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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_{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_{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_{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_{CRAC} (Hoth and Penner, 1992; Parekh and Penner, 1997). I_{CRAC} can be measured directly using the whole-cell patch-clamp technique. Until recently, I_{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_{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_{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_{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_{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_{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 (Hajnoczky *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 (Jouvaille *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_{CRAC} in weak Ca^{2+} buffer. We find that respiring mitochondria enhance the extent of I_{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_{CRAC} is reduced in the presence of energized mitochondria. Mitochondrial Ca^{2+} uptake

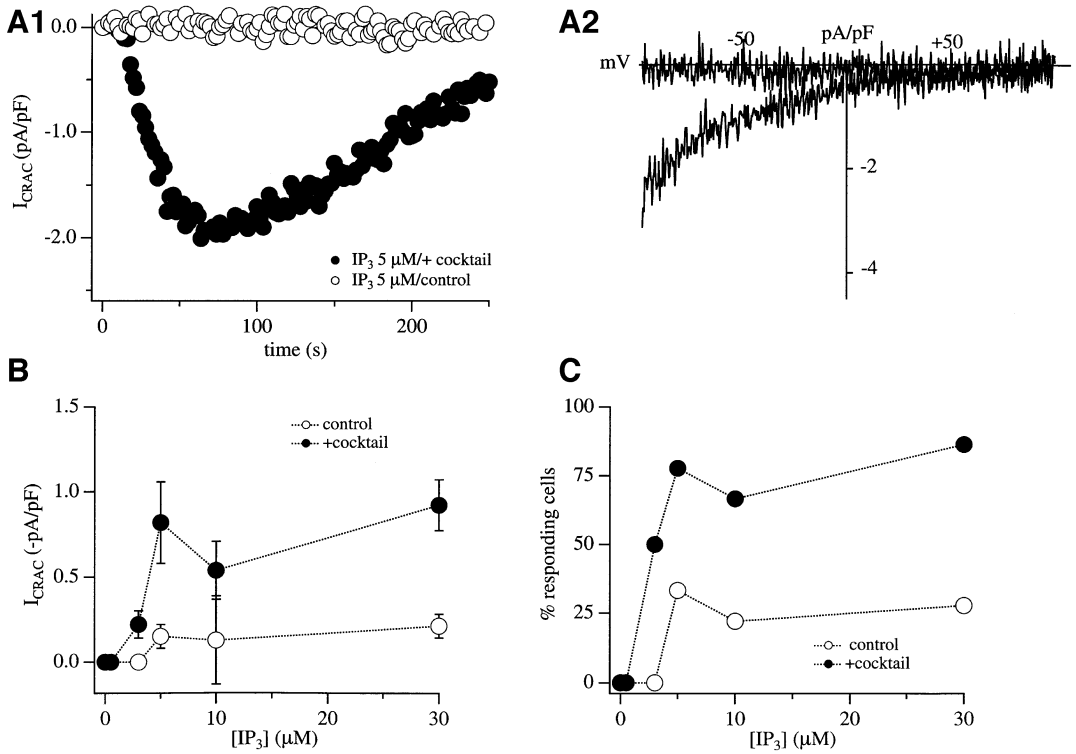

Fig. 1. Energized mitochondria increase the ability of $InsP_3$ to activate I_{CRAC} in weak intracellular Ca^{2+} buffer. (**A1**) Time course of I_{CRAC} to 5 μM $InsP_3$ in the absence (open circles) and presence (filled circles) of mitochondrial cocktail solution. (**A2**) Current–voltage relationships for the recordings shown in (A1), taken after 100 s. (**B**) Amplitude of I_{CRAC} (measured at –80 mV from the voltage ramps and normalized for cell capacitance) is plotted against $InsP_3$ concentration included in the recording pipette. Open and filled circles correspond to experiments in the absence and presence of the mitochondrial cocktail solution, respectively. (**C**) The percentage of responding cells is plotted against $InsP_3$ concentration. Note that, at 3 μM $InsP_3$, no cell responds in the absence of the mitochondrial cocktail whereas 50% do so when the cocktail is present.

therefore increases the dynamic range over which $InsP_3$ is an effective stimulus for Ca^{2+} entry and may determine the efficacy of relatively weak stimuli in promoting store-dependent Ca^{2+} influx.

Results

A range of $InsP_3$ concentrations fail to activate I_{CRAC} consistently in weak Ca^{2+} buffer

When RBL-1 cells are dialysed with a patch pipette containing strong Ca^{2+} buffer (10 mM EGTA) and a maximally effective concentration of $InsP_3$ (30 μM), virtually all cells respond by generating a relatively large store-operated Ca^{2+} 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 Ca^{2+} buffering (0.1 mM EGTA), the same concentration of $InsP_3$ 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 $InsP_3$ were similarly ineffective in weak Ca^{2+} buffer, we dialysed RBL-1 cells with different concentrations of $InsP_3$ (in 0.1 mM EGTA). In Figure 1A1, the time course of I_{CRAC} in response to 5 μM $InsP_3$, 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. $InsP_3$ at 5 μM failed to evoke any detectable I_{CRAC} in this cell. Averaged data from several cells, exposed to different concentrations of $InsP_3$, 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 $InsP_3$ ⩽3 μM consistently failed to evoke any discernible current. These concentrations nevertheless do release Ca^{2+} from the stores (Parekh *et al*., 1997). For higher $InsP_3$ 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 $InsP_3$). Hence, over a range of concentrations, $InsP_3$ is a very weak activator of I_{CRAC} in the presence of weak intracellular Ca^{2+} buffer.

$InsP_3$ 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 μM $InsP_3$ to activate I_{CRAC} in weak buffer in the presence of either Cs^+ or K^+. With Cs^+, the mean I_{CRAC} was –0.34 ± 0.04 pA/pF and in K^+ it was –0.41 ± 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 μM $InsP_3$ 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 μM $InsP_3$ 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 μM; 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 μM $InsP_3$ 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 ± 0.08 (n = 5), –1.06 ± 0.25 (n = 7), –0.81 ± 0.16 (n = 6) and –1.18 ± 0.16 (n = 21) pA/pF for 3, 5, 10 and 30 μM $InsP_3$, respectively; the only significant difference was between 3 and 30 μM $InsP_3$]. 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 μM) 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 μM fail to evoke any detectable I_{CRAC}, whereas 5 μM $InsP_3$ 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.

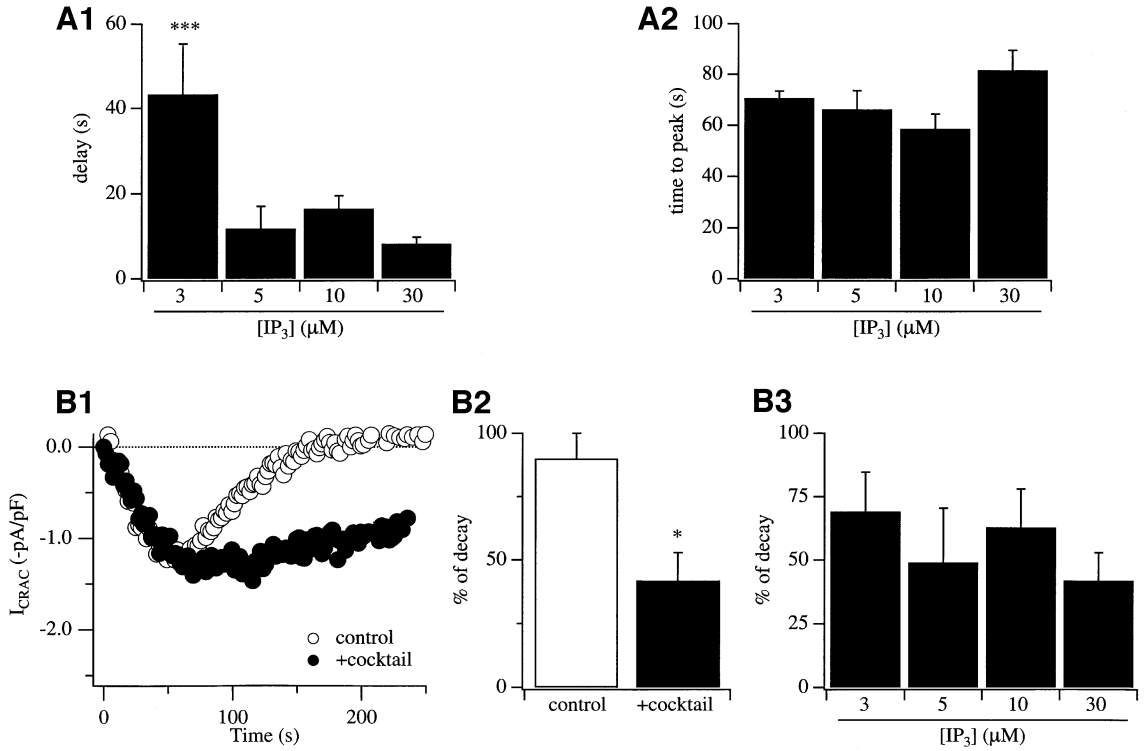

Fig. 2. Effects of mitochondrial Ca^{2+} buffering on the kinetics of I_{CRAC} under conditions where SERCA pumps are active. The delay before I_{CRAC} activates (**A1**) and the time to peak (**A2**) are plotted against $InsP_3$ concentration for cells with energized mitochondria. The time to peak was corrected for the delay. (**B1**) The time course of I_{CRAC} is shown for a cell dialysed with 30 μM $InsP_3$ in weak Ca^{2+} buffer in the absence (open circles) and presence (filled circles) of mitochondrial cocktail. Note that the amplitude of the current was similar for the two cells, but I_{CRAC} declined much more quickly when mitochondria were not energized. (**B2**) The extent of decline of I_{CRAC} is compared for cells dialysed in the absence (open bar) and presence (filled bar) of mitochondrial cocktail. (**B3**) The extent of decline of I_{CRAC} in the presence of cocktail is plotted against the different $InsP_3$ concentrations. *p <0.05 and ***p <0.001.

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

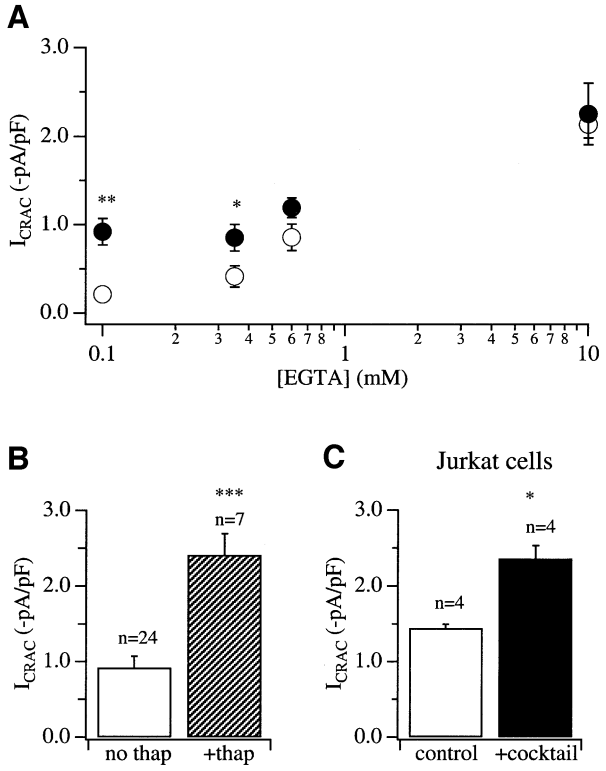

Fig. 3. Moderate concentrations of the slow Ca^{2+} chelator EGTA suppress the potentiating effects of mitochondrial cocktail on I_{CRAC}. (**A**) Amplitude of I_{CRAC} is plotted against EGTA concentration in the absence (open circles) and presence (filled circles) of mitochondrial cocktail. I_{CRAC} 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_{CRAC}. (**C**) Energized mitochondria potentiate the size of I_{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_{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_{CRAC}, and SERCA pumps promoting store refilling and therefore a reduction in the extent of activation of I_{CRAC}. To distinguish between these possibilities, we compared the size of I_{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_{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_{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_{CRAC}.

Energized mitochondria potentiate I_{CRAC} in weak Ca^{2+} buffer in Jurkat T lymphocytes

In Jurkat T lymphocytes, like RBL-1 cells, we have found that macroscopic I_{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_{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_{CRAC} following dialysis with $InsP_3$ in weak Ca^{2+} buffer.

Discussion

Our new findings demonstrate that energized mitochondria determine whether macroscopic I_{CRAC} activates over a range of stimulus intensities. By reducing the threshold concentration of $InsP_3$ required to evoke I_{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

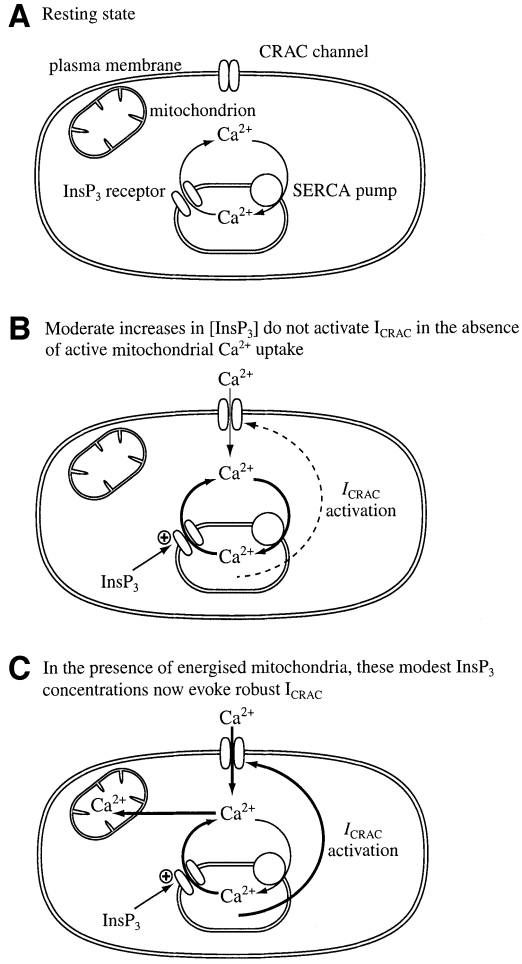

Fig. 4. Cartoon summary of the role of mitochondria in activation of I_{CRAC} for moderate increases in $InsP_3$. (**A**) The resting situation, where most CRAC channels are closed. The stores are sufficiently full that I_{CRAC} is deactivated and Ca^{2+} that leaks out of the stores is taken back up by the SERCA pumps. (**B**) Moderate increases in $InsP_3$ evoke substantial Ca^{2+} release from the stores but sufficient Ca^{2+} is resequestrated by the pumps so that the threshold for macroscopic activation of I_{CRAC} is not reached. The current therefore does not activate to detectable levels. (**C**) In the presence of energized mitochondria, these moderate concentrations of $InsP_3$ are now able to activate macroscopic I_{CRAC}. Mitochondria take up some of the Ca^{2+} that has been released by $InsP_3$. This will result in less Ca^{2+} being available for the SERCA pumps, resulting in a reduction in net re-uptake. In addition, mitochondrial Ca^{2+} buffering may reduce the extent of Ca^{2+}-dependent inactivation of the $InsP_3$ receptors, thereby enabling greater Ca^{2+} release and hence store depletion. Mitochondrial Ca^{2+} uptake will also reduce the rate and extent of Ca^{2+}-dependent slow inactivation, and this will increase the size of I_{CRAC} as well as prolong its duration (Gilabert and Parekh, 2000).

novel mechanism to dictate whether weak/moderate receptor stimulation evokes Ca^{2+} influx or not.

Because relatively high concentrations of $InsP_3$ or receptor engagement are required to activate I_{CRAC}, it has been proposed that additional Ca^{2+} entry pathways may be involved during weaker levels of stimulation (Shuttleworth, 1999). Our findings, demonstrating that energized mitochondrial Ca^{2+} uptake can reduce the amount of $InsP_3$ that is necessary to evoke I_{CRAC}, suggest instead that I_{CRAC} can be activated by lower $InsP_3$ 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^{2+} uptake by this organelle and hence a further reduction in the $InsP_3$ concentration required to activate I_{CRAC}.

In RBL-1 cells, like certain other non-excitable cells, low concentrations of $InsP_3$ can trigger Ca^{2+} release and reduce the amount of Ca^{2+} within the stores without evoking any Ca^{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_{CRAC} (Parekh *et al*., 1997; Broad *et al*., 1999; Krause *et al*., 1999). This store seems harder to deplete since somewhat higher concentrations of $InsP_3$ are required to activate Ca^{2+} influx than Ca^{2+} release. It has been suggested that low levels of $InsP_3$ fail to access these stores because the $InsP_3$ 5-phosphatase breaks down these concentrations very efficiently (Hermosura *et al*., 2000). Only high concentrations of $InsP_3$, which are well above the K_M of the phosphatase, can mobilize the stores and thus activate I_{CRAC}. An alternative explanation is that low concentrations of $InsP_3$ do access these specialized stores but that the SERCA pumps are so active that they prevent the Ca^{2+} released by these low $InsP_3$ concentrations from depleting stores sufficiently for I_{CRAC} to activate. Energized mitochondria reduce the threshold concentration of $InsP_3$ that is required to evoke I_{CRAC}. Because the activity of the 5-phosphatase is not thought to be Ca^{2+} dependent (Shears, 1992) and presumably, therefore, would not be affected by mitochondrial Ca^{2+} uptake, this indicates that low concentrations of $InsP_3$ (3 μM) probably do in fact mobilize these specialized stores and hence that metabolism of $InsP_3$ is unlikely to be the main factor preventing these $InsP_3$ concentrations from evoking the current. Instead, enhanced mitochondrial Ca^{2+} uptake would facilitate store depletion by reducing both possible Ca^{2+}-dependent inactivation of $InsP_3$ receptors and Ca^{2+} uptake via SERCA pumps.

Our results also suggest that mitochondrial Ca^{2+} uptake and Ca^{2+} ATPases of the ER, two major Ca^{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_{CRAC} is generally not activated by $InsP_3$ in weak Ca^{2+} buffer unless SERCA pumps are blocked (Fierro and Parekh, 2000; Gilabert and Parekh, 2000). This indicates that a sizeable fraction of the Ca^{2+} released by $InsP_3$ is resequestrated into the stores such that the intraluminal Ca^{2+} content does not fall sufficiently for macroscopic I_{CRAC} to activate. On the other hand, in the presence of energized mitochondria and active SERCA pumps, enough Ca^{2+} is taken up by the mitochondria (and hence away from the pumps) so that stores are depleted to an extent that macroscopic I_{CRAC} activates, albeit to a submaximal level (Gilabert and Parekh, 2000). This dynamic interplay between SERCA pumps and mitochondrial Ca^{2+} uptake sites might require close apposition between the two Ca^{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 mitochondrion has a region that is positioned very close to the SERCA pumps of the ER. It is likely

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 μM 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_{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_{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_{CRAC} activates or not (Gilabert and Parekh, 2000); (ii) they help set the time course of I_{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.

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 MΩ 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_2PO_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_{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.

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.

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