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Respiring mitochondria determine the pattern of activation and inactivation of the store-operated Ca^{2+} current I_{CRAC}

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In eukaryotic cells, hormones and neurotransmitters that engage the phosphoinositide pathway evoke a biphasic increase in intracellular free Ca^{2+} concentration: an initial transient release of Ca^{2+} from intracellular stores is followed by a sustained phase of Ca^{2+} influx. This influx is generally store dependent. Most attention has focused on the link between the endoplasmic reticulum and store-operated Ca^{2+} channels in the plasma membrane. Here, we describe that respiring mitochondria are also essential for the activation of macroscopic store-operated Ca^{2+} currents under physiological conditions of weak intracellular Ca^{2+} buffering. We further show that Ca^{2+}-dependent slow inactivation of Ca^{2+} influx, a widespread but poorly understood phenomenon, is regulated by mitochondrial buffering of cytosolic Ca^{2+}. Thus, by enabling macroscopic store-operated Ca^{2+} current to activate, and then by controlling its extent and duration, mitochondria play a crucial role in all stages of store-operated Ca^{2+} influx. Store-operated Ca^{2+} entry reflects a dynamic interplay between endoplasmic reticulum, mitochondria and plasma membrane.

Keywords: Ca^{2+} buffering/mitochondria/store-operated Ca^{2+} entry

Introduction

Store-operated or capacitative Ca^{2+} entry, in which the process of emptying the inositol 1,4,5-trisphosphate ($InsP_3$)-sensitive Ca^{2+} stores activates Ca^{2+} channels in the plasma membrane, is the major route for Ca^{2+} influx in non-excitable cells (Putney, 1986; Berridge, 1993). The best characterized and most widely distributed store-operated pathway is I_{CRAC} (Hoth and Penner, 1992; Parekh and Penner, 1997). Ca^{2+} entry through CRAC channels is important for a host of functions, including refilling the stores, regulating the period of Ca^{2+} oscillations, driving exocytosis and controlling cell growth and proliferation (Parekh and Penner, 1997). The duration of I_{CRAC} is, therefore, an important factor that determines the extent of activation of these Ca^{2+}-dependent processes. In many cells, I_{CRAC} is inactivated slowly following a global rise in intracellular Ca^{2+} (Ca^{2+}-dependent slow inactivation; Foskett and Wong, 1994; Zweifach and Lewis, 1995a; Parekh, 1998). Ca^{2+}-dependent slow inactivation is thought to contribute to the frequency of intracellular Ca^{2+} oscillations in various non-excitable cells as well as shaping the profile of the Ca^{2+} signal. The molecular mechanism underlying this slow inactivation is not known.

Under physiological conditions of weak intracellular Ca^{2+} buffering, $InsP_3$ fails to activate any whole-cell (macroscopic) I_{CRAC} (Broad *et al*., 1999; Fierro and Parekh, 2000; Glitsch and Parekh, 2000) despite substantial store emptying (Parekh *et al*., 1997). It was widely thought that Ca^{2+}-inactivation of CRAC channels was the explanation for the absence of I_{CRAC}. However, it has recently been established that this is not the case (Broad *et al*., 1999; Fierro and Parekh, 2000). Instead, it appears that the Ca^{2+} content of the stores needs to fall below a certain level in order for macroscopic I_{CRAC} to activate (Fierro and Parekh, 2000). Understanding why $InsP_3$ fails to activate I_{CRAC} consistently in the presence of weak Ca^{2+} buffer is essential if we are to place the current in a physiological context.

Using the patch–clamp technique to record directly the store-operated Ca^{2+} current I_{CRAC}, we find that mitochondria are a key determinant of the ability of I_{CRAC} to activate in weak Ca^{2+} buffer. Furthermore, we find that Ca^{2+} buffering by respiring mitochondria can reduce the extent of Ca^{2+}-dependent slow inactivation. We propose that, by determining activation and inactivation of macroscopic I_{CRAC}, mitochondria are key orchestrators of intracellular Ca^{2+} signalling.

Results

Comparison of voltage ramps versus steps on I_{CRAC} in strong Ca^{2+} buffer

I_{CRAC} was measured directly using the whole-cell patch–clamp technique. Stores were depleted rapidly and irreversibly by dialysing cells with a patch pipette solution containing a maximally effective concentration of the second messenger $InsP_3$ (30 μM; Parekh *et al*., 1997) and the SERCA pump blocker thapsigargin. Figure 1A shows the time-course of development of I_{CRAC} following dialysis with $InsP_3$, thapsigargin and strong Ca^{2+} buffer (10 mM EGTA), a protocol that activates the current to its maximal extent (Glitsch and Parekh, 2000). I_{CRAC} was monitored by applying voltage ramps (–100 to +100 mV in 50 ms) from a holding potential of 0 mV, and its amplitude was measured from the ramps at –80 mV. The inset in Figure 1A shows a typical ramp current (taken at 100 s), revealing the characteristics of I_{CRAC} (voltage independent, inwardly rectifying, Erev >+50mV). The graph in Figure 1C plots the amplitude of I_{CRAC} versus the time-constant (τ) of activation (n = 16). When I_{CRAC} was measured instead by applying voltage steps to –80 mV for 250 ms, the current developed to reach an extent similar to those obtained with ramps (time-course of a recording is shown in Figure 1B, pooled data displayed in Figure 1C,

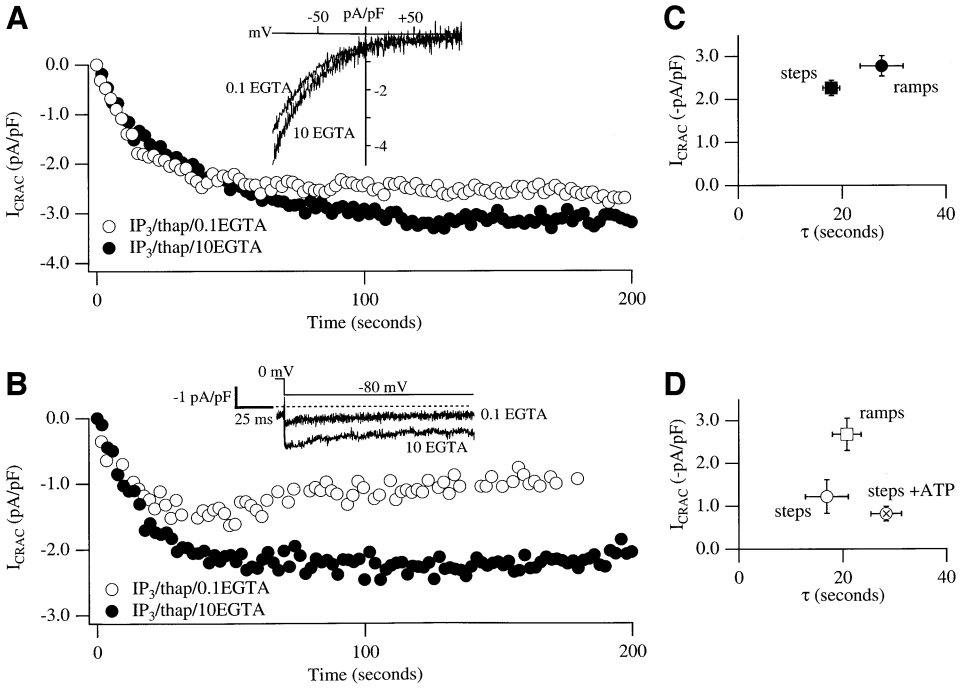

Fig. 1. I_{CRAC} is smaller using hyperpolarizing steps than ramps in weak intracellular Ca^{2+} buffer. (**A**) Time-course of I_{CRAC}, followed using voltage ramps, in strong and weak buffer (filled and open circles, respectively). Inset shows I–V relationships taken at 100 s. The current was measured at –80 mV from the ramps, and normalized for cell capacitance. (**B**) Time-course of I_{CRAC} in strong and weak buffer following voltage steps to –80 mV (inset). The current was measured between 0.8 and 0.9 ms after the step (see Materials and methods). (**C**) A plot of I_{CRAC} against activation time-constant in strong buffer using ramps or steps. (**D**) A similar plot to (C) but for low buffer. Note that inclusion of 2 mM Mg-ATP reduces the size of the current.

n = 17). In strong buffer, all cells responded irrespective of whether the ramp or step protocol was used.

Comparison of voltage ramps versus steps on I_{CRAC} in weak Ca^{2+} buffer

We then examined whether the size of I_{CRAC} was affected by using the ramp versus step protocols in the presence of weak intracellular Ca^{2+} buffer. This better represents endogenous levels of Ca^{2+} buffer found within cells. Superimposed in Figure 1A (open circles) are recordings taken using ramps for cells dialysed with weak buffer ($InsP_3$ + thapsigargin + 0.1 mM EGTA, n = 11; I–V relationship is shown in inset). The extent of the current as well as its rate of development were not significantly different from that seen in strong buffer (p = 0.6 each; Figure 1C and D). Only one cell failed to respond under these conditions. A strikingly different pattern was found, however, when I_{CRAC} was monitored by applying voltage steps (instead of ramps) in weak buffer (Figure 1B and D). The current was now significantly smaller when compared with either strong buffer (also employing the step protocol) or weak buffer (using voltage ramps), (p = 0.018 and 0.014, respectively). Furthermore, three of 11 cells failed to produce any detectable current using the step protocol in weak buffer. Supplementing the weak Ca^{2+} buffer solution with 2 mM Mg-ATP tended to reduce further the size of I_{CRAC}, when the current was measured using the voltage step protocol (but this was not significant, p = 0.29). In the presence of ATP, the rate of development of the current slowed slightly and, most dramatically, the probability that a cell would fail to generate detectable I_{CRAC} increased substantially (12/30 cells now failed to respond, Figure 2E).

During the voltage ramps, very little Ca^{2+} actually enters the cell because first, the ramp duration is so short (50 ms) and secondly, cells are at those negative potentials that favour Ca^{2+} entry only briefly. Since the amplitude of I_{CRAC} was similar using steps or ramps in strong buffer, the discrepancy in amplitudes between ramps and steps in weak buffer does not reflect the voltage protocols *per se*. Instead, in weak buffer, cytosolic Ca^{2+} would increase substantially during the step protocol. The elevated Ca^{2+} would be maintained after each voltage step because the SERCA pumps have been blocked with thapsigargin to prevent store refilling, and these pumps are very active in RBL cells (Fierro and Parekh, 1999a). In strong buffer on the other hand, the incoming Ca^{2+} during the long hyperpolarizing steps is effectively captured by the large amounts of free EGTA present in the cytosol.

The small I_{CRAC} seen in weak buffer using the step protocol represents Ca^{2+}-dependent slow inactivation, a well-documented feature of I_{CRAC} that operates over a time-frame of tens of seconds (Foskett and Wong, 1994; Zweifach and Lewis, 1995a; Parekh, 1998). Because thapsigargin was present throughout, the small current does not reflect Ca^{2+}-dependent deactivation of I_{CRAC} due to store refilling. Slow Ca^{2+}-dependent inactivation is thought to contribute to the frequency of intracellular Ca^{2+} oscillations in various non-excitable cells as well as shaping the profile of the Ca^{2+} signal (Foskett and Wong, 1994; Zweifach and Lewis, 1995a; Parekh and Penner, 1997). The molecular mechanism underlying this slow inactivation is not known but, in RBL cells, it does not

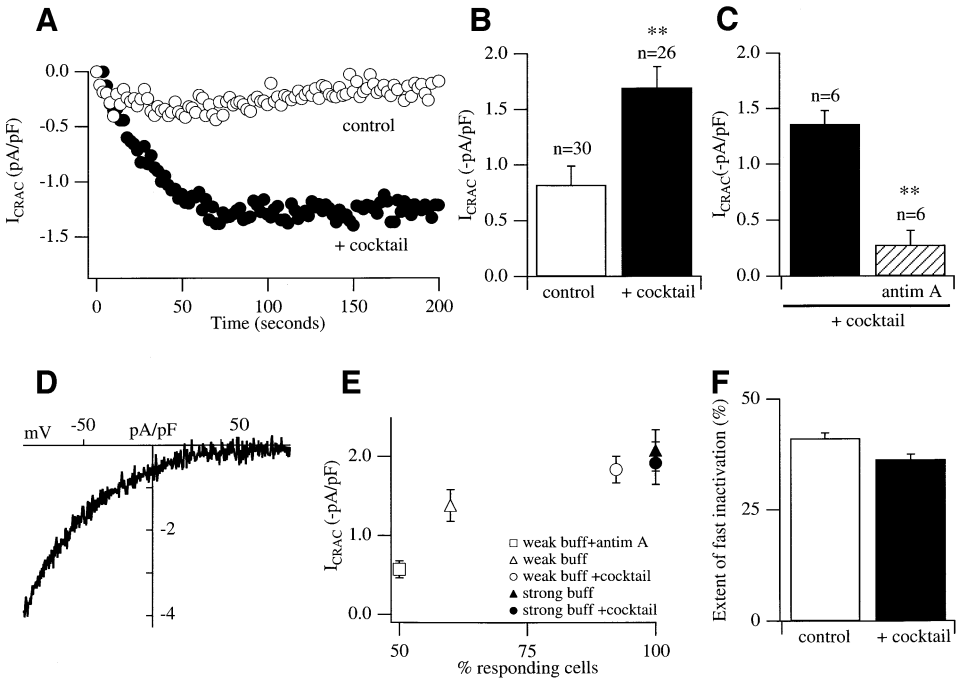

Fig. 2. Respiring mitochondria increase the size of I_{CRAC} in weak but not strong buffer. (**A**) Time-course of I_{CRAC} in weak buffer in the absence (control) and presence of the mitochondrial cocktail. (**B**) I_{CRAC} is significantly bigger in the presence of cocktail compared with control. (**C**) Pre-treatment with antimycin A and oligomycin prevents the enhancing effect of cocktail. The experiments were carried out on cells from the same preparations using the same solutions. Cells were incubated for at least 20 min in 5 μg/ml antimycin A and 0.5 μg/ml oligomycin. (**D**) I–V relationship for a cell dialysed with cocktail when I_{CRAC} had peaked. (**E**) A plot of current amplitude against % of responding cells in weak and strong buffer in the absence and presence of cocktail. (**F**) Extent of rapid inactivation in the absence (control) and presence of cocktail. For all panels, control refers to $InsP_3$ + thapsigargin + 0.1 mM EGTA + ATP.

seem to involve a protein kinase, phosphatase or GTP-binding protein (Parekh, 1998).

Respiring mitochondria reduce Ca^{2+}-dependent slow inactivation

Because mitochondria can take up significant amounts of Ca^{2+} following Ca^{2+} elevations in the cytosol (Rizzuto *et al*., 1993; Babcock *et al*., 1997; Duchen, 1999; Tinel *et al*., 1999) and can buffer Ca^{2+} that has entered through the store-operated pathway (Lawrie *et al*., 1996; Hoth *et al*., 1997; Hartmann and Verkhratsky, 1998), we investigated whether mitochondrial Ca^{2+} uptake affected the size of I_{CRAC} using the step protocol ($InsP_3$ + thapsigargin + 0.1 mM EGTA + ATP, henceforth control). We dialysed cells with a cocktail (malate, pyruvate, NaH_2PO_4, cAMP and GTP) known to be important for sustaining respiring mitochondria in the whole-cell recording configuration (Gunter and Pfeiffer, 1990; Villalba *et al*., 1994; Herrington *et al*., 1996). The effects were dramatic. Figure 2A compares the time-course of development of I_{CRAC} for cells dialysed in the absence (control) and presence of cocktail. Inclusion of cocktail significantly increased the size of the current almost 2-fold (Figure 2B, $p < 0.01$). This potentiation was due to a specific increase in I_{CRAC}, as opposed to another current, because the typical I–V relationship was preserved (Figure 2D). Furthermore, whereas only 60% of cells responded to control solution (using the step protocol), ~90% of cells responded in the presence of cocktail (Figure 2E). Inclusion of cocktail, therefore, increases the probability of obtaining macroscopic I_{CRAC}. The size of I_{CRAC} in responding cells in weak buffer was smaller than in the presence of cocktail, although this was not significant ($p = 0.10$).

Antimycin A accelerates Ca^{2+}-dependent slow inactivation

The effects of the cocktail appeared to be due entirely to an action on mitochondria because pre-treating cells with antimycin A, an inhibitor of complex III in the respiratory chain that results in mitochondrial depolarization (Duchen, 1999), together with oligomycin (to prevent the ATP synthase from operating in reverse), suppressed the enhancing effect of cocktail on I_{CRAC} (Figure 2C). The fraction of responding cells in the presence of antimycin A + cocktail, as well as the amplitude, was even less than control recordings made in the absence of cocktail (Figure 2E). This suggests that a little mitochondrial Ca^{2+} uptake is taking place even when cocktail is not present. Antimycin A + oligomycin have no effect on the extent of I_{CRAC} in strong buffer, ruling out effects on the CRAC channels themselves or their mechanism of activation (data not shown).

Effects of mitochondrial cocktail are Ca^{2+} dependent

The effects of the mitochondrial cocktail solution were Ca^{2+} dependent because no enhancing effect on I_{CRAC} was seen in strong Ca^{2+} buffer using either the ramp or step protocols [ramps: –2.13 ± 0.15 and –2.25 ± 0.35 for control and cocktail, respectively, ($n = 6$ for both, $p = 0.31$), steps: –2.07 ± 0.26 and –1.91 ± 0.27 ($n = 6$ for both, $p = 0.67$)]. Strong buffer prevents a rise in

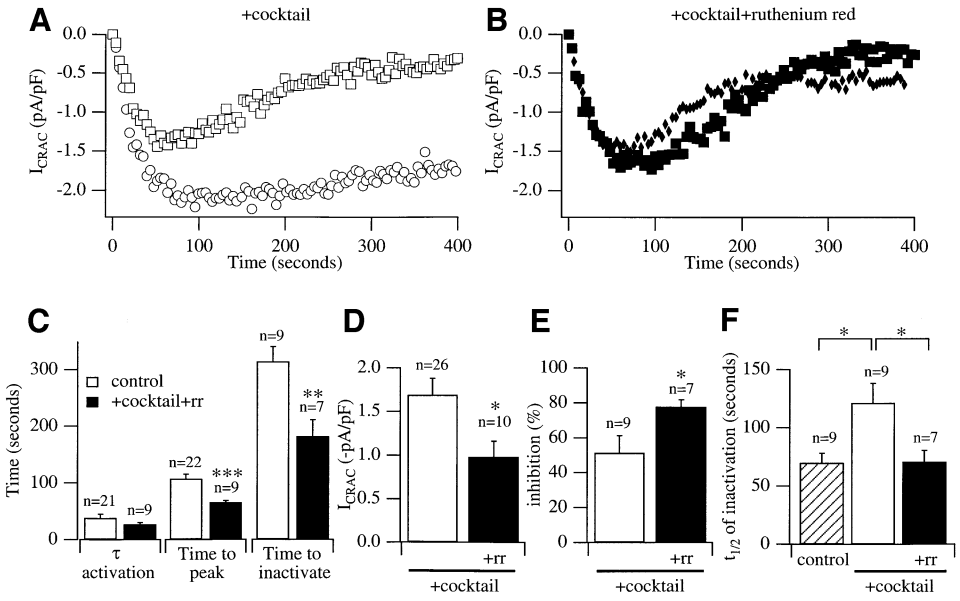

Fig. 3. Inhibition of Ca^{2+} uptake into respiring mitochondria reduces the extent of I_{CRAC} and accelerates slow inactivation. (**A**) Time-course of I_{CRAC} in the presence of the mitochondrial cocktail for two cells showing the range of slow inactivation. One showed only modest inactivation (circles), whereas the other inactivated substantially (squares). (**B**) Time-course of I_{CRAC} in two cells dialysed with 100 μM ruthenium red together with cocktail, showing substantial inactivation. We used this concentration in order to achieve high intracellular levels shortly after breaking into the cells. (**C–F**) Compare various properties of I_{CRAC} for cocktail (open bars) with cocktail + ruthenium red (filled bars). Apart from τ-activation, ruthenium red significantly reduced all parameters compared with cocktail alone. Time to inactivate was measured from the time at which I_{CRAC} had peaked to the time where inactivation had reached a steady state. The difference between peak current and steady-state current was taken as the inactivation % after normalization to peak amplitude.

intracellular Ca^{2+}, and so mitochondrial Ca^{2+} uptake will be substantially reduced.

Rapid Ca^{2+}-dependent inactivation of I_{CRAC} is not affected by mitochondrial Ca^{2+} uptake

Rapid Ca^{2+}-dependent inactivation of CRAC channels operates on a millisecond time-scale and reflects the build-up of a microdomain of Ca^{2+} in the vicinity of each open channel (Zweifach and Lewis, 1995b; Fierro and Parekh, 1999b). The Ca^{2+} binding site(s) are thought to lie <6 nm from the channel mouth (Zweifach and Lewis, 1995b). If mitochondria were physically very close to CRAC channels, then one might expect fast inactivation to be substantially reduced in the presence of cocktail. However, this was not the case (Figure 2F). Hence, mitochondria are located at least several nanometres away from the CRAC channel mouth.

Effects of ruthenium red

Mitochondrial Ca^{2+} uptake occurs through a uniporter that is blocked by ruthenium red (Duchen, 1999). To probe further the role of mitochondria on the properties of I_{CRAC}, we examined whether ruthenium red interfered with the effects of the cocktail. Figure 3A shows relatively long recordings from two cells dialysed with cocktail together with $InsP_3$ + thapsigargin + ATP in weak buffer. The current was followed using the voltage step protocol. Analysis of various features of the current is presented in Figure 3C–F (open bars). I_{CRAC} inactivated slowly over this time-frame but the extent of this was quite variable. Inclusion of ruthenium red in the pipette solution prevented the effects of the mitochondrial cocktail. The time-course of the current is shown in Figure 3B for two cells and averaged data are summarized in Figure 3C–F (filled bars). Although ruthenium red did not significantly alter τ activation (Figure 3C, $p = 0.25$), it significantly reduced (i) the time to peak of the current as well as the time to inactivate (Figure 3C), and (ii) the amplitude of I_{CRAC} such that the size was now similar to that seen without cocktail (Figure 3D). Furthermore, in the presence of ruthenium red, the extent of inactivation increased (Figure 3E) and slow inactivation developed more quickly (Figure 3F). The shorter time to peak in ruthenium red presumably reflects the faster onset of slow inactivation. Ruthenium red had no effect on the properties of I_{CRAC} in strong buffer, arguing against a direct action on CRAC channels themselves (data not shown). Ruthenium red also did not alter the extent of fast inactivation when compared with control or cocktail in weak buffer (data not shown).

Macroscopic I_{CRAC} activates in weak Ca^{2+} buffer provided mitochondria are active

In weak intracellular Ca^{2+} buffer, $InsP_3$ usually fails to activate any macroscopic I_{CRAC} unless SERCA pumps are blocked with thapsigargin (Fierro and Parekh, 2000). $InsP_3$ nevertheless reduces the Ca^{2+} content of the stores (Parekh *et al.*, 1997). Activation of macroscopic I_{CRAC}, therefore, seems to occur only when the Ca^{2+} content of the stores falls below a certain level, and the ability to reach this level depends on the prevalent SERCA pump activity. Any factor that regulates SERCA pump-mediated Ca^{2+} uptake is likely to have quite profound effects on Ca^{2+} entry under physiological levels of intracellular Ca^{2+} buffering. We hypothesized that mitochondrial Ca^{2+} uptake might be able to compete with the SERCA pumps for removing cytosolic Ca^{2+} following $InsP_3$-mediated Ca^{2+} release, as well as

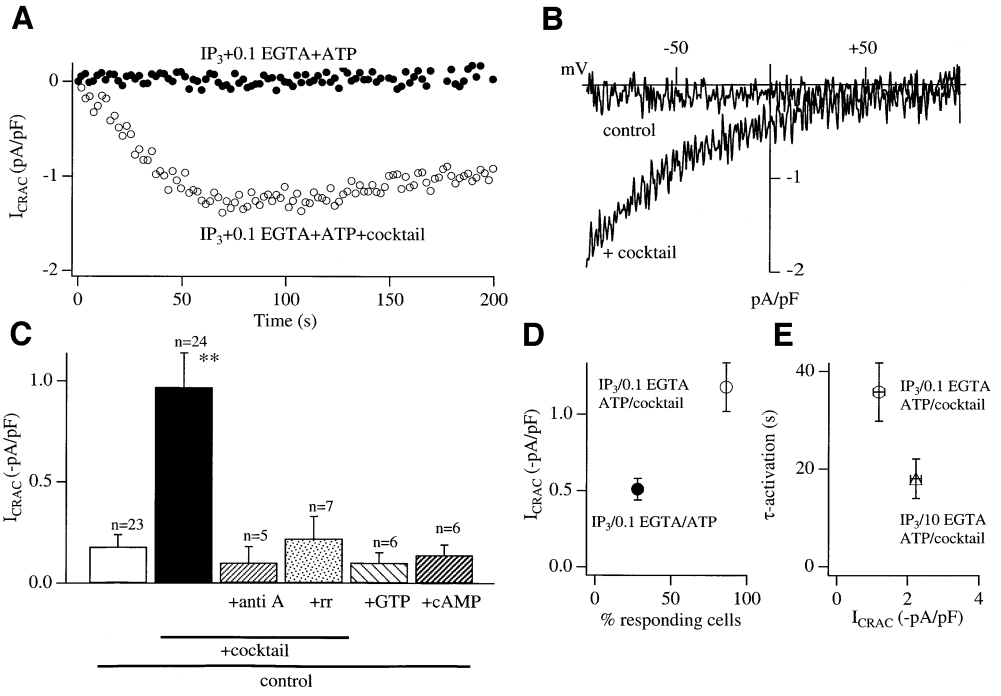

Fig. 4. I_{CRAC} activates to $InsP_3$ alone in weak buffer provided mitochondria are active. (**A**) Time-course of I_{CRAC} for a cell dialysed with $InsP_3$ + 0.1 mM EGTA + ATP (control) and then for one supplemented with cocktail. No thapsigargin was present. (**B**) I–V relationship from voltage ramps is shown (taken when after 60 s). (**C**) Bar chart depicting the enhancing effect of cocktail, and that the mitochondrial inhibitors antimycin A (+ oligomycin) and ruthenium red prevent the effects of cocktail. Control + cAMP or GTP did not mimic the effects of cocktail. Again, thapsigargin was not present. (**D**) Plot of amplitude of I_{CRAC} versus % responding cells for control and cocktail-treated cells (no thapsigargin present). Only the amplitudes of responding cells were analysed. (**E**) τ-activation versus amplitude of I_{CRAC} is plotted for cocktail-treated cells in weak buffer (no thapsigargin) and strong buffer ($InsP_3$ + thapsigargin + 10 mM EGTA + ATP). Note that in weak buffer without thapsigargin, I_{CRAC} was still sub-maximal in spite of cocktail. This reflects some SERCA-dependent refilling, because the current was larger in weak buffer when thapsigargin was included.

reducing possible Ca^{2+}-inactivation of $InsP_3$ receptors, especially since mitochondria can be spatially very close to the sites of Ca^{2+} release from the endoplasmic reticulum (Jouvaille *et al*., 1995; Rizzuto *et al*., 1998; Hajnoczky *et al*., 1999). Respiring mitochondria might, therefore, enable $InsP_3$ to deplete the stores more effectively such that the threshold for macroscopic activation of I_{CRAC} is reached. To test this, we dialysed cells with $InsP_3$ and weak buffer in the absence and presence of cocktail. Importantly, thapsigargin was not included in the pipette solution so SERCA pumps were active. $InsP_3$ in the absence of cocktail usually failed to activate any detectable I_{CRAC} (Figure 4A and B), and, in the small fraction of cells that responded, the current was very small (Figure 4C). Inclusion of cocktail had quite pronounced effects. A robust I_{CRAC} could now be activated (Figure 4A and B). Furthermore, the majority of cells now responded (Figure 4D), although the size of the current was smaller than in strong buffer and activated more slowly (Figure 4E). The effects of the cocktail were prevented by pre-treatment with the combination of antimycin A and oligomycin, or by including ruthenium red in the pipette together with $InsP_3$ and cocktail (Figure 4C). On the other hand, dialysis with only cAMP or GTP (two constituents of the cocktail) failed to enhance the current (Figure 4C).

Discussion

Our new findings demonstrate that Ca^{2+} uptake into mitochondria is essential for the normal functioning of the store-operated Ca^{2+} current under the physiological conditions of weak intracellular Ca^{2+} buffering. Our results provide a novel explanation for a long-standing problem in the calcium signalling field, namely why the Ca^{2+}-mobilizing second messenger $InsP_3$ fails to activate macroscopic I_{CRAC} in weak Ca^{2+} buffer. It was generally believed that this inability of $InsP_3$ reflected Ca^{2+}-inactivation of CRAC channels, but we and others have recently shown that this is not so (Broad *et al*., 1999; Fierro and Parekh, 2000). Instead, it seems that substantial depletion of stores is required for macroscopic I_{CRAC} to activate, and $InsP_3$ is not able to exceed this apparent threshold. We now find that Ca^{2+} uptake by respiring mitochondria is essential for I_{CRAC} to activate under physiological conditions. We propose that removal of cytosolic Ca^{2+} by mitochondria competes effectively with store refilling by SERCA pumps, and may also reduce Ca^{2+}-inactivation of $InsP_3$ receptors. Combined, this would enable $InsP_3$ to deplete the stores sufficiently for macroscopic I_{CRAC} to activate (see Figure 5 and legend for a cartoon depicting this model). Detailed studies have established that mitochondria can be physically close to endoplasmic reticulum (Rizzuto *et al*., 1998) and sense Ca^{2+} microdomains generated by open $InsP_3$ receptors (Jouvaille *et al*., 1995; Hajnoczky *et al*., 1999). Our results provide a functional explanation for this close apposition and indicate that cross-talk between the two organelles determines whether macroscopic I_{CRAC} activates under the conditions of weak intracellular buffer. In strong buffer, mitochondrial uptake will be severely attenuated, SERCA

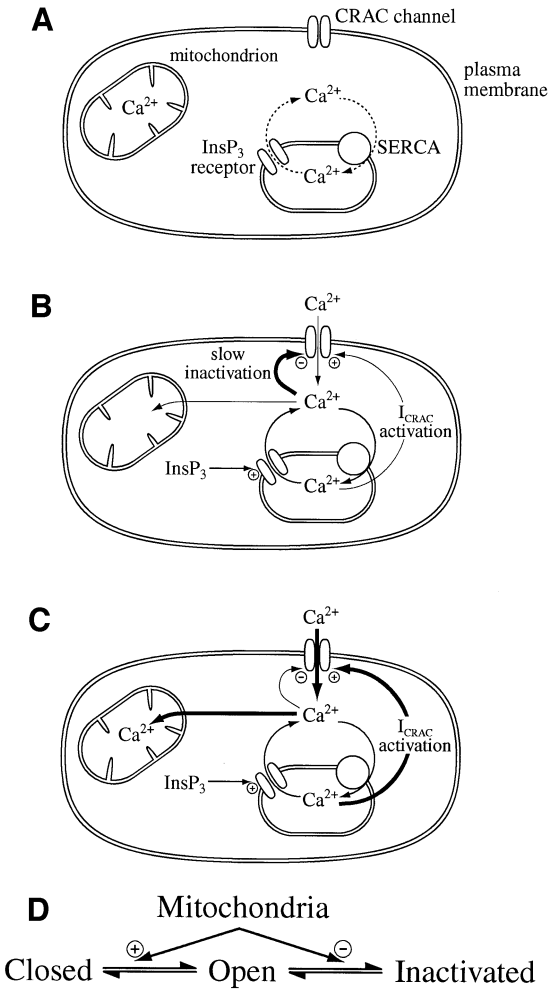

Fig. 5. Cartoon summary of mitochondrial role in I_{CRAC} in weak (physiological) intracellular Ca^{2+} buffer. (**A**) Shows the resting state, where I_{CRAC} is not functioning. Stores are full and any Ca^{2+} that leaks from the stores is re-sequestrated by the SERCA pumps. (**B**) Following an increase in the levels of the second messenger $InsP_3$ in the absence of active mitochondrial Ca^{2+} uptake, Ca^{2+} is released from the stores. However, the SERCA pumps are able to re-sequestrate sufficient Ca^{2+} to prevent the threshold for macroscopic activation of I_{CRAC} from being reached. Only a very small fraction of CRAC channels are activated (undetectable in whole-cell mode). Furthermore, the rise in cytosolic Ca^{2+} results in strong inactivation of I_{CRAC} through Ca^{2+}-dependent slow inactivation. (**C**) In the presence of respiring mitochondria, $InsP_3$ activates macroscopic I_{CRAC}. Ca^{2+} released from the stores by $InsP_3$ is taken up by mitochondria through a ruthenium red-sensitive uniporter. This reduces the amount of Ca^{2+} available to the SERCA pumps and in the vicinity of open $InsP_3$ receptors, such that the stores are depleted sufficiently for macroscopic I_{CRAC} to activate (less refilling by SERCA pumps and less inactivation of $InsP_3$ receptors). I_{CRAC} now activates. Some refilling does occur because inclusion of thapsigargin enhances the size of the current. Furthermore, mitochondrial Ca^{2+} buffering reduces the rate and extent of Ca^{2+}-dependent slow inactivation, thereby increasing the size and duration of the current. (**D**) A simplified gating scheme for CRAC channels summarizing the role of mitochondrial Ca^{2+} buffering. Mitochondria facilitate opening (Closed to Open transition) whilst simultaneously reducing inactivation (Open to Inactivated transition). In this way, mitochondria have a much larger impact on I_{CRAC} than through either transition alone.

pumps will be much less active and $InsP_3$ routinely activates I_{CRAC}.

Could mitochondria be directly involved in the activation mechanism of I_{CRAC} through a process independent of Ca^{2+} buffering? In pancreatic β cells, mitochondria release a diffusible factor (glutamate) that promotes Ca^{2+}-independent secretion of insulin (Maechler and Wollheim, 1999). However, we do not think that mitochondria release the activating signal for I_{CRAC} because pre-treating cells with antimycin/oligomycin does not prevent I_{CRAC} from developing in strong Ca^{2+} buffer.

Finally, our results provide new insight into the important, but elusive mechanism underlying slow inactivation of I_{CRAC}. Increasing Ca^{2+} uptake into mitochondria delays the onset of slow inactivation and reduces its extent, whereas inactivation develops more quickly and to a greater extent when mitochondrial uptake is compromised. It is particularly striking that the same mechanism, Ca^{2+} buffering by mitochondria, should have profound effects on both the activation and inactivation of I_{CRAC}. By facilitating activation (through ensuring more store depletion) and, at the same time, reducing slow inactivation, mitochondria will increase I_{CRAC} to a much larger extent than through an action on activation or inactivation alone. A gating scheme depicting this dual action of mitochondria on I_{CRAC} is shown in Figure 5D.

Under physiological conditions, mitochondria therefore play a pivotal role in all stages of store-operated Ca^{2+} entry, determining whether macroscopic I_{CRAC} activates, to what extent, and then for how long Ca^{2+} entry stays operational.

Materials and methods

Cell culture

Rat basophilic leukaemia cells (RBL-1) cells, which were bought from Cell Bank at the Sir William Dunn School of Pathology, Oxford University, were cultured as previously described (Fierro and Parekh, 2000).

Electrophysiology

Patch–clamp experiments were conducted in the tight-seal whole-cell configuration at room temperature (20–25°C) as previously described (Parekh, 1998; Fierro and Parekh, 2000). Sylgard-coated fire-polished pipettes had d.c. resistances of 2.9–4 MΩ when filled with standard internal solution that contained: 145 mM caesium glutamate, 8 mM NaCl, 1 mM $MgCl_2$, 0.03 mM $InsP_3$, 10 mM HEPES pH 7.2 with CsOH. EGTA was included in the pipette solution at concentrations of either 0.1 mM (weak buffer) or 10 mM (strong buffer), as indicated in the text. Thapsigargin (2 μM) was always included in the pipette solution, except for the experiments of Figure 4. A correction of +10 mV was applied for the subsequent liquid junction potential that arose from this glutamate-based internal solution. Mitochondrial cocktail contained: 2 mM pyruvic acid, 2 mM malic acid, 1 mM NaH_2PO_4, 0.5 mM cAMP, 2 mM Mg^{2+}-ATP, 0.5 mM GTP. Extracellular 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. I_{CRAC} was measured by applying either voltage ramps (–100 to +100 mV in 50 ms) or hyperpolarizing voltage steps (to –80 mV, 250 ms duration) at 0.5 Hz from a holding potential of 0 mV as previously described. 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 or averaged between 0.8 and 0.9 ms after the onset of the hyperpolarizing step) by the cell capacitance. The settling time of the clamp was ~100 μs (typical series resistance of 9 MΩ and cell capacitance of 15 pF). Peak currents were therefore measured at 0.8–0.9 ms after the step to minimize potential contributions from uncompensated capacitative currents. Furthermore, I_{CRAC} inactivates partially (40%) during a hyperpolarizing step to –80 mV under the present conditions, with time-constants of ~10 and 120 ms (Fierro and Parekh, 1999b). By measuring the current very shortly after the onset of the step, we obtained a better estimate of the true peak. Capacitative currents were compensated before each ramp by using the automatic compensation of the EPC 9 and EPC 9-2 amplifiers. All leak currents were subtracted by

averaging the first ramp current (or two), and then subtracting this from all subsequent currents. Data are presented as mean ± SEM, and statistical evaluation was carried out using both Student's *t* and Mann–Whitney non-parametric tests.

Thapsigargin was purchased from Alomone laboratories. All other chemicals were purchased from Sigma.

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