Store-operative $\text{Ca}^{2+}$ entry depends on mitochondrial
$\text{Ca}^{2+}$ uptake

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Store-operative $\text{Ca}^{2+}$ channels, which are activated by the emptying of intracellular $\text{Ca}^{2+}$ stores, provide one major route for $\text{Ca}^{2+}$ influx. Under physiological conditions of weak intracellular $\text{Ca}^{2+}$ buffering, the ubiquitous $\text{Ca}^{2+}$ releasing messenger InsP$_3$ usually fails to activate any store-operative $\text{Ca}^{2+}$ entry unless mitochondria are maintained in an energized state. Mitochondria rapidly take up $\text{Ca}^{2+}$ that has been released by InsP$_3$, enabling stores to empty sufficiently for store-operative channels to activate. Here, we report a novel role for mitochondria in regulating store-operative channels under physiological conditions. Mitochondrial depolarization suppresses store-operative $\text{Ca}^{2+}$ 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 $\text{Ca}^{2+}$-dependent inactivation of the store-operative channels and does not involve changes in intracellular ATP, oxidants, cytosolic acidification, nitric oxide or the permeability transition pore, but is suppressed when mitochondrial $\text{Ca}^{2+}$ uptake is impaired. Our results suggest that mitochondria may have a more fundamental role in regulating store-operative influx and raise the possibility of bidirectional $\text{Ca}^{2+}$-dependent crosstalk between mitochondria and store-operative $\text{Ca}^{2+}$ channels.

Keywords: $\text{Ca}^{2+}$ influx/mitochondria/store-operative $\text{Ca}^{2+}$ channels

Introduction

Store-operative (capacitative) $\text{Ca}^{2+}$ influx, in which a fall in the $\text{Ca}^{2+}$ content of the endoplasmic reticulum (ER) opens $\text{Ca}^{2+}$ 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-operative $\text{Ca}^{2+}$ entry is manifested as a non-voltage-gated, inwardly rectifying $\text{Ca}^{2+}$ current called I$_{\text{CRAC}}$ (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 $\text{Ca}^{2+}$ rapidly following physiological levels of cell stimulation, and then subsequently release this $\text{Ca}^{2+}$ slowly back into the cytosol (Pozzan et al., 1994; Duchen, 2000; Rutter and Rizzuto, 2000). Mitochondrial $\text{Ca}^{2+}$ 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 $\text{Ca}^{2+}$ that has been released from intracellular stores by the opening of either InsP$_3$- or ryanodine-sensitive $\text{Ca}^{2+}$ 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 $\text{Ca}^{2+}$ that enters the cell via voltage-gated or store-operative 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 $\text{Ca}^{2+}$ dynamics have numerous effects on cell physiology. Buffering of cytosolic $\text{Ca}^{2+}$ by respiring mitochondria is seen in many diverse cell types, where it shapes the profile of the cytosolic $\text{Ca}^{2+}$ signal (Duchen, 2000; Rutter and Rizzuto, 2000). An increase in mitochondrial matrix $\text{Ca}^{2+}$, following rapid $\text{Ca}^{2+}$ 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 thezymogen-containing secretory granules (Tinel et al., 1999). Mitochondrial $\text{Ca}^{2+}$ uptake in the belt region restricts $\text{Ca}^{2+}$ 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 $\text{Ca}^{2+}$ release channels on the stores and voltage-operated $\text{Ca}^{2+}$ channels in the plasma membrane (Montero et al., 2000). Following stimulation, rapid intra-mitochondrial $\text{Ca}^{2+}$ transients in excess of 1 mM occur and this sequestration of $\text{Ca}^{2+}$ 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 $\text{Ca}^{2+}$ buffering, the ubiquitous second messenger InsP$_3$ generally fails to evoke any detectable whole-cell (macroscopic) I$_{\text{CRAC}}$ (Broad et al., 1999; Pierro and Parekh, 2000; Glitsch and Parekh, 2000). It appears that InsP$_3$, in spite of releasing $\text{Ca}^{2+}$ from the stores, fails to empty the stores sufficiently and/or long enough for I$_{\text{CRAC}}$ 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$_{\text{CRAC}}$ in physiological buffer (Gilabert and Parekh, 2000; Gilabert et al., 2001).

Mitochondria take up some of the $\text{Ca}^{2+}$ that has been released from the stores and this results in greater store depletion and hence activation of the current. In addition,
mitochondrial Ca²⁺ uptake reduces the extent of Ca²⁺-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 InsP₃ 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 Ca²⁺-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 Ca²⁺-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 Ca²⁺-sensitive fluorescent dye Fura 2 were exposed to the SERCA pump blocker thapsigargin in Ca²⁺-free external solution for 10–20 min in order to empty the intracellular Ca²⁺ stores and open the CRAC channels in the plasma membrane. Thereafter, external Ca²⁺ was readmitted. This resulted in a rapid increase in cytosolic Ca²⁺, 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 F₀F₁ ATP synthase, respectively), subsequent readmission of Ca²⁺ generated a smaller Ca²⁺ signal, which developed more slowly (Figure 1A and B). We measured the initial rate of rise of the Ca²⁺ 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 Ca²⁺ influx, the Ca²⁺ 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 Ca²⁺ 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 fundamentally different from the effects of mitochondria in enabling InsP₃ to activate IₐCRAC under physiological conditions (Gilabert and Parekh, 2000). In this latter case, by taking up some of the Ca²⁺ released by InsP₃, mitochondria promote greater store depletion and hence activation of IₐCRAC.

**Known Ca²⁺-dependent inactivation mechanisms cannot fully explain the inhibition of Ca²⁺ influx following mitochondrial depolarization**

The reduction in Ca²⁺ influx following mitochondrial depolarization in Figure 1 cannot be explained solely by Ca²⁺-dependent inactivation of CRAC channels. In rat basophilic leukaemia (RBL-1) cells, there are at least three mechanisms whereby an increase in cytosolic Ca²⁺ can lead to inactivation of IₐCRAC. Ca²⁺-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 Ca²⁺-dependent fast inactivation is
unlikely to be involved here. Ca²⁺-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. Ca²⁺ entry-dependent but store-independent inactivation requires a more global increase in Ca²⁺ (being suppressed by the slow chelator EGTA) and develops slowly with a half-time of around 60 s (Gilbert 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 Ca²⁺-dependent inactivation mechanisms seem unlikely to fully account for the inhibition of Ca²⁺ influx that occurs following mitochondrial depolarization, we nevertheless considered other potential Ca²⁺-dependent mechanisms. One possibility is that mitochondria rapidly take up much of the Ca²⁺ that has been released from the stores by thapsigargin, but then release this Ca²⁺ upon depolarization. This release may result in a form of Ca²⁺-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 Ca²⁺-free solution failed to produce any detectable Ca²⁺ increase (9/10 cells, data not shown). Hence it appears unlikely that mitochondrial depolarization results in a global Ca²⁺ increase following slow store emptying with thapsigargin. Secondly, dialysis with a pipette solution containing 1 μM Ca²⁺ did not prevent thapsigargin from evoking clear IₐCRAC (our unpublished observations). Therefore, in order for mitochondrial Ca²⁺ release to inactivate CRAC channels, an increase in cytosolic Ca²⁺ levels exceeding 1 μM globally would be required. It is conceivable that mitochondria take up Ca²⁺ following slow release from the stores and then release this Ca²⁺ locally, following depolarization, just below the plasma membrane such that the Ca²⁺ concentration exceeds 1 μM and inactivates the CRAC channels. But this explanation does not sit easily with the finding that fast Ca²⁺-dependent inactivation, reflecting local feedback of permeating Ca²⁺ on the CRAC channels, is not affected by either energizing (Gilbert 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 IₐCRAC 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 Ca²⁺ entry through CRAC channels, as has been suggested for the effects of the protonophore (CCCP) on antigen-stimulated Ca²⁺ 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 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 Ca²⁺-free external solution. Vehicle (DMSO) or antimycin A plus oligomycin was then added in Ca²⁺-free solution (in the presence of thapsigargin) and a seal was then formed. After obtaining background currents, Ca²⁺ was readmitted to the cell and the development of IₐCRAC 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 Ca²⁺, 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 Ca²⁺ 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 Ca²⁺ 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 IC\textsubscript{CRAC} is maintained in the perforated patch configuration and is likely therefore to be of physiological relevance.

**Mitochondria are required for thapsigargin to evoke IC\textsubscript{CRAC} in the whole-cell configuration**

Thapsigargin is able to activate IC\textsubscript{CRAC} routinely in weak intracellular Ca\textsuperscript{2+} buffer (0.1 mM EGTA) in the whole-cell patch-clamp configuration, whereas InsP\textsubscript{3} is ineffective under these conditions (Fierro and Parekh, 2000). A typical recording of the activation of IC\textsubscript{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\textsuperscript{2+} across the ER. Strikingly, if mitochondria were depolarized with antimycin A and oligomycin prior to exposure to thapsigargin in weak Ca\textsuperscript{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\textsuperscript{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 IC\textsubscript{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\textsuperscript{2+} uptake plays an important role in the ability of IC\textsubscript{CRAC} to activate under physiological conditions of weak intracellular Ca\textsuperscript{2+} buffering, even when the current is evoked by depolarizing stores with thapsigargin.

**Ca\textsuperscript{2+} release from intracellular stores is not compromised 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 IC\textsubscript{CRAC} in the experiments of Figure 3. To examine this, we compared the size of the Ca\textsuperscript{2+} release transient from InsP\textsubscript{3}-sensitive stores between control cells and those exposed to antimycin A and oligomycin. In these experiments, we monitored Ca\textsuperscript{2+} release from stores by stimulating cell surface muscarinic receptors with carbachol instead of thapsigargin. Thapsigargin-evoked Ca\textsuperscript{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\textsuperscript{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\textsuperscript{2+} in Figure 4B (left panel). We also integrated the fluorescent signal arising from Ca\textsuperscript{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\textsuperscript{2+} measurements cannot give precise information about the amount of Ca\textsuperscript{2+} released from the stores. In the experiments of Figure 4, the rise in cytosolic Ca\textsuperscript{2+} concentration depends not only on the extent of Ca\textsuperscript{2+} release but also on both cytoplasmic Ca\textsuperscript{2+} buffering and the rate of Ca\textsuperscript{2+} extrusion via plasma membrane Ca\textsuperscript{2+} ATPases. It is possible that a reduced Ca\textsuperscript{2+} release from the stores could be matched exactly by reduced Ca\textsuperscript{2+} extrusion in the presence of antimycin/oligomycin, and this would produce an unchanged cytosolic Ca\textsuperscript{2+} transient. Nevertheless, the fact that the rate and extent of agonist-evoked Ca\textsuperscript{2+} release were largely unimpaired indicates that the reduction in IC\textsubscript{CRAC} in cells pre-treated with antimycin A and oligomycin (Figure 3), following dialysis with thapsigargin, is
Mitochondrial depolarization does not interfere with maximal activation of $I_{\text{CRAC}}$ in strong buffer

Another possibility as to why mitochondrial depolarization suppresses the development of $I_{\text{CRAC}}$ 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 $I_{\text{CRAC}}$ between control cells and those cells pre-incubated with antimycin A and oligomycin. $I_{\text{CRAC}}$ 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 $I_{\text{CRAC}}$ in weak buffer

Although InsP$_3$ is ineffective in activating $I_{\text{CRAC}}$ 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 $I_{\text{CRAC}}$ 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 $I_{\text{CRAC}}$ 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).

Mitochondrial depolarization reduces the extent of $I_{\text{CRAC}}$ 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 $I_{\text{CRAC}}$ 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 $I_{\text{CRAC}}$ by adenophostin A (Figure 5E and F). The extent of $I_{\text{CRAC}}$ by adenophostin A in the presence of mitochondrial cocktail was significantly reduced by antimycin A and oligomycin. Although just how adenophostin A activates $I_{\text{CRAC}}$ 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 $I_{\text{CRAC}}$ by InsP$_3$ in weak buffer can be suppressed by mitochondrial depolarization

Although InsP$_3$ is generally unable to activate $I_{\text{CRAC}}$ 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 $-1.31 \pm 0.14$ 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 $-0.21 \pm 0.06$ pA/pF; six cells, $p < 0.01$). Presumably, in these controls cells, mitochondria are energized sufficiently to support some activation of $I_{\text{CRAC}}$.

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

Mitochondrial depolarization results in reversal of the FoF$_{1}$ 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 $I_{\text{CRAC}}$ 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
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, pretreatment with oligomycin did not suppress the activation of I\(_{\text{CRAC}}\) 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 pretreated with antimycin A and oligomycin, dialysis with InsP\(_3\) together with 10 mM Mg-ATP failed to activate any detectable I\(_{\text{CRAC}}\) (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\(_{\text{CRAC}}\) 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\(_{\text{CRAC}}\). 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\(_{\text{CRAC}}\) 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\(_{\text{CRAC}}\) 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\(_{\text{CRAC}}\) 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 Ca²⁺ entry through CRAC channels. (A) Exposure to oligomycin (>15 min) after store depletion fails to affect the rate of Ca²⁺ 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 the rates of Ca²⁺ entry (initial slopes) for cells exposed to FCCP alone or oligomycin and then FCCP plus oligomycin following store depletion in Ca²⁺-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 Ca²⁺ buffer is impaired by antimycin A plus oligomycin but not oligomycin alone. Cells were dialysed with InsP₃ (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).

Discussion

Using a combination of fluorescent Ca²⁺ 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 Ca²⁺ stores (InsP₃, adenophostin A and thapsigargin). The involvement of mitochondria is especially prominent in weak intracellular Ca²⁺ buffer, suggesting that it is likely to be of considerable physiological importance.

Previously we have reported that mitochondrial Ca²⁺ uptake enables InsP₃ to deplete the stores sufficiently for I_{CRAC} to activate in weak Ca²⁺ buffer (Gilbert and Parekh, 2000; Gilbert 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 Ca²⁺ that accompany opening of InsP₃ receptors (Rizzuto et al., 1993, 1998), mitochondrial Ca²⁺ uptake would be expected to compete with SERCA for removing cytosolic Ca²⁺ as well as to reduce Ca²⁺-dependent inactivation of InsP₃ 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 Ca²⁺ leakage pathway that seems to involve translocons (Lomax et al., 2002) but that does not require heparin-sensitive InsP₃.
Ca\(^{2+}\) buffer, a condition that obviates a role for mitochondrial Ca\(^{2+}\) uptake (Gilbert and Parekh, 2000). The fact that mitochondrial depolarization impairs I\(_{\text{CRAC}}\) in weak, but not strong, Ca\(^{2+}\) buffer impacts a Ca\(^{2+}\) dependence on the underlying mechanism. Furthermore, because ruthenium red reduces the ability of thapsigargin to activate I\(_{\text{CRAC}}\) 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\(_{\text{CRAC}}\) following mitochondrial depolarization. However, we do not think that such a mechanism can wholly account for the inability of I\(_{\text{CRAC}}\) 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). Second, Ca\(^{2+}\)-dependent slow inactivation requires a global increase in Ca\(^{2+}\) and develops relatively slowly with a half-time of around 65 s (Gilbert and Parekh, 2000) in weak Ca\(^{2+}\) buffer. One might have expected therefore to see an initial increase in I\(_{\text{CRAC}}\) 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 (Gilbert 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 \(\mu\)M, the

receptors (Fierro and Parekh, 1999b; Lomax et al., 2002). Hence the mitochondrial requirement for the development of I\(_{\text{CRAC}}\) 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\(_{\text{CRAC}}\).

How does mitochondrial depolarization suppress the extent of activation of I\(_{\text{CRAC}}\)? The site of action is clearly distal to store emptying because, first of all, mitochondrial depolarization reduces I\(_{\text{CRAC}}\) 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

**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 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 + Mg (14), FCCP + L-NAME (25), FCCP + cyclosporin (12). (B) The amplitude of I\(_{\text{CRAC}}\) was not affected by the agents tested. In these experiments, I\(_{\text{CRAC}}\) was recorded in 10 nM 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 Mg, 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\(_{\text{CRAC}}\). 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.
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 \(I_{\text{CRAC}}\) 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 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 Pfirffer, 1990). This cocktail contained: 2 mM pyruvic acid, 2 mM malic acid, 1 mM Na\(_2\)HPO\(_4\), 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, amphoterin C (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. $I_{\text{CRAC}}$ 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 (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. $I_{\text{CRAC}}$ 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 mM) 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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**References**


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