the presence of 10 mM EGTA. These results are very similar to our previous findings (Glitsch and Parekh, 2000). The presence of the cocktail significantly potentiated I_{CRAC} in the presence of
Fig. 3. Moderate concentrations of the slow Ca\(^{2+}\) chelator EGTA suppress the potentiating effects of mitochondrial cocktail on I\(_{\text{CRAC}}\). (A) Amplitude of I\(_{\text{CRAC}}\) is plotted against EGTA concentration in the absence (open circles) and presence (filled circles) of mitochondrial cocktail. I\(_{\text{CRAC}}\) was potentiated by the cocktail for EGTA concentrations <0.6 mM. (B) In the presence of respiring mitochondria, 30 μM InsP\(_3\) and 0.1 mM EGTA, inhibition of SERCA pumps by thapsigargin (2 μM) results in a further increase in the amplitude of I\(_{\text{CRAC}}\). (C) Energized mitochondria potentiate the size of I\(_{\text{CRAC}}\) when Jurkat T cells are dialysed with InsP\(_3\) (30 μM) and 0.1 mM EGTA. *p < 0.05 and ***p < 0.001.

weak to moderate concentrations of EGTA (<0.6 mM EGTA; Figure 3A) and virtually all cells responded under these conditions (90% in 0.1 mM and 100% in 0.35 mM EGTA). In 0.6 mM EGTA, the current was enhanced slightly by cocktail but this was not significant (Figure 3A). No facilitatory effects of the cocktail were seen in 10 mM EGTA. These results are in good agreement with those reported by Csordas et al. (1999) who found that 0.6 mM EGTA suppressed the increase in intramitochondrial Ca\(^{2+}\) following InsP\(_3\)-mediated Ca\(^{2+}\) release in permeabilized RBL-2H3 cells. Because slow buffers such as EGTA are unable to reduce Ca\(^{2+}\) levels at distances <20 nm from open Ca\(^{2+}\)-permeable channels such as InsP\(_3\) gated channels (Neher, 1998), our results indicate that a molecular distance significantly larger than 20 nm separates the Ca\(^{2+}\) release and uptake sites on the ER and mitochondria, respectively. This is entirely consistent with the findings of Csordas et al. (1999), who calculated an average distance of 100 nm between InsP\(_3\) receptors and mitochondrial Ca\(^{2+}\) uptake sites in permeabilized RBL-2H3 cells.

**SERCA pumps can compete with mitochondria for removal of cytosolic Ca\(^{2+}\)**

Inspection of Figures 1B and 3A reveals that, in energized mitochondria and weak Ca\(^{2+}\) buffer, I\(_{\text{CRAC}}\) is generally around ~1 pA/pF. In strong buffer, the current is almost three times larger. This difference in current amplitudes could arise from some vestigial Ca\(^{2+}\)-dependent inactivation of the CRAC channels in weak buffer such that the current size is reduced. Alternatively, it could reflect some Ca\(^{2+}\)-dependent store refilling, implying that not all of the Ca\(^{2+}\) released by InsP\(_3\) is taken up by mitochondria but that some of this Ca\(^{2+}\) is resequestrated into the stores. This latter scenario would constitute a form of physiological antagonism between two major Ca\(^{2+}\) clearance mechanisms in RBL cells, with mitochondria enhancing depletion of InsP\(_3\)-sensitive Ca\(^{2+}\) stores and hence activation of I\(_{\text{CRAC}}\) and SERCA pumps promoting store refilling and therefore a reduction in the extent of activation of I\(_{\text{CRAC}}\). To distinguish between these possibilities, we compared the size of I\(_{\text{CRAC}}\) between cells dialysed with InsP\(_3\) + weak Ca\(^{2+}\) buffer + mitochondrial cocktail in the absence and presence of thapsigargin. Results are summarized in the histogram of Figure 3B. Inclusion of thapsigargin resulted in an almost 3-fold increase in the size of the current. The amplitude of I\(_{\text{CRAC}}\) now was similar to that seen in strong buffer (InsP\(_3\) + 10 mM EGTA; Figure 3A). Hence, SERCA pumps can still resequestrate sufficient Ca\(^{2+}\) even in the presence of energized mitochondria such that I\(_{\text{CRAC}}\) cannot activate to its maximum extent. This would be consistent with the notion that mitochondria and SERCA pumps compete for Ca\(^{2+}\) and that this impacts upon the extent of store depletion and subsequent activation of I\(_{\text{CRAC}}\).

**Energized mitochondria potentiate I\(_{\text{CRAC}}\) in weak Ca\(^{2+}\) buffer in Jurkat T lymphocytes**

In Jurkat T lymphocytes, like RBL-1 cells, we have found that macroscopic I\(_{\text{CRAC}}\) can be activated only weakly following dialysis with InsP\(_3\) and 0.1 mM EGTA (Fierro et al., 2000). We therefore investigated whether energized mitochondria could enhance the size of the current, as is the case in RBL cells. As shown in Figure 3C, the amplitude of I\(_{\text{CRAC}}\) was significantly larger in the presence of the mitochondrial cocktail. These results complement the reports by Hoth et al. (1997, 2000), who found that mitochondrial Ca\(^{2+}\) buffering reduced Ca\(^{2+}\)-dependent slow inactivation of CRAC channels in the Jurkat cell line. Like RBL cells, energized mitochondria in Jurkat T lymphocytes seem to increase the size of I\(_{\text{CRAC}}\) following dialysis with InsP\(_3\) in weak Ca\(^{2+}\) buffer.

**Discussion**

Our new findings demonstrate that energized mitochondria determine whether macroscopic I\(_{\text{CRAC}}\) activates over a range of stimulus intensities. By reducing the threshold concentration of InsP\(_3\) required to evoke I\(_{\text{CRAC}}\), mitochondria increase the dynamic range over which this ubiquitous second messenger is able to control, via store depletion, the extent of Ca\(^{2+}\) influx. A cartoon summarizing this effect is shown in Figure 4 (see legend for explanation). The increased sensitivity to lower levels of InsP\(_3\) may be an important factor that helps determine whether weak/moderate stimulation of cell surface receptors can promote store-operated Ca\(^{2+}\) entry. It is intriguing to consider that regulation of mitochondrial Ca\(^{2+}\) uptake and/or spatial distribution of mitochondria relative to the ER might be a
A novel mechanism to dictate whether weak/moderate receptor stimulation evokes Ca\textsuperscript{2+} influx or not.

Because relatively high concentrations of InsP\textsubscript{3} or receptor engagement are required to activate I\textsubscript{CRAC}, it has been proposed that additional Ca\textsuperscript{2+} entry pathways may be involved during weaker levels of stimulation (Shuttleworth, 1999). Our findings, demonstrating that energized mitochondrial Ca\textsuperscript{2+} uptake can reduce the amount of InsP\textsubscript{3} that is necessary to evoke I\textsubscript{CRAC}, suggest instead that I\textsubscript{CRAC} can be activated by lower InsP\textsubscript{3} concentrations when care is taken to maintain mitochondria in an energized state. It is likely that addition to our pipette solution of further components that support respiring mitochondria may result in greater Ca\textsuperscript{2+} uptake by this organelle and hence a further reduction in the InsP\textsubscript{3} concentration required to activate I\textsubscript{CRAC}.

In RBL-1 cells, like certain other non-excitatory cells, low concentrations of InsP\textsubscript{3} can trigger Ca\textsuperscript{2+} release and reduce the amount of Ca\textsuperscript{2+} within the stores without evoking any Ca\textsuperscript{2+} influx (Parekh et al., 1997; Hartmann and Verkhratsky, 1998; Liu et al., 1998). Growing evidence from RBL-1 cells points towards a specialized subcompartment of the ER that is involved specifically in the activation of I\textsubscript{CRAC} (Parekh et al., 1997; Broad et al., 1999; Krause et al., 1999). This store seems harder to deplete since somewhat higher concentrations of InsP\textsubscript{3} are required to activate Ca\textsuperscript{2+} influx than Ca\textsuperscript{2+} release. It has been suggested that low levels of InsP\textsubscript{3} fail to access these stores because the InsP\textsubscript{3} 5-phosphatase breaks down these concentrations very efficiently (Hermosura et al., 2000). Only high concentrations of InsP\textsubscript{3}, which are well above the K\textsubscript{M} of the phosphatase, can mobilize the stores and thus activate I\textsubscript{CRAC}. An alternative explanation is that low concentrations of InsP\textsubscript{3} do access these specialized stores but that the SERCA pumps are so active that they prevent the Ca\textsuperscript{2+} released by these low InsP\textsubscript{3} concentrations from depleting stores sufficiently for I\textsubscript{CRAC} to activate. Energized mitochondria reduce the threshold concentration of InsP\textsubscript{3} that is required to evoke I\textsubscript{CRAC}. Because the activity of the 5-phosphatase is not thought to be Ca\textsuperscript{2+} dependent (Shears, 1992) and presumably, therefore, would not be affected by mitochondrial Ca\textsuperscript{2+} uptake, this indicates that low concentrations of InsP\textsubscript{3} (3 μM) probably do in fact mobilize these specialized stores and hence that metabolism of InsP\textsubscript{3} is unlikely to be the main factor preventing these InsP\textsubscript{3} concentrations from evoking the current. Instead, enhanced mitochondrial Ca\textsuperscript{2+} uptake would facilitate store depletion by reducing both possible Ca\textsuperscript{2+}-dependent inactivation of InsP\textsubscript{3} receptors and Ca\textsuperscript{2+} uptake via SERCA pumps.

Our results also suggest that mitochondrial Ca\textsuperscript{2+} uptake and Ca\textsuperscript{2+} ATPases of the ER, two major Ca\textsuperscript{2+} removal mechanisms in these and other cells (Herrington et al., 1996; Tinel et al., 1999), can functionally antagonize one another. In the absence of energized mitochondria, I\textsubscript{CRAC} is generally not activated by InsP\textsubscript{3} in weak Ca\textsuperscript{2+} buffer unless SERCA pumps are blocked (Fierro and Parekh, 2000; Gilabert and Parekh, 2000). This indicates that a sizeable fraction of the Ca\textsuperscript{2+} released by InsP\textsubscript{3} is resequestered into the stores such that the intraluminal Ca\textsuperscript{2+} content does not fall sufficiently for macroscopic I\textsubscript{CRAC} to activate. On the other hand, in the presence of energized mitochondria and active SERCA pumps, enough Ca\textsuperscript{2+} is taken up by the mitochondria (and hence away from the pumps) so that stores are depleted to an extent that macroscopic I\textsubscript{CRAC} activates, albeit to a submaximal level (Gilabert and Parekh, 2000). This dynamic interplay between SERCA pumps and mitochondrial Ca\textsuperscript{2+} uptake sites might require close apposition between the two Ca\textsuperscript{2+} removal mechanisms so that effective competition can take place. Recent morphological evidence strongly suggests that this is the case in RBL cells. Csordas and Hajnoczky (2001) have found that almost every mitochondria has a region that is positioned very close to the SERCA pumps of the ER. It is likely

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**Fig. 4.** Cartoon summary of the role of mitochondria in activation of I\textsubscript{CRAC} for moderate increases in InsP\textsubscript{3}. (A) The resting situation, where most CRAC channels are closed. The stores are sufficiently full that I\textsubscript{CRAC} is deactivated and Ca\textsuperscript{2+} that leaks out of the stores is taken back up by the SERCA pumps. (B) Moderate increases in InsP\textsubscript{3} evoke substantial Ca\textsuperscript{2+} release from the stores but sufficient Ca\textsuperscript{2+} is resequestered by the pumps so that the threshold for macroscopic activation of I\textsubscript{CRAC} is not reached. The current therefore does not activate to detectable levels. (C) In the presence of energized mitochondria, these moderate concentrations of InsP\textsubscript{3} are now able to activate macroscopic I\textsubscript{CRAC}. Mitochondria take up some of the Ca\textsuperscript{2+} that has been released by InsP\textsubscript{3}. This will result in less Ca\textsuperscript{2+} being available for the SERCA pumps, resulting in a reduction in net uptake. In addition, mitochondrial Ca\textsuperscript{2+} buffering may reduce the extent of Ca\textsuperscript{2+}-dependent inactivation of the InsP\textsubscript{3} receptors, thereby enabling greater Ca\textsuperscript{2+} release and hence store depletion. Mitochondrial Ca\textsuperscript{2+} uptake will also reduce the rate and extent of Ca\textsuperscript{2+}-dependent slow inactivation, and this will increase the size of I\textsubscript{CRAC} as well as prolong its duration (Gilabert and Parekh, 2000).
therefore that at these mitochondria–ER junctions, competition between the two organelles for removing Ca\(^{2+}\) would be particularly strong.

At moderate InsP\(_3\) concentrations (nM to low \(\mu\)M range), Ca\(^{2+}\) inactivation of InsP\(_3\) receptors becomes more prominent (Mak et al., 1998). Through their effects on buffering cytosolic Ca\(^{2+}\), mitochondria might reduce this inactivation process and thereby promote further Ca\(^{2+}\) release from the stores. If the pumps are now inhibited, then the amplitude of \(I_{\text{CRAC}}\) increases to the maximal extent. Hence, in respiring mitochondria, some of the released Ca\(^{2+}\) is still taken back up into the InsP\(_3\)-sensitive stores. Coordinated regulation of the Ca\(^{2+}\) transport capacities of these two organelles would therefore have quite marked effects on store depletion and subsequent Ca\(^{2+}\) influx.

Mitochondrial Ca\(^{2+}\) buffering prolongs the time course of \(I_{\text{CRAC}}\) even when the powerful SERCA pumps are active (Figure 2B), and therefore it is an important factor that helps determine the extent of Ca\(^{2+}\) influx under conditions where other Ca\(^{2+}\) removal mechanisms are still operational. Under physiological conditions, therefore, mitochondria are powerful intracellular Ca\(^{2+}\) buffering organelles that help prolong the duration of Ca\(^{2+}\) influx.

Our results demonstrate that mitochondria are key orchestrators of store-operated Ca\(^{2+}\) entry in RBL cells. Mitochondria are involved in three crucial aspects of Ca\(^{2+}\) influx: (i) they determine whether macroscopic \(I_{\text{CRAC}}\) activates or not (Gilabert and Parekh, 2000); (ii) they help set the time course of \(I_{\text{CRAC}}\) following its activation; and (iii) they reduce the levels of intracellular InsP\(_3\) required to activate the current. By sensitizing cells to lower InsP\(_3\), mitochondria may determine whether relatively weak stimuli are capable of evoking Ca\(^{2+}\) influx or not.

Data are presented as the mean ± SEM, and statistical analysis was carried out using both Student’s t and Mann–Whitney non-parametric tests. Thapsigargin was purchased from Alomone Laboratories. All other chemicals were from Sigma.

**Acknowledgements**

J.A.G. is supported by a Marie Curie EU Postdoctoral Fellowship. D.B. holds a British Heart Foundation Prize Studentship. A.B.P. is a Lister Institute Research Fellow. Early stages of this work was supported by the Wellcome Trust (Career Development Fellowship award to A.B.P.).

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**Materials and methods**

**Cell culture**

RBL-1 cells and Jurkat T lymphocytes, which were bought from Cell Bank at the Sir William Dunn School of Pathology, Oxford University, were cultured as previously described (Fierro and Parekh, 2000; Fierro et al., 2000).

**Electrophysiology**

Patch-clamp experiments were conducted in the tight-seal whole-cell configuration at room temperature (20–23°C) as previously described (Parekh et al., 1997; Fierro and Parekh, 1999). Sylgard-coated, fire-polished pipettes had DC resistances of 2.5–4 M\(\Omega\) when filled with standard internal solution that contained 145 mM Cs glutamate, 8 mM NaCl, 1 mM MgCl\(_2\), 0.1 mM EGTA, 2 mM Mg-ATP and 10 mM HEPES pH 7.2 with CsOH. A correction of +10 mV was applied for the subsequent liquid junction potential that arose from this glutamate-based internal solution. In some experiments, Cs\(^+\) was replaced with K\(^+\) (see text). Mitochondrial cocktail contained 2 mM pyruvic acid, 2 mM malic acid, 1 mM NaH\(_2\)PO\(_4\), 0.5 mM cAMP, 0.5 mM GTP and 0.5 mM MgCl\(_2\). Extracellular solution contained 145 mM NaCl, 2.8 mM KCl, 10 mM CaCl\(_2\), 2 mM MgCl\(_2\), 10 mM CsCl, 10 mM glucose and 10 mM HEPES pH 7.4 with NaOH. \(I_{\text{CRAC}}\) was measured by applying voltage ramps (−100 to +100 mV in 50 ms) at 0.5 Hz from a holding potential of 0 mV as previously described (Parekh et al., 1997). Currents were filtered using an eight-pole Bessel filter at 2.5 kHz and digitized at 100 μs. Currents were normalized by dividing the amplitudes (measured from the voltage ramps at −80 mV) by the cell capacitance. Capacitative currents were compensated before each ramp by using the automatic compensation of the EPC 9-2 amplifier. All leak currents were subtracted by averaging the first few ramp currents (usually two), and then subtracting this from all subsequent currents.


Received February 23, 2001; revised and accepted April 3, 2001