Mitochondrial control of calcium-channel gating: A mechanism for sustained signaling and transnational activation in T lymphocytes

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In addition to their well-known functions in cellular energy transduction, mitochondria play an important role in modulating the amplitude and time course of intracellular Ca2⁺ signals. In many cells, mitochondria act as Ca2⁺ buffers by taking up and releasing Ca2⁺, but this simple buffering action by itself often cannot explain the organelle's effects on Ca2⁺ signaling dynamics. Here we describe the functional interaction of mitochondria with store-operated Ca2⁺ channels in T lymphocytes as a mechanism of mitochondrial Ca2⁺ signaling. In Jurkat T cells with functional mitochondria, prolonged depletion of Ca2⁺ stores causes sustained activation of the store-operated Ca2⁺ current, I_{CRAC} (CRAC, Ca2⁺ release-activated Ca2⁺). Inhibition of mitochondrial Ca2⁺ uptake by compounds that dissipate the intramitochondrial potential unmasks Ca2⁺-dependent inactivation of I_{CRAC}. Thus, functional mitochondria are required to maintain CRAC-channel activity, most likely by preventing local Ca2⁺ accumulation near sites that govern channel inactivation. In cells stimulated through the T-cell antigen receptor, acute blockade of mitochondrial Ca2⁺ uptake inhibits the nuclear translocation of the transcription factor NFAT in parallel with CRAC channel activity and [Ca2⁺]i elevation, indicating a functional link between mitochondrial regulation of I_{CRAC} and T-cell activation. These results demonstrate a role for mitochondria in controlling Ca2⁺ channel activity and signal transmission from the plasma membrane to the nucleus.

Mitochondria are becoming widely recognized as multifunctional organelles that are essential not only for cellular energy transduction but also for pathways of Ca2⁺ signaling, homeostasis, and cell death (1–7). As Ca2⁺ signaling organelles, they can rapidly take up and slowly release large amounts of Ca2⁺, enabling them not only to protect cells against cytosolic Ca2⁺ overload under pathophysiological conditions but also to shape and prolong physiological Ca2⁺ signals (1, 2, 8, 9).

Recent evidence suggests that mitochondria can also modulate the flux of Ca2⁺ across cell membranes. In a variety of nonexcitable cells, mitochondria have been shown to influence signals generated by Ca2⁺ release from the endoplasmic reticulum (ER), presumably through intimate interactions with the inositol 1,4,5-trisphosphate (IP3) receptor (1, 4–7, 10, 11). In T lymphocytes, functional mitochondria act to sustain store-operated Ca2⁺ entry after prolonged depletion of intracellular Ca2⁺ stores (12). These examples show clearly how mitochondria may extend their influence on Ca2⁺ signaling by modulating Ca2⁺ transport pathways; however, the mechanisms underlying these phenomena are unknown. Mitochondria could alter Ca2⁺ channel activity itself or they could alter indirectly net Ca2⁺ fluxes by regulating Ca2⁺-ATPases or by regulating the K⁺ or Cl⁻ channels that set the driving force for Ca2⁺ entry. Furthermore, these effects in principle could be mediated by changes in local or global cell [Ca2⁺], or by changes in the ATP/ADP ratio, both of which are known to affect the function of IP3 receptors (13) and store-operated Ca2⁺ channels (14–18). In addition, the physiological significance of these phenomena, if any, has not been demonstrated clearly in any system.

In this study, we examine the interaction of mitochondria with store-operated Ca2⁺ channels in T lymphocytes. Antigenic stimulation of T cells triggers intracellular Ca2⁺ release by IP3 and the consequent opening of Ca2⁺ release-activated Ca2⁺ (CRAC) channels that in turn generate the prolonged elevation of cytosolic Ca2⁺ ([Ca2⁺]) required for T-cell activation (19–21). Using whole-cell and perforated-patch recording techniques, we find that mitochondria prevent the inactivation of CRAC channels that results from prolonged Ca2⁺ influx. Moreover, after stimulation through the T-cell antigen receptor, mitochondrial inhibition reduces the [Ca2⁺]i signal and in parallel inhibits the nuclear import of NFAT, a critical transcription factor for T-cell activation (22, 23). The results show that mitochondria regulate an ion channel, identify the mechanism for their interaction, and illustrate implications for downstream cellular events.

Materials and Methods

Cell Culture and Solutions. Jurkat cell clone E6–1 [American Type Culture Collection, Rockville, MD (ATCC 1378)] and a parental diphertheria toxin-resistant Jurkat clone (21) were grown in culture medium consisting of RPMI 1640 supplemented with 10% FCS and penicillin–streptomycin (21). Normal Ringer’s solution contained (in mM): 155 NaCl, 4.5 KCl, 2 CaCl2, 1 MgCl2, 10 D-glucose, and 5 Heps (pH 7.4 with NaOH). CaCl2 was replaced by MgCl2 in the 0-Ca2⁺ Ringer’s solution. The standard pipette solution for whole-cell patch-clamp recordings contained (in mM): 140 cesium aspartate, 2 MgCl2, 0.66 CaCl2, 1.2 EGTA ([Ca2⁺]i ≈ 5 nM), 0.1 indo-1 pentapotassium salt, and 10 Heps (pH 7.2 with CsOH). The pipette solution for perforated-patch recordings contained (in mM): 115 NaCl, 4.5 KCl, 1 MgCl2, 10 NaCl, 10 Heps (pH 7.2 with CsOH), and nystatin (200–300 µg/ml, diluted 1:500 from a DMSO stock solution made daily). Reagents include thapsigargin (TG, LC Laboratories, Woburn, MA), carbonyl cyanide m-chlorophenylhydrazone (CCCP, Sigma), antimycin A1 (Sigma), and oligomycin (Sigma). OKT3 ascites was a generous gift from Gerald Crabtree (Stanford University, Stanford, CA).

Combined Patch-Clamp and [Ca2⁺]i Measurements. Patch pipettes were pulled from 100-µl capillaries, coated with Sylgard, and fire polished. Membrane currents were recorded at 22–25°C with an
Two methods were used to deplete fully intracellular Ca2+ during experiments by staining with Hoechst 33258 (not shown). Extent of depletion varied with the number of cells. Nuclear location was confirmed in several experiments by measuring EGFP intensity to facilitate analysis of a much larger number of cells. Nuclear and cytoplasmic EGFP signals are expected from out-of-focus fluorescence. All data are given as mean ± SEM (number of experiments).

Results

Mitochondrial Inhibitors Evoke CRAC-Channel Inactivation. CRAC current (I_{CRAC}) was measured in Jurkat T cells using the noninvasive perforated-patch technique, with no exogenous cytosolic Ca2+ buffers applied other than a small amount of indo-1/AM for the purpose of measuring [Ca2+]i. The ER Ca2+-store was depleted by treatment for 3 min with 1 μM TG in Ca2+-free Ringer's solution. The subsequent addition of 2 mM Ca2+ to the bath induced a small sustained inward current (average current density of −0.44 ± 0.21 pA/pF, n = 8) accompanied by an increase in [Ca2+]i to a supramicromolar level (Fig. 1A). The current was identified as I_{CRAC} by its dependence on store depletion and extracellular Ca2+ and by its inwardly rectifying current-voltage relation (Fig. 1B) (19, 24).

Fig. 1 C and D show the results from a similar experiment conducted in the presence of 1 μM CCCP, a lipophilic protonophore that blocks mitochondrial Ca2+ uptake by dissipating the negative mitochondrial potential (8, 9). With CCCP present, I_{CRAC} inactivated with a time constant of ~40 s after Ca2+ readdition, declining to a steady-state level of ~50% within 100 s (Fig. 1C). The shape and reversal potential of the current-voltage relation remained constant during the experiment (Fig. 1D), showing that the current's decline was not caused by induction of an outward current, but rather by a reduction of I_{CRAC}. The degree of inactivation in CCCP-treated cells relative to control did not correlate with the peak current density, which on average was not affected significantly by CCCP (peak current density of −0.59 ± 0.25 pA/pF in 8 CCCP-treated cells: −0.44 ± 0.21 pA/pF in 8 control cells). As a consequence of the decrease in I_{CRAC}, [Ca2+]i also declined from a peak of >1 μM to a lower plateau. We obtained similar results with antimycin A1 and oligomycin, structurally unrelated compounds that depolarize mitochondria and inhibit their uptake of Ca2+ through a different mechanism involving inhibition of electron transport and the F0–F1 ATPase (see summary in Fig. 1E). Again, no correlation was found between peak current density and degree of inactivation.

The Mechanism of Coupling Between Mitochondria and CRAC Channels. The slow decline of I_{CRAC} in the presence of mitochondrial inhibitors is similar in kinetics and extent to the slow Ca2+-dependent inactivation of I_{CRAC} that has been described in whole-cell recordings from Jurkat (15) and RBL cells (16). Slow inactivation occurs under conditions in which store refilling is prevented by TG and [Ca2+]i is allowed to rise (i.e., in the presence of low exogenous Ca2+ buffering in the recording pipette). To test whether mitochondrial inhibitors promote this type of inactivation, we conducted a series of whole-cell recordings starting with the tonic conditions of our original study (15). After establishing the whole-cell configuration with a weakly Ca2+-buffered internal solution (see Fig. 2 legend), each cell was treated for 3–4 min in 0-Ca2+ Ringer's solution containing 1 μM TG to achieve complete store depletion. As reported previously (15), readdition of Ca2+ to the cell evoked large CRAC currents that rapidly overcame the low intracellular buffering capacity, increasing [Ca2+]i to supramicromolar levels and causing I_{CRAC} to inactivate by ~50% within 100 sec (Fig. 2A and B). This result contrasts markedly with the results of perforated-patch recordings in which little or no inactivation was seen (Fig. 1A). Because inhibition of mitochondrial Ca2+ uptake leads to inactivation in the perforated-patch configuration, we reasoned that the similar inactivation seen in whole-cell recordings might have resulted from the presence of extracellular Ca2+.
from a loss of mitochondrial function, possibly because of washout of oxidative substrates. To test this hypothesis, we supplemented the pipette solution with several compounds to help maintain the mitochondrial potential (9, 25): 2.5 mM malic acid, 2.5 mM Na pyruvate, 1 mM MgATP, 0.5 mM Tris-GTP. Under these “energized” whole-cell conditions, CRAC channel activity was sustained (Fig. 2 C and D). To rule out the possibility that the added compounds prevent inactivation through a nonmitochondrial mechanism, energized cells were treated with CCCP, antimycin A1, and oligomycin (Fig. 2 E and F). The inhibitors clearly unmask inactivation despite the presence of the mitochondrial substrates, supporting the conclusion that the energy state of the mitochondria controls the extent of Ca2+-dependent ICRAC inactivation.

Fig. 2. Energized mitochondria prevent Ca2+-dependent inactivation of CRAC channels. ICRAC was measured in whole-cell recordings with 1.2 mM EGTA in the recording pipette. Stimulus protocol was identical to that used in Fig. 1. (A) ICRAC measured at −100 mV before and after addition of 22 mM Ca2+ using the standard whole-cell internal solution (see Materials and Methods). (B) Leak-corrected ramp currents collected at the times indicated by the triangles in A. (C, D) ICRAC recorded as in A and B with the addition of 2.5 mM malic acid/2.5 mM Na pyruvate/1 mM Na2HPO4/5 mM MgATP/0.5 mM Tris-GTP to the recording pipette to support mitochondrial function (energized conditions). (E, F) ICRAC recorded as in C and D with addition of 1 μM antimycin A1/1 μM oligomycin to the bath. (G) CRAC channel inactivation under various conditions measured as described in Fig. 1. Inactivation under energized conditions or in the presence of 12 mM internal EGTA (last two bars) was significantly less than under the other conditions shown (unpaired Student’s t test, P < 0.007). The number of cells for each condition is indicated.
or antimycin + oligomycin) but does not inactivate when mitochondrial Ca\(^{2+}\) uptake can occur (e.g., in perforated-patch configuration or under “energized” whole-cell conditions). Moreover, addition of 12 mM EGTA intracellularly to prevent the rise in \([Ca^{2+}]_i\) (15) also prevents \(I_{\text{CRAC}}\) inactivation caused by mitochondrial inhibitors, demonstrating that the inactivation is in fact \(Ca^{2+}\)-dependent (Fig. 2G). The notion that mitochondria may reduce \([Ca^{2+}]_i\), locally near CRAC channels is supported by previous findings that \(Ca^{2+}\) uptake by mitochondria in T cells and other cells is known to occur rapidly and with high capacity (12, 26), and that mitochondria are close enough to apparently sense local \(Ca^{2+}\) gradients near open CRAC channels in T cells (12). Two \(Ca^{2+}\)-dependent inactivation processes for mitochondrial inhibitors were also considered. The most obvious is that these poisons might diminish \(I_{\text{CRAC}}\) by depleting cellular ATP (17, 18). Such a mechanism is unlikely to explain our results for several reasons. First, \(I_{\text{CRAC}}\) inactivated in the presence of the inhibitors despite the presence of 5 mM ATP supplied through the whole-cell recording pipette (Fig. 2E). Second, luciferase measurements on Jurkat cell suspensions indicated that intracellular [ATP] was stable over a period of 30 min in cells treated with TG and either antimycin A1 or CCCP in the presence of oligomycin (data not shown); this result is consistent with the known ability of glycolysis to supply the energy needs of tumor cells (27). Third, oligomycin did not diminish the effect of CCCP or antimycin A1 on inactivation (data not shown), even though it prevents ATP consumption resulting from reverse-mode operation of the \(F_0 - F_1\) ATPase (28). A second alternative explanation we have considered is that the mitochondrial inhibitors may block \(I_{\text{CRAC}}\) through a side effect unrelated to their effects on mitochondria. This possibility is also unlikely, however, as CRAC channel inactivation in the presence of CCCP or antimycin is prevented fully by the 12 mM EGTA added intracellularly (Fig. 2G). This result confirms that the mitochondrial inhibitors unmask \(Ca^{2+}\)-dependent inactivation of \(I_{\text{CRAC}}\) rather than inhibiting the channels directly.

**Physiological Consequences of Mitochondrial Regulation of CRAC Channels.** Because sustained elevation of \([Ca^{2+}]_i\) plays a crucial role in T-cell activation, it is important to determine how mitochondrial regulation of \(I_{\text{CRAC}}\) may influence downstream effector pathways. We examined the acute effects of mitochondrial inhibitors on the nuclear translocation of NFATc1, a critical transcription factor in T cells whose translocation to the nucleus is triggered by the \(Ca^{2+}\)-dependent phosphatase calcineurin (22, 23). Cytosolic \([Ca^{2+}]_i\) and the localization of an NFATc1-EGFP chimera were measured simultaneously in Jurkat cells using video microscopy (Fig. 3A). As shown in Fig. 3A, addition of \(Ca^{2+}\) to TG-treated cells caused a sustained \([Ca^{2+}]_i\), rise and a progressive accumulation of NFATc1-EGFP in the nucleus mirrored by a decrease in cytosolic intensity, indicating nuclear import. In contrast, \(Ca^{2+}\) readdition in the presence of CCCP resulted in CRAC-channel inactivation and an early decline in \([Ca^{2+}]_i\) from its initial peak level (Fig. 3C). Although the initial rate for nuclear translocation of NFATc1-EGFP was similar to that of control cells, accumulation ceased shortly after the fall in \([Ca^{2+}]_i\). The average increase in nuclear EGFP intensity 500 s after \(Ca^{2+}\) readdition was reduced by about half in CCCP-treated cells (29 ± 4% vs. n = 25) compared with control (59 ± 6%, n = 40; unpaired Student’s t test, \(P < 0.01\)). CCCP apparently does not interfere directly with nuclear import because normal nuclear translocation of NFATc1-EGFP was observed in those CCCP-treated cells (6 of 25) in which \(Ca^{2+}\) readdition evoked normal sustained \([Ca^{2+}]_i\) elevation.

One concern about the use of TG as the \(Ca^{2+}\)-mobilizing stimulus in these experiments is that blockade of SERCA pumps may obscure the contributions of \(Ca^{2+}\) sequestration by the ER, possibly leading to an overestimate of the role of mitochondria. For this reason, we also tested the ability of mitochondria to promote NFAT translocation under more physiological conditions. Cells were treated at 37°C with OKT3, an anti-CD3 monoclonal antibody that activates T cells by crosslinking the T-cell receptor complex (29). As shown in Fig. 3D, mitochondrial inhibitors attenuated the plateau phase of the \([Ca^{2+}]_i\) response by 50%, from 437 ± 88 nM in control cells (7 experiments on 527 cells) to 214 ± 58 nM in cells treated with 1 μM CCCP with or without 1 μM antimycin A1 + 1 μM oligomycin (6 experiments on 486 cells; unpaired Student’s t test, \(P < 0.001\)). Mitochondrial inhibition also impaired the nuclear translocation of NFATc1-EGFP measured in the same cells. After a brief phase of accumulation during the initial \([Ca^{2+}]_i\) rise, nuclear import slowed dramatically in the presence of CCCP (Fig. 3D). After 15 min of stimulation with OKT3, nuclear levels of NFATc1-EGFP increased significantly more in control cells (25 ± 7%) than in cells treated with CCCP + antimycin A1/oligomycin (15 ± 4%; unpaired Student’s t test, \(P < 0.01\)), consistent with the sustained elevated plateau in \([Ca^{2+}]_i\). Thus, mitochondria enhance the activation of NFAT even when \(Ca^{2+}\) uptake systems are operational, suggesting that the ability of mitochondria to prevent \(I_{\text{CRAC}}\) inactivation may serve to optimize \(Ca^{2+}\)-dependent transcriptional events in vivo.

**Discussion**

This study identifies store-operated \(Ca^{2+}\) channels as a target for mitochondrial modulation and describes the mechanism and a functional consequence of the interaction. The regulation of \(Ca^{2+}\) channel activity by mitochondria enables mitochondria to have a more profound impact on \(Ca^{2+}\) signaling and downstream signaling cascades than would be possible through simple \(Ca^{2+}\) buffering effects alone. Although mitochondrial inhibition of \(Ca^{2+}\)-channel inactivation has been proposed previously for ligand-gated (30), voltage-gated (28), and store-operated \(Ca^{2+}\) channels (12), and therefore might be a widespread phenomenon, the present study represents the first experimental test of this idea. Functional interactions between mitochondria and intracellular \(Ca^{2+}\) channels have also been inferred from the effects of mitochondrial inhibitors on \(Ca^{2+}\) release from the ER (10) or the propagation of cytosolic \(Ca^{2+}\) waves (1, 6, 7, 31–33). These effects have been attributed to changes in \(Ca^{2+}\) feedback on IP3 receptors in the ER, but this hypothesis has been difficult to test directly because of the inaccessibility of IP3 receptors in intact cells.

The functional interaction between mitochondria and CRAC channels is mediated through the control of \(Ca^{2+}\)-dependent \(I_{\text{CRAC}}\) inactivation. Mitochondria probably produce these effects by taking up \(Ca^{2+}\) and locally reducing its concentration near sites that govern inactivation rather than by reducing \([Ca^{2+}]_i\), globally, as mitochondrial inhibitors initiate the inactivation process at a time when global \([Ca^{2+}]_i\) is similar to that of control cells (e.g., compare Fig. 1A and C). Local accumulation of \(Ca^{2+}\) may also help explain why \(I_{\text{CRAC}}\) inactivation in the presence of CCCP persists even after the global \([Ca^{2+}]_i\) has declined (Fig. 1C); alternatively, prolonged inhibition of \(I_{\text{CRAC}}\) could result from a slow rate of recovery from inactivation. We cannot distinguish between these two possibilities at present. A physical basis for local interactions is indicated by the close proximity of mitochondria to CRAC channels; in electron micrographs of Jurkat cells, the majority of mitochondrial profiles lie within 500 nm of the plasma membrane (M.H., J. Buchanan, and R.L., unpublished data), similar to the intimate physical coupling between mitochondria and the ER reported in other cell types (5–7). Controlling \(Ca^{2+}\) channel activity and the resultant cytosolic \(Ca^{2+}\) signals through the localization, abundance, and metabolic state of mitochondria may therefore be a general cell biological mechanism for tuning \(Ca^{2+}\) signals and subsequent cell responses. In this context, it is intriguing that mitochondrial
activity increases severalfold during the first few days of T cell activation (34). Thus, increased resistance to ICRAC inactivation, together with increased expression of CRAC channels (35) and Ca²⁺-activated K⁺ channels (36), may contribute to the enhancement of Ca²⁺ signaling observed during this time period (37).

In a previous study of intact single T cells not under voltage clamp, mitochondrial inhibitors were found to reduce store-operated Ca²⁺ entry by increasing the likelihood of abrupt transitions from a high rate of Ca²⁺ influx to a low rate (12). These transitions were more rapid than the decreases in [Ca²⁺], we observe in voltage-clamped cells under otherwise similar conditions (compare Fig. 1B with figure 9C of ref. 12). One possible explanation for this difference is that in unclamped cells, slow inactivation of ICRAC causes Ca²⁺-activated K⁺ channels to deactivate, leading to membrane depolarization. The ensuing reduction in the driving force for Ca²⁺ entry would further deactivate IKCa and accelerate the transition from high to low [Ca²⁺].

As shown in this study, one important consequence of mitochondrial regulation of CRAC channels in T cells is to enable the sustained elevation of [Ca²⁺], that is required to drive nuclear translocation of NFAT. Maintained nuclear localization of NFAT is an essential prerequisite for the expression of interleukin-2 and other T-cell activation genes after exposure to antigen (22, 23). Thus, the close communication between mitochondria and CRAC channels may be important for optimizing early transcriptional events. Furthermore, these interactions are expected to have additional effects on cell signaling and metabolism, such as strengthening the link between CRAC channel activation and enhancement of ATP generation (3, 38, 39) and expanding the role of mitochondria as a pathway for Ca²⁺ redistribution from entry sites to other locations within the cell. An important goal for the future will be to determine how the appropriate coupling of mitochondria to CRAC channels is controlled, and how it contributes to setting the stimulation threshold for T cell activation and other downstream signaling events.

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