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Store-operated Ca^{2+} entry depends on mitochondrial Ca^{2+} uptake

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Store-operated Ca^{2+} channels, which are activated by the emptying of intracellular Ca^{2+} stores, provide one major route for Ca^{2+} influx. Under physiological conditions of weak intracellular Ca^{2+} buffering, the ubiquitous Ca^{2+} releasing messenger $InsP_3$ usually fails to activate any store-operated Ca^{2+} entry unless mitochondria are maintained in an energized state. Mitochondria rapidly take up Ca^{2+} that has been released by $InsP_3$, enabling stores to empty sufficiently for store-operated channels to activate. Here, we report a novel role for mitochondria in regulating store-operated channels under physiological conditions. Mitochondrial depolarization suppresses store-operated Ca^{2+} influx independently of how stores are depleted. This role for mitochondria is unrelated to their actions on promoting $InsP_3$-sensitive store depletion, can be distinguished from Ca^{2+}-dependent inactivation of the store-operated channels and does not involve changes in intracellular ATP, oxidants, cytosolic acidification, nitric oxide or the permeability transition pore, but is suppressed when mitochondrial Ca^{2+} uptake is impaired. Our results suggest that mitochondria may have a more fundamental role in regulating store-operated influx and raise the possibility of bidirectional Ca^{2+}-dependent crosstalk between mitochondria and store-operated Ca^{2+} channels.

Keywords: Ca^{2+} influx/mitochondria/store-operated Ca^{2+} channels

Introduction

Store-operated (capacitative) Ca^{2+} influx, in which a fall in the Ca^{2+} content of the endoplasmic reticulum (ER) opens Ca^{2+} channels in the plasma membrane, is one of the more widespread mechanisms whereby mammalian non-excitable cells can increase their intracellular free calcium concentration (Putney, 1986; Parekh and Penner, 1997). In many cell types, store-operated Ca^{2+} entry is manifested as a non-voltage-gated, inwardly rectifying Ca^{2+} current called I_{CRAC} (Hoth and Penner, 1992; Parekh and Penner, 1997).

In addition to their role as the main energy-producing centres of eukaryotic cells, much recent work has established that mitochondria can and do take up Ca^{2+} rapidly following physiological levels of cell stimulation, and then subsequently release this Ca^{2+} slowly back into the cytosol (Pozzan *et al*., 1994; Duchen, 2000; Rutter and Rizzuto, 2000). Mitochondrial Ca^{2+} uptake occurs via a relatively low affinity ruthenium red-sensitive uniporter that is driven by the large inner mitochondrial membrane potential (Pozzan *et al*., 1994). Mitochondria can take up Ca^{2+} that has been released from intracellular stores by the opening of either $InsP_3$- or ryanodine-sensitive Ca^{2+} channels on the stores (Rizzuto *et al*., 1993, 1998; Jouaville *et al*., 1995; Hajnoczky *et al*., 1999; Tinel *et al*., 1999; Montero *et al*., 2000; Park *et al*., 2000). Alternatively, they can sequester Ca^{2+} that enters the cell via voltage-gated or store-operated CRAC channels (Lawrie *et al*., 1996; Babcock *et al*., 1997; Hoth *et al*., 1997; Gilabert and Parekh, 2000; Park *et al*., 2000).

Changes in mitochondrial Ca^{2+} dynamics have numerous effects on cell physiology. Buffering of cytosolic Ca^{2+} by respiring mitochondria is seen in many diverse cell types, where it shapes the profile of the cytosolic Ca^{2+} signal (Duchen, 2000; Rutter and Rizzuto, 2000). An increase in mitochondrial matrix Ca^{2+}, following rapid Ca^{2+} uptake, stimulates key rate-limiting dehydrogenase enzymes of the Krebs cycle (McCormack *et al*., 1990; Hajnoczky *et al*., 1995), resulting in an increase in ATP production (Jouaville *et al*., 1999). In the polarized pancreatic acinar cell, mitochondria form a belt that surrounds the apical area, the latter containing the zymogen-containing secretory granules (Tinel *et al*., 1999). Mitochondrial Ca^{2+} uptake in the belt region restricts Ca^{2+} signals from propagating out of the apical zone and hence exocytosis is restricted to the apical area. In chromaffin cells, mitochondria seem to exist in functional units with ryanodine-sensitive Ca^{2+} release channels on the stores and voltage-operated Ca^{2+} channels in the plasma membrane (Montero *et al*., 2000). Following stimulation, rapid intra-mitochondrial Ca^{2+} transients in excess of 1 mM occur and this sequestration of Ca^{2+} determines the pattern of the secretory response.

Respiring mitochondria are also very important in determining the pattern of activation and inactivation of CRAC channels (Gilabert and Parekh, 2000; Gilabert *et al*., 2001). Under physiological conditions of weak intracellular Ca^{2+} buffering, the ubiquitous second messenger $InsP_3$ generally fails to evoke any detectable whole-cell (macroscopic) I_{CRAC} (Broad *et al*., 1999; Fierro and Parekh, 2000; Glitsch and Parekh, 2000). It appears that $InsP_3$, in spite of releasing Ca^{2+} from the stores, fails to empty the stores sufficiently and/or long enough for I_{CRAC} to activate (Parekh *et al*., 1997). However, if precautions are taken to maintain mitochondria in an energized state, $InsP_3$ is now able to activate I_{CRAC} in physiological buffer (Gilabert and Parekh, 2000; Gilabert *et al*., 2001). Mitochondria take up some of the Ca^{2+} that has been released from the stores and this results in greater store depletion and hence activation of the current. In addition,

mitochondrial Ca^{2+} uptake reduces the extent of Ca^{2+}-dependent inactivation of CRAC channels and slows its rate of development.

Here, we find that mitochondria play a central role in the ability of CRAC channels to activate under physiological conditions, even when stores are emptied irreversibly and independently of $InsP_3$ receptors. The involvement of mitochondria under these conditions is unrelated to actions in promoting store depletion. The requirement for respiring mitochondria can also be distinguished from Ca^{2+}-dependent inactivation of the CRAC channels. Instead, our results suggest that mitochondria seem to have a more fundamental role in regulating CRAC channels than has been hitherto suspected. Although the underlying mechanism is as yet unclear, it is not affected by anti-oxidants, acidification of the cytosol, nitric oxide or the permeability transition pore. One possibility is that mitochondria release factor(s), in a Ca^{2+}-dependent manner, which are particularly important in regulating CRAC channels under physiological conditions. Our results support a novel role for mitochondria in regulating the activity of the widely expressed CRAC channels.

Results

Mitochondrial depolarization reduces calcium entry after store depletion

Cells loaded with the Ca^{2+}-sensitive fluorescent dye Fura 2 were exposed to the SERCA pump blocker thapsigargin in Ca^{2+}-free external solution for 10–20 min in order to empty the intracellular Ca^{2+} stores and open the CRAC channels in the plasma membrane. Thereafter, external Ca^{2+} was readmitted. This resulted in a rapid increase in cytosolic Ca^{2+}, which then decayed slowly (see controls in Figure 1A and B). However, if mitochondria were depolarized after store depletion by exposure to either the protonophore FCCP (5 μM, which collapses the proton motive force across the inner mitochondrial membrane) or a mixture of antimycin A and oligomycin (to inhibit complex III of the respiratory chain and the mitochondrial F_0F_1 ATP synthase, respectively), subsequent readmission of Ca^{2+} generated a smaller Ca^{2+} signal, which developed more slowly (Figure 1A and B). We measured the initial rate of rise of the Ca^{2+} signal (initial slope) as this is a better indicator of the number of open CRAC channels than the amplitude of the peak signal. The slope was significantly reduced by either FCCP or antimycin A plus oligomycin (Figure 1E), compared with control recordings from the same cell preparations. To obtain more quantitative information on the effects of mitochondrial depolarization upon Ca^{2+} influx, the Ca^{2+} entry signal for both control cells and those exposed to either FCCP (Figure 1A) or antimycin A plus oligomycin (Figure 1B) was differentiated, and the corresponding graphs are shown in Figure 1C and D, respectively. The peak of the differentiated signal is another indicator of the maximal rate of Ca^{2+} entry and, like the slope, this parameter was also significantly reduced by mitochondrial depolarization (Figure 1F).

Because thapsigargin was used to deplete the stores irreversibly and mitochondrial depolarization occurred after thapsigargin had emptied the stores, the role of mitochondria is clearly distal to store emptying. This is

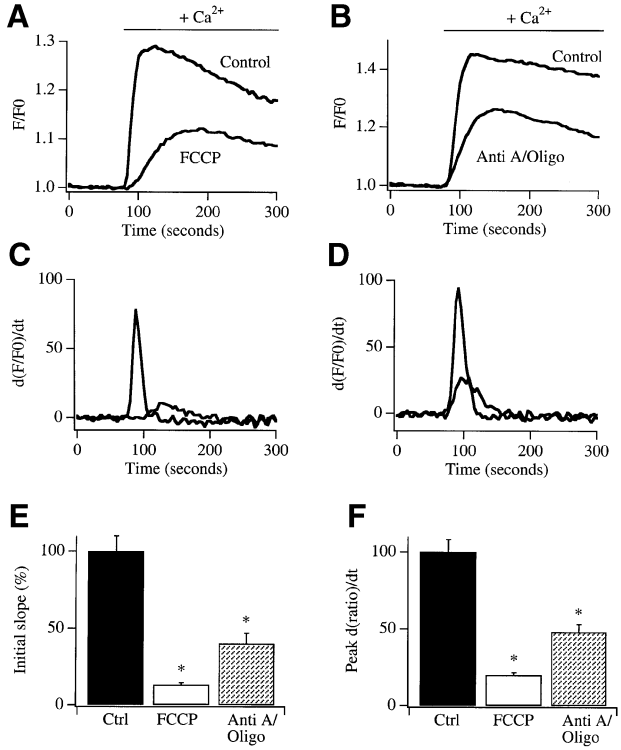

Fig. 1. Mitochondrial depolarization reduces the rate of Ca^{2+} entry in intact cells. After depleting stores with thapsigargin in Fura 2-loaded cells, readmission of external Ca^{2+} (2 mM) resulted in a rapid increase in cytosolic Ca^{2+} concentration (control). This was substantially reduced by depolarizing mitochondria with either 5 μM FCCP (**A**) or antimycin A (5 μg/ml) plus oligomycin (0.5 μg/ml) (**B**). The differentiated signals are shown in (**C**) and (**D**). (**E**) Bar chart comparing the initial slope of the Ca^{2+} signal upon Ca^{2+} readmission in control cells with those pre-exposed to either FCCP or antimycin A plus oligomycin. Numbers of cells (*n*) for each condition are: control (ctrl) 40, FCCP 38, antimycin A plus oligomycin (Anti A/Oligo) 35. (**F**) Bar chart plotting the peak rate of Ca^{2+} increase (obtained from the differentiated signal) for the conditions shown. $*p < 0.01$.

fundamentally different from the effects of mitochondria in enabling $InsP_3$ to activate I_{CRAC} under physiological conditions (Gilabert and Parekh, 2000). In this latter case, by taking up some of the Ca^{2+} released by $InsP_3$, mitochondria promote greater store depletion and hence activation of I_{CRAC}.

Known Ca^{2+}-dependent inactivation mechanisms cannot fully explain the inhibition of Ca^{2+} influx following mitochondrial depolarization

The reduction in Ca^{2+} influx following mitochondrial depolarization in Figure 1 cannot be explained solely by Ca^{2+}-dependent inactivation of CRAC channels. In rat basophilic leukaemia (RBL-1) cells, there are at least three mechanisms whereby an increase in cytosolic Ca^{2+} can lead to inactivation of I_{CRAC}. Ca^{2+}-dependent fast inactivation occurs at a site within a few nanometres of the pore (Fierro and Parekh, 1999a) and is unaffected either by energizing mitochondria (Gilabert and Parekh, 2000) or by depolarizing them (extent of inactivation at the end of a 200 ms hyperpolarizing pulse to –100 mV was 53.0 ± 1% after exposure to antimycin A plus oligomycin, versus 50.0 ± 1% for control cells; five cells each condition). Hence Ca^{2+}-dependent fast inactivation is

unlikely to be involved here. Ca^{2+}-dependent store refilling deactivates the current but this is prevented by thapsigargin (Bakowski *et al*., 2001). Because we had exposed cells continuously to thapsigargin (Figure 1), it is unlikely that refilling took place to any appreciable extent. Ca^{2+} entry-dependent but store-independent inactivation requires a more global increase in Ca^{2+} (being suppressed by the slow chelator EGTA) and develops slowly with a half-time of around 60 s (Gilabert and Parekh, 2000). One would therefore expect it to be manifest after a prominent initial rate of calcium entry, but this was clearly not the case (Figure 1). Although these well-characterized Ca^{2+}-dependent inactivation mechanisms seem unlikely to fully account for the inhibition of Ca^{2+} influx that occurs following mitochondrial depolarization, we nevertheless considered other potential Ca^{2+}-dependent mechanisms. One possibility is that mitochondria rapidly take up much of the Ca^{2+} that has been released from the stores by thapsigargin, but then release this Ca^{2+} upon depolarization. This release may result in a form of Ca^{2+}-dependent slow inactivation of the channels. Two arguments are hard to reconcile with this. First, application of FCCP to cells pre-exposed to thapsigargin in Ca^{2+}-free solution failed to produce any detectable Ca^{2+} increase (9/10 cells, data not shown). Hence it appears unlikely that mitochodrial depolarization results in a global Ca^{2+} increase following slow store emptying with thapsigargin. Secondly, dialysis with a pipette solution containing 1 μM Ca^{2+} did not prevent thapsigargin from evoking clear I_{CRAC} (our unpublished observations). Therefore, in order for mitochondrial Ca^{2+} release to inactivate CRAC channels, an increase in cytosolic Ca^{2+} levels exceeding 1 μM globally would be required. It is conceivable that mitochondria take up Ca^{2+} following slow release from the stores and then release this Ca^{2+} locally, following depolarization, just below the plasma membrane such that the Ca^{2+} concentration exceeds 1 μM and inactivates the CRAC channels. But this explanation does not sit easily with the finding that fast Ca^{2+}-dependent inactivation, reflecting local feedback of permeating Ca^{2+} on the CRAC channels, is not affected by either energizing (Gilabert and Parekh, 2000) or depolarizing mitochondria.

Collectively, the effects of mitochondrial depolarizing agents suggest a new role for mitochondria in regulating CRAC channel activity in intact cells. The following experiments were designed to establish the nature of this regulation.

Mitochondrial depolarization suppresses I_{CRAC} even when the membrane potential is controlled

In the experiments of Figure 1, the cell membrane potential was not controlled but was free to fluctuate. It is therefore possible that mitochondrial depolarization somehow depolarizes the membrane potential, thereby reducing the electric driving force for Ca^{2+} entry through CRAC channels, as has been suggested for the effects of the protonophore (CCCP) on antigen-stimulated Ca^{2+} influx (Mohr and Fewtrell, 1987). To test this directly, we carried out patch–clamp experiments where the membrane potential was experimentally controlled. Figure 2 summarizes experiments using the perforated patch technique, which enables the current to be recorded with minimal perturbation of the cytoplasm under voltage

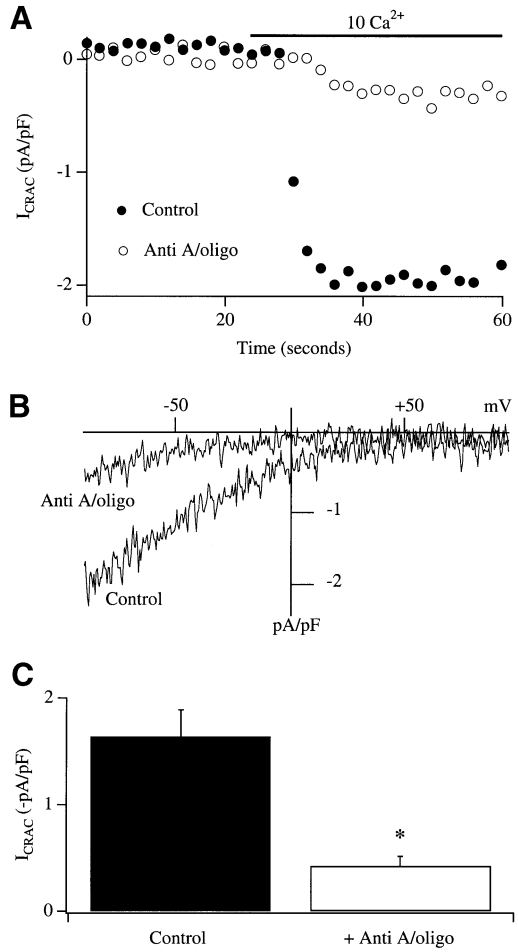

Fig. 2. Mitochondrial depolarization reduces I_{CRAC} in the perforated patch configuration. (**A**) Time course of I_{CRAC} in a control cell is compared with one in which mitochondria were depolarized by a 20 min exposure to antimycin A plus oligomycin, prior to Ca^{2+} readmission. (**B**) The current–voltage relationships for the cells shown in (A) are depicted. These were taken after 50 s. (**C**) Aggregate data comparing the size of I_{CRAC} between control cells and those with depolarized mitochondria. In these experiments, mitochondria were depolarized after store depletion with thapsigargin.

clamp conditions. The protocol employed was identical to that used above for Fura 2-loaded cells and was as follows. Cells were first exposed to 2 μM thapsigargin in Ca^{2+}-free external solution. Vehicle (DMSO) or antimycin A plus oligomycin was then added in Ca^{2+}-free solution (in the presence of thapsigargin) and a seal was then formed. After obtaining background currents, Ca^{2+} was readmitted to the cell and the development of I_{CRAC} was followed. A typical control recording is shown in Figure 2A (filled circles), the current–voltage relationship in Figure 2B, and aggregate data in Figure 2C ($n = 5$). However, if mitochondria were depolarized prior to the readmission of external Ca^{2+}, the size of the current was significantly reduced (Figure 2; five cells, $p < 0.002$). Two conclusions can be drawn from these experiments. First, the reduction in Ca^{2+} influx following mitochondrial depolarization after store depletion cannot be explained simply by a reduction in electric driving force at the plasma membrane. Although this effect may contribute to the slightly greater reduction in the rate of Ca^{2+} influx seen after exposure to FCCP compared with antimycin A and oligomycin

(Figure 1E), it is clearly not the dominant mechanism. Secondly, mitochondrial regulation of I_{CRAC} is maintained in the perforated patch configuration and is likely therefore to be of physiological relevance.

Mitochondria are required for thapsigargin to evoke I_{CRAC} in the whole-cell configuration

Thapsigargin is able to activate I_{CRAC} routinely in weak intracellular Ca^{2+} buffer (0.1 mM EGTA) in the whole-cell patch–clamp configuration, whereas $InsP_3$ is ineffective under these conditions (Fierro and Parekh, 2000). A typical recording of the activation of I_{CRAC} by thapsigargin is shown in Figure 3A (filled circles, labelled 0.1 EGTA + thap.), the current–voltage relationship in Figure 3B, and aggregate data in Figure 3C ($n = 7$). The current developed relatively slowly as expected, since it is determined by the passive leakage of Ca^{2+} across the ER. Strikingly, if mitochondria were depolarized with antimycin A and oligomycin prior to exposure to thapsigargin in weak Ca^{2+} buffer, the subsequent size of the current was dramatically reduced (open diamonds in Figure 3A, ramp I–V in Figure 3B and pooled data in Figure 3C, $n = 9$; $p < 0.001$ compared with control). In the whole-cell configuration, mitochondrial Ca^{2+} uptake can be maintained by including a cocktail solution that maintains mitochondria in an energized state (Gilabert and Parekh, 2000). To see if the size of I_{CRAC} evoked by thapsigargin could be enhanced by energized mitochondria, we dialysed cells with thapsigargin plus mitochondrial cocktail in weak buffer. Under these conditions, the size of the current was increased significantly (Figure 3, $n = 8$, $p < 0.01$ relative to control). Mitochondrial Ca^{2+} uptake plays an important role in the ability of I_{CRAC} to activate under physiological conditions of weak intracellular Ca^{2+} buffering, even when the current is evoked by depleting stores with thapsigargin.

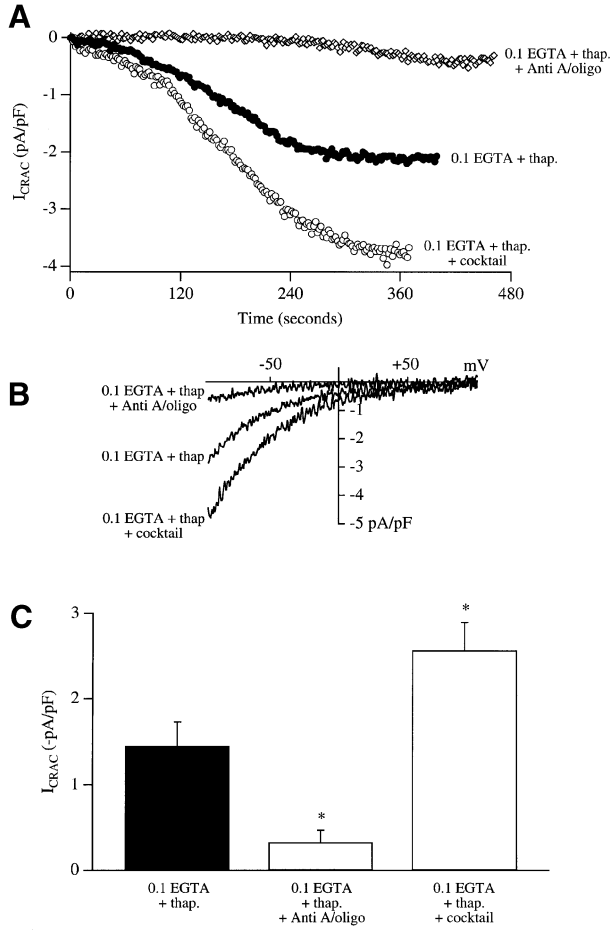

Fig. 3. Mitochondrial Ca^{2+} buffering regulates the extent of I_{CRAC} evoked by thapsigargin in weak Ca^{2+} buffer. (**A**) Time courses of development of I_{CRAC} following dialysis with thapsigargin alone (filled circles), thapsigargin plus cocktail (open circles) and thapsigargin alone after exposure to antimycin A and oligomycin (open diamonds) are shown. (**B**) Current–voltage relationships for the cells shown in (A) are depicted, taken when the whole-cell currents had reached steady state. (**C**) Bar chart plotting mean data for the three conditions. $*p < 0.01$.

Ca^{2+} release from intracellular stores is not compromized by mitochondrial depolarization

The results of Figure 3 are consistent with those summarized in Figures 1 and 2, although the experimental protocols employed differ slightly. In Figures 1 and 2, mitochondria were depolarized after store depletion, whereas in Figure 3, they were depolarized prior to store emptying. We considered that mitochondrial depolarization might somehow interfere with the ability of stores to empty. If so, then this would result in less store depletion and hence could account for the reduced I_{CRAC} in the experiments of Figure 3. To examine this, we compared the size of the Ca^{2+} release transient from $InsP_3$-sensitive stores between control cells and those exposed to antimycin A and oligomycin. In these experiments, we monitored Ca^{2+} release from stores by stimulating cell surface muscarinic receptors with carbachol instead of thapsigargin. Thapsigargin-evoked Ca^{2+} release in RBL cells is extremely variable in rate, rendering it difficult to quantify accurately (Parekh *et al*., 1997). Because the agonist- and thapsigargin-sensitive stores overlap completely in RBL cells (Ali *et al*., 1994; our unpublished data), receptor stimulation is a reasonable approach to use to probe the store Ca^{2+} content. Results are summarized in Figure 4. A typical control recording and one taken after exposure to antimycin A and oligomycin for 30 min are shown in Figure 4A, and averaged data for the peak increase in Ca^{2+} in Figure 4B (left panel). We also integrated the fluorescent signal arising from Ca^{2+} release from the stores (Figure 4B, right panel), but there was no significant difference between control cells and those pre-exposed to antimycin A and oligomycin for either method of analysis. It is important to note that such cytosolic Ca^{2+} measurements cannot give precise information about the amount of Ca^{2+} released from the stores. In the experiments of Figure 4, the rise in cytosolic Ca^{2+} concentration depends not only on the extent of Ca^{2+} release but also on both cytoplasmic Ca^{2+} buffering and the rate of Ca^{2+} extrusion via plasma membrane Ca^{2+} ATPases. It is possible that a reduced Ca^{2+} release from the stores could be matched exactly by reduced Ca^{2+} extrusion in the presence of antimycin/oligomycin, and this would produce an unchanged cytosolic Ca^{2+} transient. Nevertheless, the fact that the rate and extent of agonist-evoked Ca^{2+} release were largely unimpaired indicates that the reduction in I_{CRAC} in cells pre-treated with antimycin A and oligomycin (Figure 3), following dialysis with thapsigargin, is

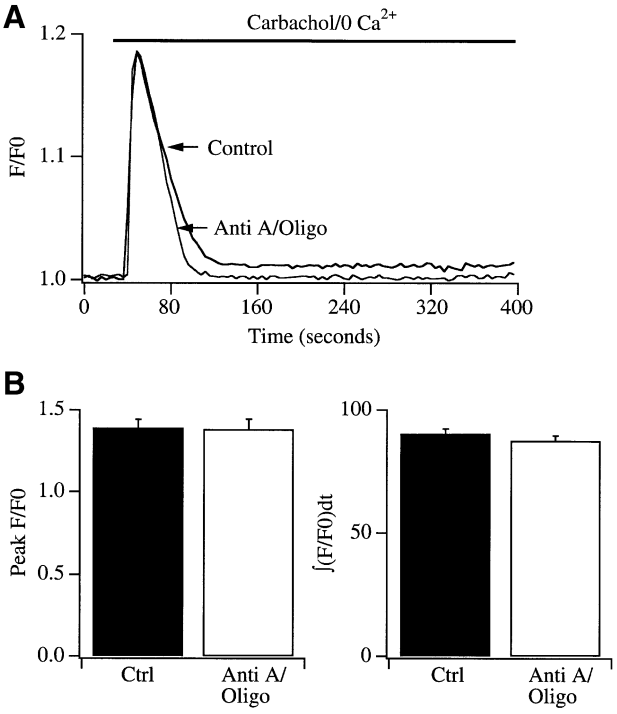

Fig. 4. Mitochondrial depolarization does not compromize Ca^{2+} release from $InsP_3$-sensitive Ca^{2+} stores. (**A**) The Ca^{2+} signal is shown for a control cell and for one pre-treated with antimycin A and oligomycin for 30 min. Carbachol was applied in Ca^{2+}-free external solution in order to selectively monitor Ca^{2+} release from the stores. (**B**) Aggregate data plotting the peak increase in the Ca^{2+} signal (left panel) and integrated Ca^{2+} response (right panel) are compared for the conditions indicated. $n = 11$ for each condition.

unlikely to reflect an inability to release Ca^{2+} from the stores.

Mitochondrial depolarization does not interfere with maximal activation of I_{CRAC} in strong buffer

Another possibility as to why mitochondrial depolarization suppresses the development of I_{CRAC} might reflect a need for mitochondria in the activation mechanism of the current. To investigate this, we dialysed cells with a solution containing $InsP_3$ in strong buffer (10 mM EGTA), which results in rapid and maximal activation of the current. We compared the size of I_{CRAC} between control cells and those cells pre-incubated with antimycin A and oligomycin. I_{CRAC} activated rapidly in both conditions and reached an overall extent not significantly different between the two conditions (Figure 5A and B). Hence mitochondrial depolarization *per se* does not impede the activation mechanism. Furthermore, these results argue against a direct CRAC channel-blocking action of antimycin A and oligomycin.

Mitochondrial depolarization interferes with maximal activation of I_{CRAC} in weak buffer

Although $InsP_3$ is ineffective in activating I_{CRAC} in weak buffer (0.1 mM EGTA), the combination of $InsP_3$ with thapsigargin is very effective (Fierro and Parekh, 2000). The current is activated to its maximal extent, in that the amplitude of I_{CRAC} is not significantly different between $InsP_3$ and thapsigargin in 0.1 mM EGTA versus $InsP_3$ in 10 mM EGTA, or $InsP_3$ and thapsigargin in 10 mM EGTA (Fierro and Parekh, 2000; Glitsch and Parekh, 2000). Figure 5C and D summarizes experiments designed to test whether mitochondrial depolarization interfered with the activation of I_{CRAC} by $InsP_3$ and thapsigargin in weak buffer. Whereas dialysis with $InsP_3$ and thapsigargin in 0.1 mM EGTA activated a large current (not significantly different from that in strong buffer; compare filled histograms in Figure 5B and D), pre-treatment with antimycin A and oligomycin significantly reduced the current by almost 80% (Figure 5C and D).

Mitochochondrial depolarization reduces the extent of I_{CRAC} evoked by adenophostin A

The non-metabolizable fungal metabolite adenophostin A, which has a several-fold higher affinity for $InsP_3$ receptors than $InsP_3$, can activate I_{CRAC} in weak buffer under conditions where $InsP_3$ is largely ineffective (Parekh *et al*., 2002). As with $InsP_3$, the size of the current in weak buffer is increased by maintaining mitochondria in an energized state. We examined whether mitochondrial depolarization impaired the activation of I_{CRAC} by adenophostin A (Figure 5E and F). The extent of I_{CRAC} by adenophostin A in the presence of mitochondrial cocktail was significantly reduced by antimycin A and oligomycin. Although just how adenophostin A activates I_{CRAC} is unclear (Parekh *et al*., 2002), the present results demonstrate that mitochondrial depolarization regulates the overall extent of the current in weak buffer when stores are emptied with a structurally distinct analogue of $InsP_3$.

The relatively infrequent activation of I_{CRAC} by $InsP_3$ in weak buffer can be suppressed by mitochondrial depolarization

Although $InsP_3$ is generally unable to activate I_{CRAC} in weak buffer, we have found on rare occasions (around 8% of the cells) that the current can develop partially. The mean amplitude of the current was -1.31 ± 0.14 pA/pF (six cells that responded under these conditions). If mitochondria were depolarized by pre-treatment with antimycin A and oligomycin, however, no such responses were seen in cells from the same preparations (mean amplitude -0.21 ± 0.06 pA/pF; six cells, $p < 0.01$). Presumably, in these controls cells, mitochondria are energized sufficiently to support some activation of I_{CRAC}.

Mitochondrial effects on I_{CRAC} in physiological buffer are not explained by changes in intracellular ATP

Mitochondrial depolarization results in reversal of the F_0F_1 ATP synthase and hence breakdown of cellular ATP. This can be prevented by inhibiting the ATP synthase with oligomycin. We carried out the following experiments to address whether the reduction in I_{CRAC} by mitochondrial depolarization reflected a fall in intracellular ATP. First, we compared the rate of Ca^{2+} influx following Ca^{2+} readmission to cells with stores depleted by pre-treatment with thapsigargin in control cells, with those pre-exposed to oligomycin alone in the absence of mitochondrial depolarization. The rate of entry was not significantly different between the two conditions (Figure 6A and B). Secondly, after store depletion, Fura 2-loaded cells were exposed to oligomycin for 15 min and then to oligomycin

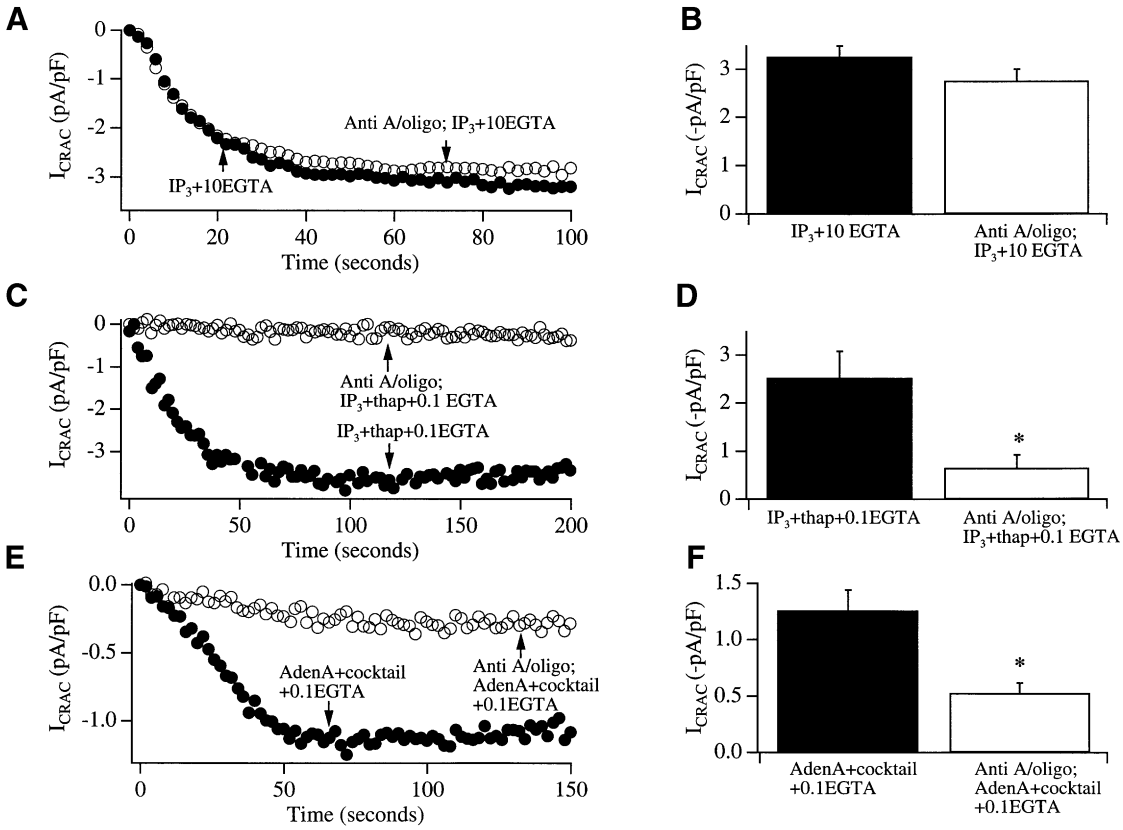

Fig. 5. Mitochondrial depolarization impairs I_{CRAC} in weak, but not strong, intracellular Ca^{2+} buffer. (**A**) The time course of development of I_{CRAC} in strong buffer (10 mM EGTA) is compared between a control cell (filled circles) and one exposed to antimycin A plus oligomycin (open circles). (**B**) Aggregate data for the two conditions (five cells each) are summarized. (**C**) The development of I_{CRAC} in weak buffer (0.1 mM EGTA) is significantly reduced by antimycin A and oligomycin. In these experiments, stores were emptied by the combination of $InsP_3$ (30 μM) and thapsigargin (2 μM), which activates I_{CRAC} maximally in control cells. (**D**) Summary of pooled data for the two conditions (six cells each). (**E**) The development of I_{CRAC} following store emptying by adenophostin A (1 μM) in the presence of mitochondrial cocktail is reduced by antimycin A and oligomycin. (**F**) Pooled data for the two conditions (six cells each).

plus FCCP. Ca^{2+} influx was still reduced following readmission of external Ca^{2+}, and there was no significant difference between FCCP-treated cells and those first exposed to oligomycin and then FCCP and oligomycin (Figure 6B). Thirdly, pre-treatment with oligomycin did not suppress the activation of I_{CRAC} following whole-cell dialysis with $InsP_3$ in the presence of mitochondrial cocktail in weak buffer, whereas the current was inhibited by pre-exposure to antimycin A and oligomycin (Figure 6C). Fourthly, in cells pre-treated with antimycin A and oligomycin, dialysis with $InsP_3$ together with 10 mM Mg-ATP failed to activate any detectable I_{CRAC} (Figure 6D). Hence high global levels of intracellular ATP did not seem to rescue the current when mitochondria were depolarized. Collectively, these results indicate that the reduction in I_{CRAC} in weak buffer by mitochondrial depolarization is unlikely to be explained simply by changes in intracellular ATP levels.

Effects of anti-oxidants, nitric oxide inhibitors, cyclosporin A and cytosolic pH on Ca^{2+} entry following mitochondrial depolarization

Mitochondrial depolarization can elicit a variety of intracellular changes, including oxidative stress, changes in the levels of nitric oxide (NO), opening of the permeability transition pore and acidification of the cytosol. We designed experiments to test whether such changes could account for the ability of mitochondrial depolarization to suppress I_{CRAC}. Pre-exposure to the anti-oxidants ascorbate (3 mM) or 2-MPG (*N*-2-mercaptopropionylglycine; 2 mM), to the NO synthase blocker *N*-nitro-L-arginine methyl ester (l-NAME; 500 μM), or to the permeability transition pore inhibitor cyclosporin A (5 μM) all failed to rescue the rate of calcium influx in cells with depolarized mitochondria, when compared with FCCP alone (Figure 7A). The inability of these agents to rescue Ca^{2+} entry might be masked if the drugs were directly blocking CRAC channels themselves. To check this, we compared the extent of activation of I_{CRAC} in strong buffer between control cells and those exposed to the various agents. However, the activation of the current was unimpaired (Figure 7B). Dialysis with an acidic intracellular pipette solution (pH 6.8) failed to interfere with the ability of thapsigargin to activate I_{CRAC} in weak buffer (Figure 7C), indicating that the slight acidification of cytosolic pH following mitochondrial depolarization that has been described is unlikely to underlie the inhibition of the current.

Ruthenium red interferes with the ability of thapsigargin to activate I_{CRAC} in weak buffer

Depolarization of mitochondria reduces the electric driving force for Ca^{2+} uptake through the mitochondrial Ca^{2+}-

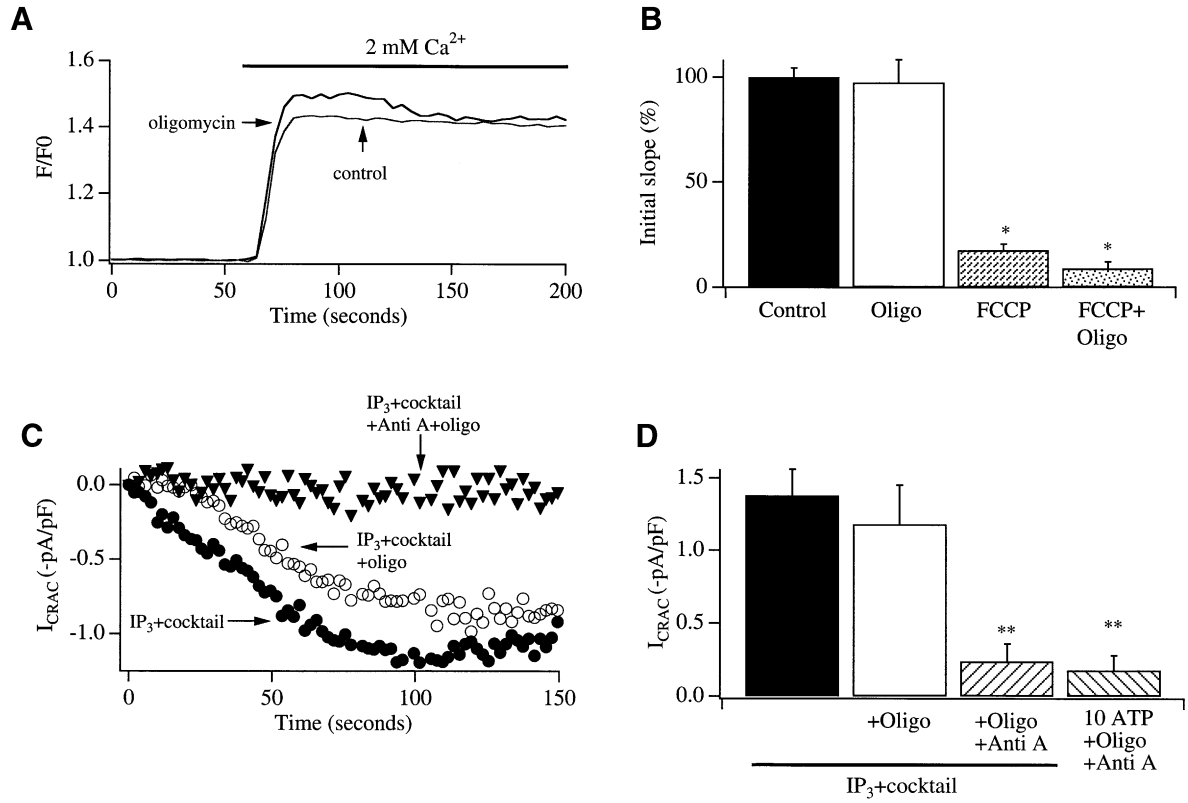

Fig. 6. Changes in intracellular ATP levels do not seem to underlie the effects of mitochondrial depolarization on Ca^{2+} entry through CRAC channels. (**A**) Exposure to oligomycin (>15 min) after store depletion fails to affect the rate of Ca^{2+} entry compared with a control cell. (**B**) Aggregate data are depicted from experiments carried out as in Figure 1 (14 cells each). Also shown are are the rates of Ca^{2+} entry (initial slopes) for cells exposed to FCCP alone or oligomycin and then FCCP plus oligomycin following store depletion in Ca^{2+}-free solution. The slopes were both significantly different from control, but not from each other. Oligomycin was added 15 min before FCCP plus oligomycin. (**C**) The activation of I_{CRAC} in weak Ca^{2+} buffer is impaired by antimycin A plus oligomycin but not oligomycin alone. Cells were dialysed with $InsP_3$ (30 μM) in the presence of mitochondrial cocktail. Pre-treatment with antimycin A and oligomycin (filled triangles) reduced the size of the current compared with a control recording (filled circles), but pre-treatment with oligomycin alone did not interfere with the development of I_{CRAC} (open circles). (**D**) Aggregate data for the three conditions in (C) are shown (seven cells for control, seven for oligomycin and five for antimycin A plus oligomycin). Also shown are averaged data showing that the inhibition of I_{CRAC} by pre-treatment with antimycin A plus oligomycin cannot be rescued by including 10 mM Mg-ATP in the pipette solution (six cells).

permeable uniporter and this impairs the ability of mitochondria to function as Ca^{2+} buffers. To determine whether reduced mitochondrial Ca^{2+} uptake could account for the inhibition of Ca^{2+} entry, we sought to separate Ca^{2+} uptake from mitochondrial depolarization by blocking the uniporter with ruthenium red. Dialysis with ruthenium red together with thapsigargin in weak buffer significantly reduced the size of the current compared with control recordings taken in the absence of the inhibitor (Figure 8). Unfortunately, specific inhibitors of the uniporter are lacking and ruthenium red has several additional targets. But ruthenium red does not interfere with the ability of I_{CRAC} to activate in strong buffer (Fierro and Parekh, 1999b), ruling out non-specific actions on the activation mechanism.

Discussion

Using a combination of fluorescent Ca^{2+} measurements in intact single cells, perforated patch recordings and whole-cell measurements of I_{CRAC}, our major new finding is that functional mitochondria are required for the activation of CRAC channels, and this is observed using rather diverse methods for emptying the intracellular Ca^{2+} stores ($InsP_3$, adenophostin A and thapsigargin). The involvement of mitochondria is especially prominent in weak intracellular Ca^{2+} buffer, suggesting that it is likely to be of considerable physiological importance.

Previously we have reported that mitochondrial Ca^{2+} uptake enables $InsP_3$ to deplete the stores sufficiently for I_{CRAC} to activate in weak Ca^{2+} buffer (Gilabert and Parekh, 2000; Gilabert *et al*., 2001). Because SERCA pumps in the ER membrane in RBL cells are located close to mitochondria (Csordas and Hajnoczky, 2001), and also because mitochondria sense microdomains of elevated Ca^{2+} that accompany opening of $InsP_3$ receptors (Rizzuto *et al*., 1993, 1998), mitochondrial Ca^{2+} uptake would be expected to compete with SERCA for removing cytosolic Ca^{2+} as well as to reduce Ca^{2+}-dependent inactivation of $InsP_3$ receptors. These mechanisms, alone or in combination, would lead to more extensive store depletion and hence activation of I_{CRAC}. Our new results demonstrate that the development of I_{CRAC} is significantly impaired by mitochondrial depolarization even when stores are emptied with thapsigargin. Thapsigargin depletes stores slowly, by relying on an endogenous Ca^{2+} leakage pathway that seems to involve translocons (Lomax *et al*., 2002) but that does not require heparin-sensitive $InsP_3$

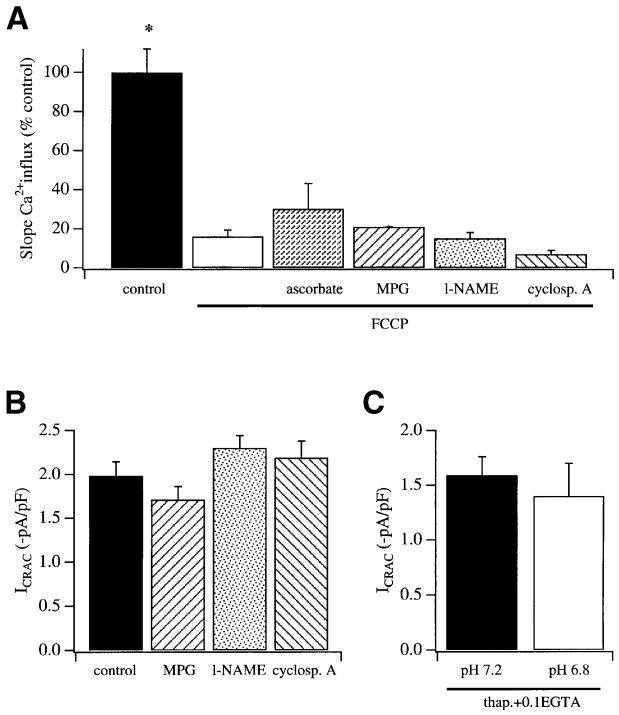

Fig. 7. (**A**) Following mitochondrial depolarization with FCCP, the rate of Ca^{2+} influx (slope) was significantly reduced compared with control cells. We then examined whether agents known to interfere with signals generated by mitochondria could overcome the reduction in Ca^{2+} influx by FCCP. However, none of these agents increased the rate of Ca^{2+} influx significantly when compared with FCCP alone. The experimental protocol was to deplete stores by applying thapsigargin in Ca^{2+}-free solution, and then expose cells to each agent (+thapsigargin) for >30 min before adding FCCP (+agent+thapsigargin) in Ca^{2+}-free solution. The number of cells for each condition was as follows: control (21), FCCP (30), FCCP + ascorbate (11), FCCP + MPG (14), FCCP + l-NAME (25), FCCP + cyclosporin (12). (**B**) The amplitude of I_{CRAC} was not affected by the agents tested. In these experiments, I_{CRAC} was recorded in 10 mM external Ca^{2+} and stores were depleted by including $InsP_3$ and 10 mM EGTA in the recording pipette. $n = 5$ cells for control, 3 for MPG, 6 for l-NAME and 5 for cyclosporin A. (**C**) Lowering pH from 7.2 (five cells) to 6.8 (seven cells) failed to affect the extent of activation of I_{CRAC}. Stores were depleted by dialysing cells with thapsigargin in 0.1 mM EGTA. The current activated slowly (after a delay of ~40 s), taking around 200 s to peak. The rate of development was similar for both conditions.

receptors (Fierro and Parekh, 1999b; Lomax *et al*., 2002). Hence the mitochondrial requirement for the development of I_{CRAC} is not restricted to $InsP_3$-mediated store depletion but instead appears to be a more general property under physiological conditions in RBL cells; a model system for studying I_{CRAC}.

How does mitochondrial depolarization suppress the extent of activation of I_{CRAC}? The site of action is clearly distal to store emptying because, first of all, mitochondrial depolarization reduces I_{CRAC} when administered after stores have already been emptied irreversibly with thapsigargin and, secondly, the amount of mobilizable Ca^{2+} within the $InsP_3$-sensitive stores is not significantly affected by mitochondrial depolarization. But mitochondria are unlikely to be involved in the activation mechanism of CRAC channels *per se* because mitochondrial depolarization did not interfere with the ability of CRAC channels to develop in the presence of strong intracellular Ca^{2+} buffer, a condition that obviates a role for mitochondrial Ca^{2+} uptake (Gilabert and Parekh, 2000). The fact that mitochondrial depolarization impairs I_{CRAC} in weak, but not strong, Ca^{2+} buffer imparts a Ca^{2+} dependence on the underlying mechanism. Furthermore, because ruthenium red reduces the ability of thapsigargin to activate I_{CRAC} in weak buffer, the Ca^{2+} dependence might require Ca^{2+} entry into the mitochondrial matrix via the ruthenium red-sensitive Ca^{2+} uniporter. Mitochondrial depolarization would reduce the organelle's role as an intracellular Ca^{2+} buffer and this could result in a larger and/or more sustained cytosolic Ca^{2+} increase following opening of CRAC channels. An increase in Ca^{2+} induces inactivation of CRAC channels, and this might explain the inhibition of I_{CRAC} following mitochondrial depolarization. However, we do not think that such a mechanism can wholly account for the inability of I_{CRAC} to activate following mitochondrial depolarization in weak intracellular Ca^{2+} buffer for the following reasons. First, Ca^{2+}-dependent deactivation of CRAC channels, due to increased refilling of stores, is unlikely to be operating under our conditions because the stores have been emptied by blocking the SERCA pumps with thapsigargin, and the $InsP_3$- and thapsigargin-sensitive stores overlap completely in these cells (Ali *et al*., 1994; our unpublished data). Secondly, Ca^{2+}-dependent slow inactivation requires a global increase in Ca^{2+} and develops relatively slowly with a half-time of around 65 s (Gilabert and Parekh, 2000) in weak Ca^{2+} buffer. One might have expected therefore to see an initial increase in I_{CRAC} followed by a subsequent slow inactivation as the Ca^{2+} levels rise to the level required to evoke such an inactivation process. However, this was not the case (see Figures 2 and 3). Furthermore, when Ca^{2+} was readmitted to cells maintained in thapsigargin/Ca^{2+}-free external solution in the presence of depolarized mitochondria, the rate of Ca^{2+} entry was significantly reduced. No rapid initial increase followed by a reduced rate of influx was observed. Thirdly, changes in Ca^{2+}-dependent fast inactivation are also unlikely to play a prominent role. Fast inactivation reflects negative feedback by permeating Ca^{2+} ions on the associated CRAC channel, and the binding site is thought to lie within 5 nm of the pore (Zweifach and Lewis, 1995; Fierro and Parekh, 1999a). Fast inactivation is still pronounced in the presence of the slow Ca^{2+} chelator EGTA. The rate and extent of fast inactivation were unaffected by either depolarizing mitochondria or energizing them with a mitochondrial cocktail solution included in the patch pipette solution (Gilabert and Parekh, 2000). Finally, Ba^{2+} influx in store-depleted cells (measured using fura 2) was suppressed by FCCP pre-treatment (data not shown). Since Ba^{2+} permeates CRAC channels but does not activate the three Ca^{2+}-dependent inhibitory pathways described above, this provides further evidence against a major role for Ca^{2+}-dependent inactivation in the reduction in the rate of Ca^{2+} influx following mitochondrial depolarization. It is possible that mitochondria first take up Ca^{2+} that is released slowly from the stores by thapsigargin, and then, upon their depolarization, release this Ca^{2+} locally onto CRAC channels, thereby inducing a form of Ca^{2+}-dependent inactivation. Such local release would not be detectable in our global Ca^{2+} measurements but would have to attain levels well in excess of 1 μM, the

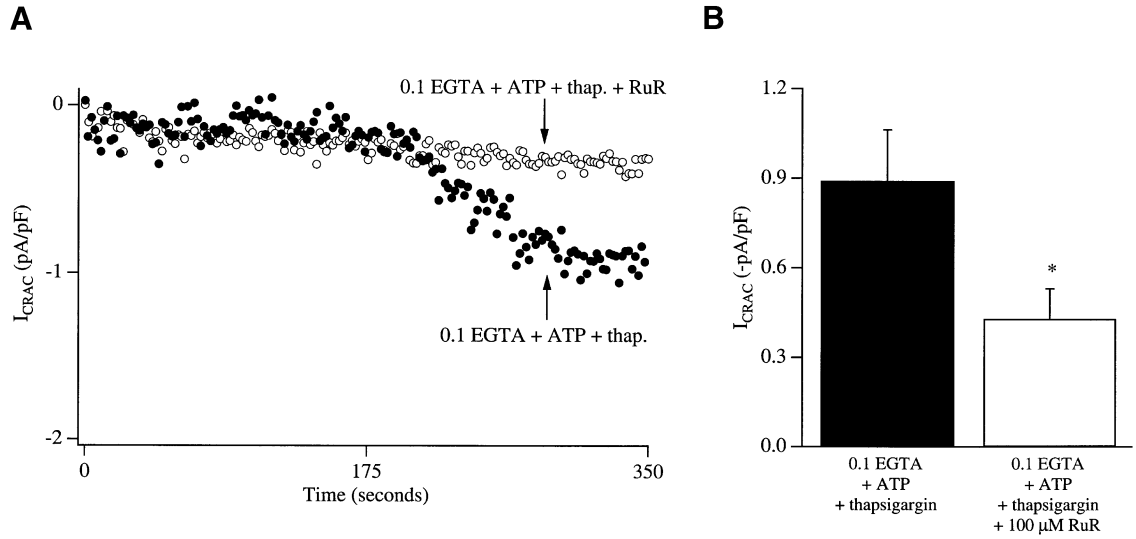

Fig. 8. The mitochondrial Ca^{2+} uptake inhibitor ruthenium red reduces the ability of I_{CRAC} to activate in weak intracellular Ca^{2+} buffer following store depletion with thapsigargin. (**A**) The time course of activation of I_{CRAC} is compared between a control cell (filled circles; dialysed with thapsigargin and 0.1 mM EGTA) and one in which ruthenium red (100 μM) had been added to this pipette solution (open circles). (**B**) Aggregate data for the two conditions are summarized [nine cells for control and eight for ruthenium red (RuR)].

latter concentration failing to inactivate CRAC channels in whole-cell recording. However, such a mechanism would require the mitochondria below the plasma membrane to take up Ca^{2+} that has been released from the stores and then release this Ca^{2+}, but not to be able to take up Ca^{2+} entering through CRAC channels (as there is no change in Ca^{2+}-dependent fast inactivation upon mitochondrial depolarization), which are presumably located in close proximity to such mitochondria. To date, there is no evidence to support the concept that mitochondria can selectively take up Ca^{2+} from one source but not another, despite being exposed to both. In fact, detailed studies by Montero *et al*. (2000) have found quite the opposite: mitochondria located close to the plasma membrane can avidly take up Ca^{2+} released from ryanodine-sensitive stores and Ca^{2+} channels in the plasma membrane.

An alternative explanation is that mitochondria release factors in a Ca^{2+}-dependent manner and such factors regulate CRAC channel activity. These factors are clearly not required for the activation of I_{CRAC} *per se* (since the current develops in strong buffer in spite of depolarized mitochondria) but may be more important under physiological conditions of weak intracellular Ca^{2+} buffering. The identity of such factors is, as yet, unclear. An increase in Ca^{2+} within the mitochondrial matrix stimulates key regulatory enzymes of the Krebs cycle, resulting in increased aerobic metabolism and hence production of intermediary metabolites and reducing equivalents (McCormack *et al*., 1990). In addition, Ca^{2+}-dependent transporters like the aspartate/glutamate shuttle are found in the inner mitochondrial membrane (Palmieri *et al*., 2001). Mitochondrial release of glutamate, for example, has been reported to prime secretory granules for exocytosis in pancreatic beta cells (Maechler and Wollheim, 1999).

Ca^{2+} entering through CRAC channels can be taken up by mitochondria (Lawrie *et al*., 1996; Hoth *et al*., 1997; Gilabert and Parekh, 2000) and this likely increases mitochondrial metabolism and hence ATP levels, which may be important in ensuring adequate energy supply following cell stimulation. Our findings raise the intriguing possibility for a reciprocal Ca^{2+}-dependent signal, emanating from mitochondria, which may regulate CRAC channels in the plasma membrane under physiological conditions. Further work is needed to establish whether such a link exists and, if so, its molecular identity.

Materials and methods

Cell preparation and solutions

RBL-1 cells, which were bought from Cell Bank at the Sir William Dunn School of Pathology, Oxford University, were grown in culture medium containing DMEM supplemented with 10% FCS and penicillin–streptomycin, as previously described (Fierro and Parekh, 2000). Cells were plated onto glass coverslips and used between 24 and 60 h after plating. Standard external solution contained: 145 mM NaCl, 2.8 mM KCl, 2 mM $CaCl_2$, 2 mM $MgCl_2$, 10 mM D-glucose, 10 mM HEPES pH 7.4 with NaOH. In Ca^{2+}-free solution, Ca^{2+} was simply omitted. For whole-cell patch–clamp recordings, the external solution contained: 145 mM NaCl, 2.8 mM KCl, 10 mM $CaCl_2$, 2 mM $MgCl_2$, 10 mM CsCl, 10 mM D-glucose, 10 mM HEPES pH 7.4 with NaOH. The standard pipette solution for whole-cell patch–clamp recordings contained: 145 mM Cs glutamate, 8 mM NaCl, 1 mM $MgCl_2$, 2 mM Mg-ATP, 0.1 mM EGTA, 10 mM HEPES pH 7.2 with CsOH. In a few experiments, EGTA was raised to 10 mM (strong buffer), as indicated in the text. In some experiments (described in the text), the standard pipette solution was supplemented with a mitochondrial cocktail solution, which maintains mitochondria in an energized state (Gunter and Pfeiffer, 1990). This cocktail contained: 2 mM pyruvic acid, 2 mM malic acid, 1 mM NaH_2PO_4, 0.5 mM cAMP, 0.5 mM GTP, 0.5 mM $MgCl_2$. The pipette solution for perforated patch recordings contained: 145 mM K glutamate, 8 mM NaCl, 5 mM $MgCl_2$, 10 mM HEPES, amphotericin B (250 μg/ml, diluted 1:500 from a frozen DMSO stock solution pH 7.2 with KOH). All reagents were from Sigma (Poole, UK) except cyclosporin A and l-NAME (both from Calbiochem).

Whole-cell patch–clamp experiments

Patch–clamp experiments were conducted in the tight-seal whole-cell configuration at room temperature (20–25°C), as described previously (Glitsch and Parekh, 2000; Bakowski *et al*., 2001). Sylgard-coated, fire-polished pipettes had d.c. resistances of 2.9–4 MΩ when filled with standard pipette solution. A correction of +10 mV was applied for the

subsequent liquid junction potential that arose from this glutamate-based internal solution. Mean series resistance was 7.0 ± 2.2 MΩ; 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 (Glitsch and Parekh, 2000; Bakowski *et al*., 2001). The currents were corrected for leak by averaging the first few ramps after break-in and then subtracting this from all subsequent currents. Currents were filtered using an 8-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 and EPC 9 –2 amplifiers.

Perforated patch recordings

Perforated patch recordings were carried out with amphotericin B. Mean series resistance in perforated patch recordings stabilized at 25.1 ± 4.4 MΩ within 5 min of forming a high-resistance seal (>10 GΩ). To deplete the stores fully in perforated patch recordings, external solution for whole-cell patch–clamp experiments was used but with Ca^{2+} omitted and 2 µM thapsigargin added. In some experiments, mitochondria were depolarized by applying antimycin A and oligomycin (added to Ca^{2+}-free-containing external solution containing thapsigargin) after 10 min pre-incubation with thapsigargin/Ca^{2+}-free external solution. I_{CRAC} was recorded in the perforated patch configuration by subsequent readmission of calcium-containing external solution.

Cytosolic calcium measurements

Calcium imaging experiments were carried out at room temperature using the IMAGO system from TILL Photonics (Bakowski *et al*., 2001). Cells were alternately excited at 356 and 380 nm (30 ms exposures) and images were acquired using the TILLVision software once every 2–4 s. The images were analysed off-line using IGOR Pro for Windows (Wavemetrics, Lake Oswego, OR). Cells were loaded with Fura 2-AM (1 µM) for 40 min at room temperature in standard external solution, as previously described (Bakowski *et al*., 2001). After loading, cells were washed three times and then left for 15 min to allow for further de-esterification. Results are presented as F/F0, where F0 denotes the ratio (356 nm/380 nm) prior to stimulation (averaged over 10 s) and F represents the change in the ratio as a function of time. Each image was corrected for background fluorescence.

Data are presented as mean ± SEM (number of experiments) and statistical evaluation was carried out using both Student's *t* and Mann–Whitney non-parametric tests.

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