

Mitochondrial Regulation of Store-operated Calcium Signaling in T Lymphocytes

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Abstract. Mitochondria act as potent buffers of intracellular Ca^{2+} in many cells, but a more active role in modulating the generation of Ca^{2+} signals is not well established. We have investigated the ability of mitochondria to modulate store-operated or “capacitative” Ca^{2+} entry in Jurkat leukemic T cells and human T lymphocytes using fluorescence imaging techniques. Depletion of the ER Ca^{2+} store with thapsigargin (TG) activates Ca^{2+} release-activated Ca^{2+} (CRAC) channels in T cells, and the ensuing influx of Ca^{2+} loads a TG-insensitive intracellular store that by several criteria appears to be mitochondria. Loading of this store is prevented by carbonyl cyanide *m*-chlorophenylhydrazone or by antimycin A1 + oligomycin, agents that are known to inhibit mitochondrial Ca^{2+} import by dissipating the mitochondrial membrane potential. Conversely, intracellular Na^+ depletion, which inhibits Na^+ -dependent Ca^{2+} export from mitochondria, enhances store loading. In addition, we find that rhod-2

labels mitochondria in T cells, and it reports changes in Ca^{2+} levels that are consistent with its localization in the TG-insensitive store. Ca^{2+} uptake by the mitochondrial store is sensitive (threshold is <400 nM cytosolic Ca^{2+}), rapid (detectable within 8 s), and does not readily saturate. The rate of mitochondrial Ca^{2+} uptake is sensitive to extracellular $[\text{Ca}^{2+}]$, indicating that mitochondria sense Ca^{2+} gradients near CRAC channels. Remarkably, mitochondrial uncouplers or Na^+ depletion prevent the ability of T cells to maintain a high rate of capacitative Ca^{2+} entry over prolonged periods of >10 min. Under these conditions, the rate of Ca^{2+} influx in single cells undergoes abrupt transitions from a high influx to a low influx state. These results demonstrate that mitochondria not only buffer the Ca^{2+} that enters T cells via store-operated Ca^{2+} channels, but also play an active role in modulating the rate of capacitative Ca^{2+} entry.

MITOCHONDRIA fill several essential physiological roles in cells. Their best recognized function is to produce the bulk of the cell's ATP through oxidative metabolism (Nicholls and Ferguson, 1992). As described by the chemiosmotic theory, this is made possible by a series of mitochondrial enzymes in the respiratory chain that pump protons out of the mitochondrial matrix and establish a large electrical and proton gradient across the inner membrane. The high driving force for proton re-entry drives a proton pump (the F_1F_0 ATPase, or ATP synthase) in the mitochondria in reverse, causing it to synthesize ATP as it allows protons to reenter the mitochondrion. In addition to this fundamental role as the cell's primary energy supplier, mitochondria have become increasingly well known in recent years as potent buffers of cytosolic Ca^{2+} . The large negative potential across the inner membrane (up to -180 mV) drives Ca^{2+} into mitochondria through a uniporter located in the inner mem-

brane, and export is achieved via multiple mechanisms, including an electroneutral $\text{Na}^+/\text{Ca}^{2+}$ exchanger (Crompton et al., 1976; Carafoli, 1987; Gunter and Pfeiffer, 1990; Thayer and Miller, 1990; Cox and Matlib, 1993; Gunter et al., 1994).

Studies of excitable and nonexcitable cells have suggested several important consequences of Ca^{2+} uptake by mitochondria. First, increases in mitochondrial Ca^{2+} concentration ($[\text{Ca}^{2+}]_m$)¹ are believed to modulate the production of ATP. Several of the dehydrogenases that supply substrates for the electron transport chain are stimulated by Ca^{2+} (Gunter et al., 1994; Hansford, 1994; Nichols and Denton, 1995), and recent studies have verified that mitochondrial uptake of Ca^{2+} in vivo is accompanied by increased levels of NADH or NADPH (Rizzuto et al., 1994; Hajnoczky et al., 1995) and activation of the matrix enzyme pyruvate dehydrogenase (Rutter et al., 1996). This

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1. *Abbreviations used in this paper:* BCECF, 2',7'-bis(2-carboxylethyl)-5(6)-carboxyfluorescein; $[\text{Ca}^{2+}]_i$, cytosolic Ca^{2+} concentration; $[\text{Ca}^{2+}]_m$, mitochondrial Ca^{2+} concentration; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; CRAC, Ca^{2+} release-activated Ca^{2+} ; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; TG, thapsigargin.

behavior is thought to couple the rate of ATP production to demand such that, during periods of high cytosolic Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$), the increased rate of ATP hydrolysis by Ca^{2+} pumps is balanced by enhanced ATP generation.

In addition, uptake and release of Ca^{2+} by mitochondria has a powerful impact on Ca^{2+} signaling. Several characteristics are important in this regard. $[\text{Ca}^{2+}]_m$ is generally low (~ 200 nM) in resting cells (Rizzuto et al., 1992, 1994; Sheu and Jou, 1994; Babcock et al., 1997), but significant uptake occurs at modestly elevated levels of $[\text{Ca}^{2+}]_i$ ($> \sim 0.5$ μM) and does not saturate readily (Gunter et al., 1994; Sparagna et al., 1995). In HeLa cells and hepatocytes, it has been shown that mitochondria have the capacity to sequester and export Ca^{2+} at physiological $[\text{Ca}^{2+}]_i$ probably by sensing microdomains of high $[\text{Ca}^{2+}]_i$ close to sources such as IP_3 receptors in the ER or Ca^{2+} channels in the plasma membrane (Rizzuto et al., 1993; Hajnoczky et al., 1995; Lawrie et al., 1996). Mitochondrial uptake may help to avert excessive increases in $[\text{Ca}^{2+}]_i$ that may damage the cell or trigger apoptosis (Ankarcrona et al., 1995). Their high rate of uptake enables mitochondria to play a dominant role among several mechanisms (including $\text{Na}^+/\text{Ca}^{2+}$ exchange and Ca^{2+} -ATPases) for clearing large loads of cytosolic Ca^{2+} (Mohr and Fewtrell, 1990; Ali et al., 1994; Werth and Thayer, 1994; Herrington et al., 1996; Park et al., 1996). By slowly releasing stored Ca^{2+} , mitochondria can prolong the period of $[\text{Ca}^{2+}]_i$ elevation in response to a transient episode of Ca^{2+} influx (Thayer and Miller, 1990; Friel and Tsien, 1994; Babcock et al., 1997). These actions may have important effects on such Ca^{2+} -dependent processes as exocytosis and synaptic transmission. Thus, mitochondria act like a slow, nonsaturable, nonlinear buffer for intracellular Ca^{2+} ; they sequester Ca^{2+} during periods of rapid Ca^{2+} entry and release it slowly after Ca^{2+} entry has ceased.

While there is now considerable evidence for the passive Ca^{2+} buffering actions of mitochondria, few studies have addressed the possibility that they may actively influence the generation of Ca^{2+} signals. In *Xenopus* oocytes, mitochondrial inhibitors affect the generation and propagation of IP_3 -triggered Ca^{2+} waves, leading to the notion that intact mitochondria may reduce the local Ca^{2+} concentration near IP_3 receptors and thereby influence their Ca^{2+} -dependent modulation (Jouaville et al., 1995). In cerebellar granule cells, the uptake of Ca^{2+} by mitochondria has been proposed to enhance Ca^{2+} influx by reducing the Ca^{2+} -dependent inactivation of voltage-gated Ca^{2+} channels in the plasma membrane (Budd and Nicholls, 1996).

In this study we examine the ability of mitochondria to modulate Ca^{2+} influx through store-operated Ca^{2+} channels in T lymphocytes. In T cells and many other nonexcitable cells, depletion of the ER Ca^{2+} stores leads to the activation of voltage-independent, store-operated channels that conduct Ca^{2+} across the plasma membrane (for reviews see Penner et al., 1993; Putney and Bird, 1993; Fasolato et al., 1994; Berridge, 1995; Lewis and Cahalan, 1995). Store-operated channels are believed to constitute a family of related channels, of which the Ca^{2+} release-activated Ca^{2+} (CRAC) channel in T cells and mast cells is the most extensively characterized (Hoth and Penner, 1993; Zweifach and Lewis, 1993). After recognition of antigen by the

T cell receptor and the resulting depletion of stores by IP_3 , CRAC channels in T cells open and act to elevate $[\text{Ca}^{2+}]_i$ for an extended period of minutes to hours. Prolonged Ca^{2+} influx through CRAC channels appears to be required to induce interleukin-2 gene expression and to commit T cells to become activated (Goldsmith and Weiss, 1988; Negulescu et al., 1994; Partiseti et al., 1994; Fanger et al., 1995). Here we report the initial characterization of a high capacity mitochondrial Ca^{2+} store in human T cells. We find that mitochondria play a major role in sustaining high rates of Ca^{2+} influx through CRAC channels, and that this function depends on their ability to take up and export Ca^{2+} . Thus, mitochondria act not only as passive Ca^{2+} buffers in T cells, but also contribute actively to the mechanisms that control store-operated Ca^{2+} entry. A preliminary account of this work has appeared in abstract form (Hoth, M., C.M. Fanger, A.B. Nelson, and R.S. Lewis, 1996. *Biophys. J.* 66:181a).

Materials and Methods

Cell Lines and Reagents

Parental Jurkat T cells and the mutant Jurkat cell line CJ-4 have been previously described and characterized (Fanger et al., 1995). Cells were grown in complete medium consisting of RPMI 1640 (Mediatech, Herndon, VA) supplemented with 10% FCS (Gemini Bio-Products, Inc., Calabasas, CA), 2 mM glutamine (Mediatech), and penicillin-streptomycin (50 U/ml and 50 $\mu\text{g}/\text{ml}$; Mediatech). Cells were continuously maintained in log-phase growth at 37°C with 6% CO_2 . Human peripheral blood T cells were isolated as previously described (Dolmetsch and Lewis, 1994). Reagents include thapsigargin (TG; LC Laboratories, Woburn, MA; 1 mM stock in DMSO); ionomycin-free acid (Calbiochem-Novabiochem Corp., La Jolla, CA; 4 mM stock in DMSO); carbonyl cyanide *m*-chlorophenylhydrazone (CCCP; Sigma Chemical Co., St. Louis, MO; 1 mM stock in ethanol); carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP; Sigma Chemical Co.; 10 mM stock in ethanol); antimycin A1 (Sigma Chemical Co.; 2 mM stock in ethanol); oligomycin (Sigma Chemical Co., 5 mg/ml stock in DMSO); and *N*-methyl-D-glucamine (NMDG; Sigma Chemical Co.).

Video Microscopic Measurements of Cytosolic $[\text{Ca}^{2+}]$ and pH

Cells were loaded at 20°–25°C for 30 min with 1 μM fura-2/AM (Molecular Probes, Eugene, OR) in complete medium, washed with fresh medium, and stored in the dark at room temperature until use. Loaded cells were allowed to adhere to poly-L-ornithine-coated glass coverslip chambers on the stage of an Axiovert 35 microscope equipped with a $\times 40$ Achromat objective (NA 1.3) (Carl Zeiss, Inc., Thornwood, NY). Imaging experiments were performed as previously described (Dolmetsch and Lewis, 1994). Cells were alternately illuminated at 350 ± 5 nm and 380 ± 6 nm (bandpass interference filters; Omega Optical, Brattleboro, VT), and the fluorescence emissions at $\lambda > 480$ nm (longpass interference filter; Chroma Technology Corp., Brattleboro, VT) were captured with an intensified CCD camera (Hamamatsu Corp., Bridgewater, NJ) and digitized and analyzed using a VideoProbe imaging system (ETM Systems, Irvine, CA). Ratio images were recorded at intervals of 0.5–5 s. $[\text{Ca}^{2+}]_i$ was estimated from the relation $[\text{Ca}^{2+}]_i = K^* (R - R_{\min}) / (R_{\max} - R)$, where the values of K^* , R_{\min} , and R_{\max} were determined from an in situ calibration of fura-2 in Jurkat T cells loaded by intracellular dialysis as described (Lewis and Cahalan, 1989). Ringer's solution had the following composition (mM): 155 NaCl, 4.5 KCl, 2 CaCl_2 , 1 MgCl_2 , 10 D-glucose, and 5 Hepes (pH 7.4 with NaOH). Ca^{2+} -free Ringer's solution was prepared by substituting MgCl_2 for CaCl_2 and adding 1 mM EGTA. All experiments were conducted at 22°–25°C.

For qualitative measurements of mitochondrial Ca^{2+} , cells were loaded with rhod-2/AM (Molecular Probes) in Ringer's solution at a concentration of 4–8 μM (2 mM stock in DMSO) for 30–40 min at 20°–25°C. Cells were illuminated at 540 ± 12 nm (bandpass filter; Chroma Technology

Corp.), and the fluorescence emission at 605 ± 28 nm (bandpass filter; Chroma Technology Corp.) was captured and analyzed as described above for fura-2. For these experiments, a $\times 63$ Zeiss Neofluar objective (NA 1.25) was used. Movement of mitochondria posed a serious problem for time-lapse imaging. To minimize movement artifacts, we first flattened the cells by attaching them to poly-ornithine-coated coverslips followed by incubation for 30 min with $10 \mu\text{g/ml}$ phytohemagglutinin in Ringer's solution. In control experiments using fura-2-loaded cells, phytohemagglutinin did not appear to affect Ca^{2+} plateaus or responses to mitochondrial inhibitors, implying that it does not alter mitochondrial Ca^{2+} uptake. The microscope was focused on the mitochondria immediately before collecting each set of data (see Fig. 5, B and C). Rhod-2 fluorescence was not calibrated in terms of $[\text{Ca}^{2+}]_{\text{m}}$ but is expressed relative to the initial fluorescence intensity.

For pH_i measurements, cells were loaded with 2',7'-bis(2-carboxyethyl)-5(6)-carboxyfluorescein (BCECF; Molecular Probes) at a concentration of $2 \mu\text{M}$ (2 mM stock in DMSO) for 45 min at $20^\circ\text{--}25^\circ\text{C}$ in complete medium. Cells were illuminated at 440 ± 5 nm and 495 ± 5 nm (bandpass filters; Omega Optical), and the emission at $\lambda > 515$ nm (Schott glass filter; Omega Optical) was captured and analyzed as described above for fura-2. Calibration of BCECF ratios was performed using nigericin (a K^+/H^+ ionophore) and high $[\text{K}^+]_o$ as follows (Thomas et al., 1979). Cells were perfused with a high $[\text{K}^+]$ Ringer's solution (close to the assumed intracellular $[\text{K}^+]$) of the following composition (mM): 29.5 NaCl, 130 KCl, 2 CaCl_2 , 1 MgCl_2 , 10 D-glucose, and 5 Hepes. The pH of this solution was set to 6.05, 6.68, 7.25, or 7.63, respectively, with NaOH, and $10 \mu\text{M}$ nigericin (Molecular Probes; 10 mM stock in ethanol) was added. Under conditions of high internal and external $[\text{K}^+]$, nigericin equilibrates transmembrane pH. A plot of the fluorescence ratio (495/440) against pH_o resulted in a linear calibration curve. Cells were alkalized by external application of 5 mM NH_4Cl , which allows NH_3 to quickly diffuse into the cells and bind intracellular protons (Roos and Boron, 1981).

Confocal Microscopy

Jurkat cells were doubly labeled with rhod-2/AM as described above and with 100 nM MitoTrackerTM Green FM (Molecular Probes) for 30 min at $20^\circ\text{--}25^\circ\text{C}$. Excess dye was removed by washing three times. Cells were imaged with a MultiProbe 2010 confocal laser scanning microscope (Molecular Dynamics, Sunnyvale, CA) using a Diaphot 200 inverted microscope and $\times 60$ Plan Apo objective (NA 1.4) (Nikon Inc., Garden City, NY). Cells were illuminated by the 488 nm and 568 nm emission lines of a krypton/argon laser at 10% power, and fluorescence was collected simultaneously at 515–545 nm (bandpass filter; Chroma Technology Corp.) for MitoTrackerTM Green FM and at $\lambda > 590$ nm (longpass interference filter; Chroma Technology Corp.) for rhod-2. A $100\text{-}\mu\text{m}$ pinhole was used.

Data Analysis

Data were analyzed using Igor Pro (WaveMetrics, Lake Oswego, OR). Ca^{2+} clearance rates were measured from several hundred TG-treated cells displaying different $[\text{Ca}^{2+}]_i$ magnitudes in the presence of $\text{Ca}^{2+}_o \pm$ mitochondrial inhibitors. In each cell, the rate of ongoing Ca^{2+} clearance from the cytosol was measured from the rate of decline in $[\text{Ca}^{2+}]_i$ after rapid perfusion with $0\text{-}\text{Ca}^{2+}$ (EGTA) Ringer's. The recording chamber (vol $\sim 60 \mu\text{l}$) had a 90% exchange time of ~ 2 s when perfused with 3 ml of solution in 10 s. The rate of $[\text{Ca}^{2+}]_i$ decline ($\Delta[\text{Ca}^{2+}]_i/\Delta t$) was measured from the steepest slope after addition of EGTA, which occurred within 3–6 s of the solution change.

To quantify loading of the TG-insensitive store (see Figs. 2 and 3), cells were chosen for analysis if they satisfied two criteria: (a) $[\text{Ca}^{2+}]_i$ was approximately constant ($\Delta[\text{Ca}^{2+}]_i/\Delta t$ between -0.1 and 0.1 nM/s) over the final 800 s of the Ca^{2+} readdition period; and (b) $[\text{Ca}^{2+}]_i$ did not oscillate or undergo spontaneous transitions (e.g., see Fig. 9). 33–53% of the cells met these two criteria. The relative store content was estimated in these cells from the amount of Ca^{2+} released by $4 \mu\text{M}$ ionomycin in three different ways: (a) from the maximum rate of $[\text{Ca}^{2+}]_i$ rise, (b) from the peak $[\text{Ca}^{2+}]_i$ as fitted by a polynomial function, and (c) by the integral of $[\text{Ca}^{2+}]_i$ during the ionomycin release transient after correction for the ongoing clearance rate (Bergling, S., R. Dolmetsch, R.S. Lewis, and J. Keizer, manuscript in preparation). Upon addition of ionomycin, the rate at which $[\text{Ca}^{2+}]_i$ changes is proportional to the rate of Ca^{2+} release from the TG-insensitive store minus the rate of Ca^{2+} clearance across the plasma membrane. The clearance rate in Jurkat cells is an approximately linear function of $[\text{Ca}^{2+}]_i$, up to $1.5 \mu\text{M}$ (rate constant = 0.057 s^{-1}); thus the clearance rate as a function of time can be estimated by scaling $[\text{Ca}^{2+}]_i$ by this rate constant.

By adding the clearance rate at each time point to the corresponding time derivative of $[\text{Ca}^{2+}]_i$, we obtained the rate of release from the store. This curve was then integrated to give a measure of total mitochondrial Ca^{2+} released. This method is equivalent to a summation of the amount of Ca^{2+} irreversibly pumped across the plasma membrane, which reflects the total Ca^{2+} released from mitochondria under the given conditions.

Results

A TG-insensitive Ca^{2+} Store in T Cells

In all experiments described below, Jurkat T cells or human T cells were treated with TG to activate capacitance Ca^{2+} entry, and the lipophilic ionophore ionomycin was applied to probe the content of TG-insensitive Ca^{2+} stores. Exposure to $1 \mu\text{M}$ TG in Ca^{2+} -free Ringer's solution fully inhibits the sarco-endoplasmic reticulum Ca^{2+} -ATPases of the ER (Thastrup et al., 1990), causing a transient rise in $[\text{Ca}^{2+}]_i$ due to the unopposed leakage of Ca^{2+} from stores followed by extrusion across the plasma membrane. As shown in Fig. 1 A (*dotted trace*), ionomycin releases very little stored intracellular Ca^{2+} when applied after TG, indicating that TG almost completely depletes the ionomycin-releasable Ca^{2+} pool in resting Jurkat T cells. Readdition of Ca^{2+} after store depletion causes an initial rapid increase of $[\text{Ca}^{2+}]_i$ to a peak, which falls slowly thereafter to a plateau level. In cells that have been exposed to a low Ca^{2+} concentration (0.5 mM ; Fig. 1 A, *solid trace*), the average plateau level is $< 500 \text{ nM}$, and subsequent removal of Ca^{2+}_o evokes a rapid decline of $[\text{Ca}^{2+}]_i$ to the basal level as Ca^{2+} is cleared from the cytosol. Ionomycin-releasable stores remain nearly empty under these conditions, as shown by the small response to ionomycin.

In contrast, Ca^{2+} removal does not quickly reduce $[\text{Ca}^{2+}]_i$ to baseline in cells previously exposed to a higher level of Ca^{2+}_o (Fig. 1 B). Addition of 2 mM Ca^{2+} elicits a $[\text{Ca}^{2+}]_i$ plateau $> 1 \mu\text{M}$, and, upon removal of Ca^{2+}_o , $[\text{Ca}^{2+}]_i$ falls to a slowly declining plateau significantly higher than the resting level. In this case, subsequent application of ionomycin releases a large bolus of stored Ca^{2+} (Fig. 1 B, *solid trace*), indicating that Ca^{2+} was taken up by a TG-insensitive store during the high Ca^{2+} plateau. The amount of Ca^{2+} released by ionomycin, and hence the store content, declines in parallel with the low plateau during exposure to Ca^{2+} -free Ringer's solution (*dotted trace*). This result suggests that Ca^{2+} is exported from the TG-insensitive store, and the gradual depletion of this source of Ca^{2+} is responsible for the slowly declining plateau. This conclusion is further supported by the observation that the duration of the low Ca^{2+} plateau is correlated with the length of time the store was allowed to fill (i.e., the duration of the high Ca^{2+} plateau; Fig. 1 C). Thus, after Ca^{2+} removal, the low Ca^{2+} plateau declines with an exponential time constant τ that varies with loading duration: $\tau = 232 \text{ s}$ (for 100 s loading time; Fig. 1 C, *dotted trace*), $\tau = 378 \text{ s}$ (for 500 s loading time; Fig. 1 B, *dotted trace*), and $\tau = 543 \text{ s}$ (for $1,000 \text{ s}$ loading time; Fig. 1 C, *solid trace*). In contrast, the average initial value of the low plateau appears to be independent of the degree of loading (*dashed line* in Fig. 1 C). These results clearly demonstrate that a TG-insensitive store can rapidly sequester Ca^{2+} that has entered the T cell via the capacitance Ca^{2+} entry mechanism, I_{CRAC} .

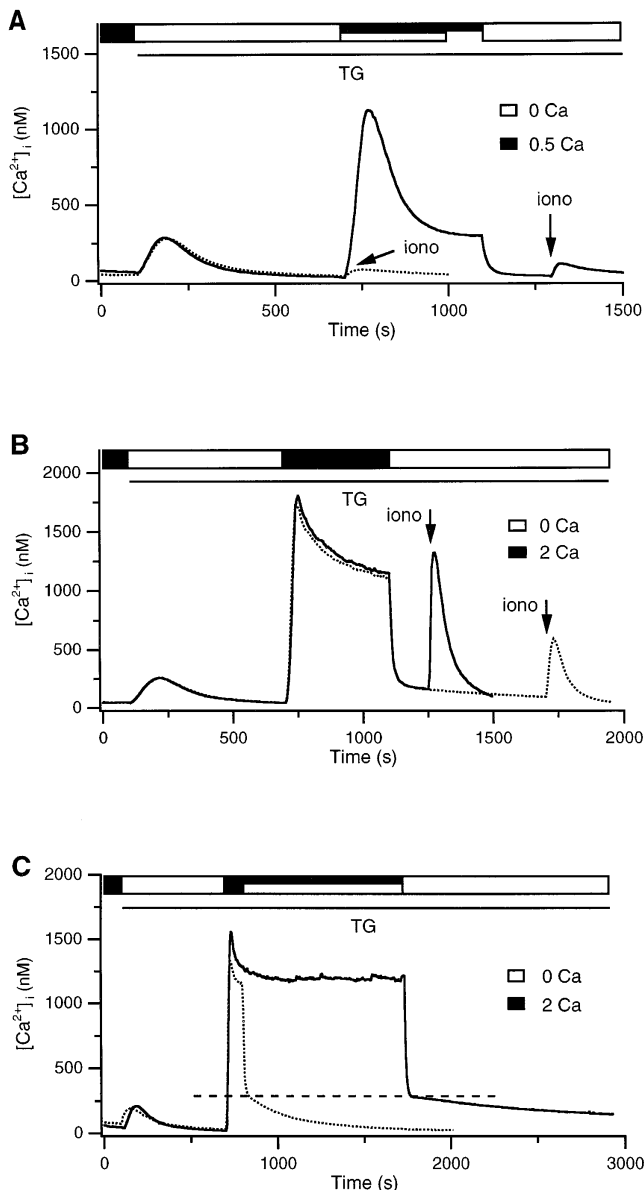


Figure 1. A TG-insensitive Ca^{2+} store in Jurkat T cells. (A–C) fura-2-loaded cells were treated with 1 μ M TG in Ca^{2+} -free Ringer's solution to deplete TG-sensitive stores (open bar). Ca^{2+} was subsequently readded to elicit capacitative Ca^{2+} entry (filled bar). Where indicated, 4 μ M ionomycin was applied in Ca^{2+} -free Ringer's solution to assay the content of intracellular stores. Each trace represents the average response of \sim 300 cells. (A) Readdition of 0.5 mM Ca^{2+} is not sufficient to load the TG-insensitive store. After exposure to 0.5 mM Ca^{2+} , ionomycin releases a small amount of Ca^{2+} (solid trace) that is not significantly greater than the amount released before Ca^{2+} readdition (dotted trace). (B) Readdition of 2 mM Ca^{2+} loads a TG-insensitive store that is releasable by ionomycin. Two experiments are superimposed, showing that the content of the TG-insensitive store and the low Ca^{2+} plateau decline in parallel. (C) The TG-insensitive store is loaded during long (1,000 s; solid trace) and short (100 s; dotted trace) exposure to 2 mM Ca^{2+} . In each case, after removal of Ca^{2+} , $[Ca^{2+}]_i$ returns to baseline with a biphasic time course. The slow recovery phase is lengthened by increasing the loading period.

The loading of the TG-insensitive store appears to depend on the cytosolic Ca^{2+} concentration since the ionomycin-releasable pool is larger in cells with higher Ca^{2+} plateaus (compare Fig. 1, A and B). We analyzed the content of the store as a function of the plateau $[Ca^{2+}]_i$ in single cells to determine quantitatively the $[Ca^{2+}]_i$ dependence of loading. After depleting stores in Ca^{2+} -free Ringer's solution with TG, 2 mM Ca^{2+} was applied for 1,000 s. This long period was chosen to assure that loading occurred primarily at a single well-defined level of $[Ca^{2+}]_i$. The loading period was terminated by applying Ca^{2+} -free Ringer's for 150 s, followed by 4 μ M ionomycin to assess store content. To simplify analysis of the 250–300 single cells of each Ca^{2+} imaging experiment, we analyzed only those cells (33–53%) that displayed a relatively constant plateau $[Ca^{2+}]_i$ (see Materials and Methods). In the three cells meeting this criterion shown in Fig. 2 A, the amount of ionomycin-releasable Ca^{2+} increased with the size of the Ca^{2+} plateau. Relative store content was estimated from the maximum rate of $[Ca^{2+}]_i$ rise, the peak $[Ca^{2+}]_i$, or

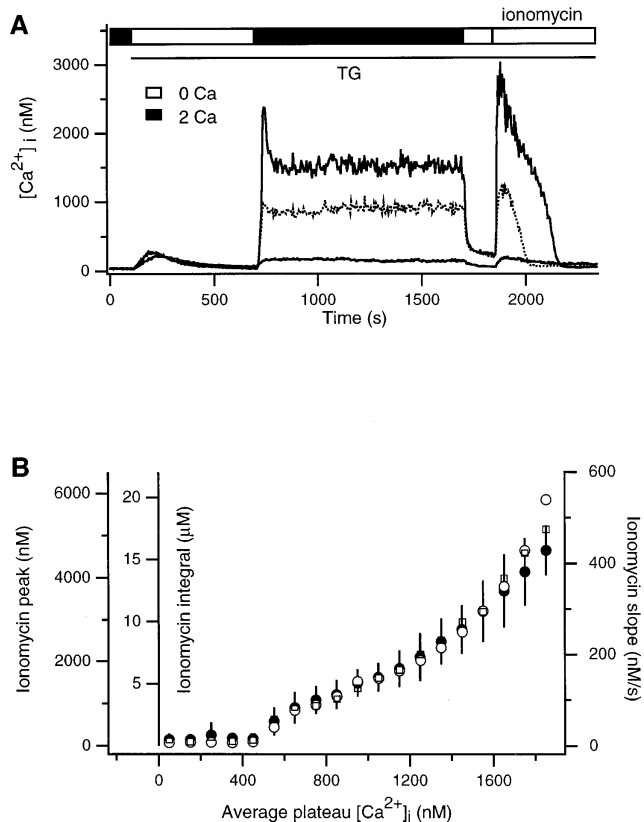


Figure 2. Ca^{2+} dependence of uptake by the TG-insensitive Ca^{2+} store. (A) Cells were stimulated as in Fig. 1 B to load the TG-insensitive store. The loading period was terminated by removing extracellular Ca^{2+} , and 4 μ M ionomycin was added subsequently to assay store content. The responses of three single cells from one experiment are superimposed. (B) Store content plotted as a function of the average magnitude of the Ca^{2+} plateau for individual cells. Three measures of store content are plotted: the peak $[Ca^{2+}]_i$ (●), the maximum rate of $[Ca^{2+}]_i$ rise (○), and the integral of released Ca^{2+} (corrected for clearance rate, see Materials and Methods; □) in response to ionomycin. Results were obtained from 964 cells in 11 experiments and binned into 100-nM intervals of $[Ca^{2+}]_i$.

the integral of released Ca^{2+} (after correction for the ongoing clearance rate; see Materials and Methods) in response to ionomycin + EGTA. All three measures of store content gave similar estimates (Fig. 2 B). The content of the TG-insensitive store appears to increase in a biphasic fashion, beginning at $[\text{Ca}^{2+}]_i > 400 \text{ nM}$ and increasing without signs of saturation to $[\text{Ca}^{2+}]_i > 1.8 \mu\text{M}$. It should be noted that the quantitation of store content in these experiments is complicated by the fact that ionomycin evoked $[\text{Ca}^{2+}]_i$ elevations as large as $6 \mu\text{M}$, far greater than the effective K_d of fura-2 for Ca^{2+} (200–1,000 nM, depending on conditions; Uto et al., 1991). Thus, it is possible that the nonlinear shape of the loading curve is a consequence of a systematic error in fura-2 measurements at high $[\text{Ca}^{2+}]_i$. In this regard it is encouraging that the store measurements based on the maximum rate of $[\text{Ca}^{2+}]_i$ rise gave similar results, as they were made at $[\text{Ca}^{2+}]_i < 1 \mu\text{M}$. Some uptake also occurred at $[\text{Ca}^{2+}]_i < 400 \text{ nM}$; a close examination revealed that in cells with plateau $[\text{Ca}^{2+}]_i$ between 100 and 500 nM, ionomycin evoked an average $[\text{Ca}^{2+}]_i$ peak of $\sim 200 \text{ nM}$, which did not increase with $[\text{Ca}^{2+}]_i$ but was significantly greater than the peak Ca^{2+} released by ionomycin before store loading (40–80 nM).

The TG-insensitive Store Senses Local Ca^{2+} near CRAC Channels

In several cell types it has been shown that Ca^{2+} uptake by organelles such as mitochondria is greatly enhanced by close proximity to sites of Ca^{2+} release or influx into the cytoplasm (Rizzuto et al., 1993; Hajnoczky et al., 1995;

Lawrie et al., 1996). This behavior is thought to result from microdomains of high $[\text{Ca}^{2+}]_i$ close to Ca^{2+} sources. If the sites of influx and uptake are closely apposed, uptake should be determined primarily by the local influx rate rather than by global $[\text{Ca}^{2+}]_i$ far from the influx site. Therefore, to determine whether the TG-insensitive store can sense local Ca^{2+} accumulation near CRAC channels, we asked whether store loading is sensitive to the driving force for Ca^{2+} entry under conditions where the global $[\text{Ca}^{2+}]_i$ is held constant. Store loading was assessed by measurements of the peak $[\text{Ca}^{2+}]_i$ or release rate in response to ionomycin (both gave identical results). In each set of experiments, Ca^{2+} flux through individual CRAC channels was increased by raising $[\text{Ca}^{2+}]_o$ from 2 to 20 mM, while one of several methods was used to equalize the global Ca^{2+} plateau under the two conditions. In the first set of experiments, 5–10 mM Ni^{2+} was added to the external medium containing 20 mM Ca^{2+} . Under these conditions, the partial blockade of CRAC channels by Ni^{2+} compensates for the increased single-channel rate of influx, making the average magnitude of the global Ca^{2+} plateau comparable to that observed with 2 mM extracellular Ca^{2+} alone (Fig. 3 A). However, the content of the ionomycin-releasable store was significantly higher in the cells exposed to 20 mM Ca^{2+} . As illustrated by the single-cell analysis in Fig. 3 B, loading of the TG-insensitive store was enhanced at all plateau $[\text{Ca}^{2+}]_i$ levels $> 500 \text{ nM}$. Similar results were obtained in a second set of experiments that compared store loading in normal Jurkat cells with that in CJ-4, a mutant Jurkat cell line that exhibits $\sim 40\%$ of the normal level of CRAC channel function (Fanger et al.,

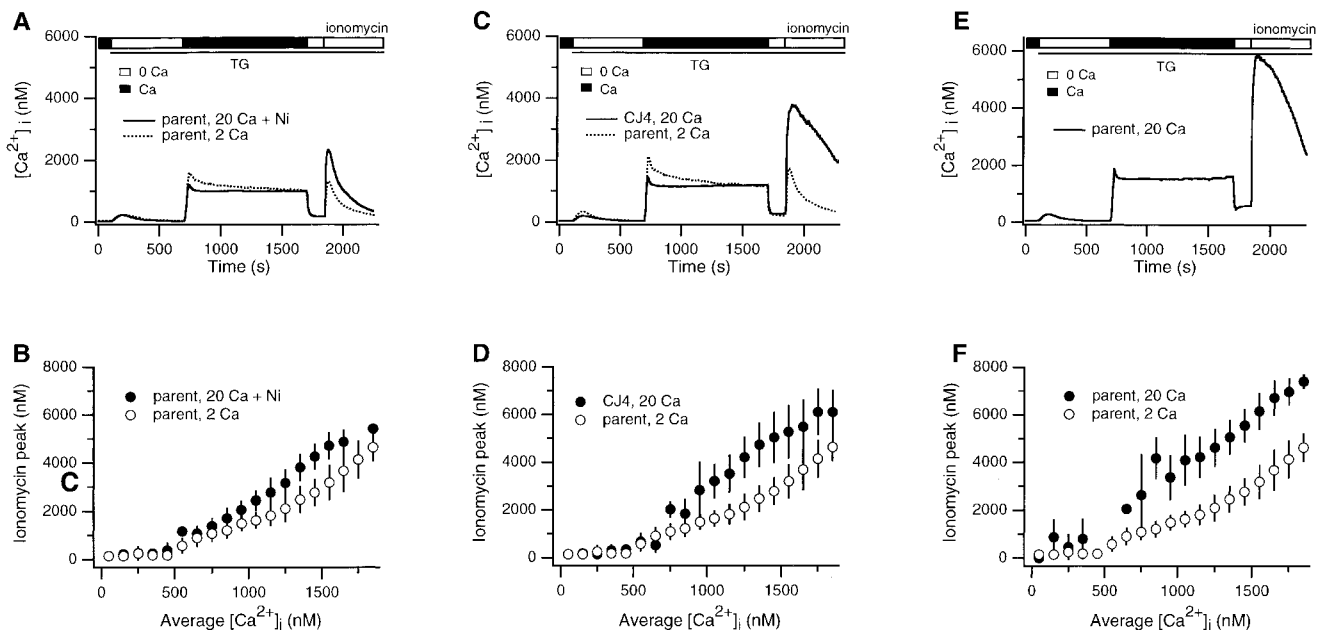


Figure 3. The TG-insensitive store senses Ca^{2+} near CRAC channels. Experimental conditions and analysis were identical to Fig. 2 with the exception that conditions during the plateau phase were varied. (A and B) 2 mM Ca^{2+} (dotted trace, \circ) or 20 mM Ca^{2+} with 8 mM Ni^{2+} (solid trace, \bullet) or 5–10 mM Ni^{2+} (\bullet) was added to create plateaus of comparable magnitude. (C and D) 2 mM Ca^{2+} was applied to parental Jurkat cells (dotted trace, \circ), and 20 mM Ca^{2+} was applied to CJ-4 cells (solid trace, \bullet), a Jurkat mutant with reduced CRAC channel function. (E and F) 20 mM Ca^{2+} was added (solid trace, \bullet) and compared to the response to 2 mM Ca^{2+} (\circ , data from Fig. 2 B). Graphs show mean values \pm SD: parent cells, 2 mM Ca^{2+} (964 cells/11 experiments), 20 mM Ca^{2+} + Ni^{2+} (604/6), 20 mM Ca^{2+} (439/3); CJ-4 cells, 20 mM Ca^{2+} (900/5). Each of the experiments in A, C, and E represents the average response of ~ 300 cells.

1995). Although the average $[Ca^{2+}]_i$ plateau in CJ-4 cells with 20 mM Ca^{2+}_o is slightly lower than that in control cells with 2 mM Ca^{2+}_o , ionomycin releases significantly more Ca^{2+} from the stores in CJ-4 (Fig. 3 C). As was observed in the Ni^{2+} experiments, the content of the ionomycin-releasable store was increased in the presence of 20 mM extracellular Ca^{2+} over a wide range of $[Ca^{2+}]_i$ levels (Fig. 3 D). As a third test, we compared store content in single normal cells exposed to 2 or 20 mM Ca^{2+} that happened to display the same plateau $[Ca^{2+}]_i$. For any given plateau level >100 nM, the loading of the ionomycin-releasable pool was enhanced by previous exposure to 20 mM Ca^{2+} (Fig. 3, E and F). Together, these results demonstrate that the uptake of Ca^{2+} into the TG-insensitive store increases with the driving force for Ca^{2+} entry, irrespective of global $[Ca^{2+}]_i$. Hence, the store uptake mechanism appears able to sense local $[Ca^{2+}]_i$ gradients that exist in the proximity of open CRAC channels.

Calcium Uptake by the TG-insensitive Store Is Rapid

In order for the TG-insensitive store to contribute to Ca^{2+} signaling under a range of physiological conditions, it must be able to take up Ca^{2+} during transient as well as sustained periods of $[Ca^{2+}]_i$ elevation. We therefore determined the $[Ca^{2+}]_i$ dependence of store loading during short applications of Ca^{2+} (8–10 s). This short pulse of Ca^{2+} elicited a brief spike of $[Ca^{2+}]_i$ that declined as Ca^{2+} was pumped across the plasma membrane (Fig. 4 A). After complete recovery, Ca^{2+} was applied again; immediately after the end of the pulse, ionomycin was applied to release any Ca^{2+} that had accumulated in the TG-insensitive store. By aligning the initial falling phases of the two Ca^{2+} spikes (Fig. 4 B), it is possible to detect Ca^{2+} released

by ionomycin as a result of store loading. As shown by the average response of 40 cells (Fig. 4 B) and in two single cells (Fig. 4 C), $[Ca^{2+}]_i$ elevation for 8–10 s causes significant loading of the TG-insensitive store. Results from 62 cells are summarized in Fig. 4 D as a plot of the Ca^{2+} released by ionomycin against the peak $[Ca^{2+}]_i$ immediately before ionomycin (a rough measure of the Ca^{2+} accessible to the store for loading). The amount of Ca^{2+} released by ionomycin after all Ca^{2+} spikes >300 nM exceeded the amount released in resting cells (40–80 nM; shaded line), and loading increased nonlinearly above ~ 1.5 μ M cytosolic Ca^{2+} . The ability of the TG-insensitive store to sequester Ca^{2+} in response to small, brief Ca^{2+} spikes suggests that it may participate in modulating transient as well as sustained Ca^{2+} signals.

Rhod-2 Reports Changes in Mitochondrial Free Calcium

The behavior of the TG-insensitive Ca^{2+} store suggests that it may consist of mitochondria. The store is relatively empty under resting conditions and takes up Ca^{2+} rapidly and without signs of saturation at $[Ca^{2+}]_i > \sim 400$ nM, much like mitochondria as studied in other cells (Carafoli, 1987; Gunter and Pfeiffer, 1990; Thayer and Miller, 1990; Friel and Tsien, 1994; Gunter et al., 1994; Herrington et al., 1996). We tested this idea using the Ca^{2+} -sensitive fluorescent indicator rhod-2 (Minta et al., 1989), which has been shown in some cells to accumulate preferentially in mitochondria (Hajnóczky et al., 1995; Rutter et al., 1996; Babcock et al., 1997). After loading cells with rhod-2/AM, we observed diffusely labeled cytoplasm punctuated with brightly fluorescent structures of variable organization among different cells. Confocal microscopy was used to

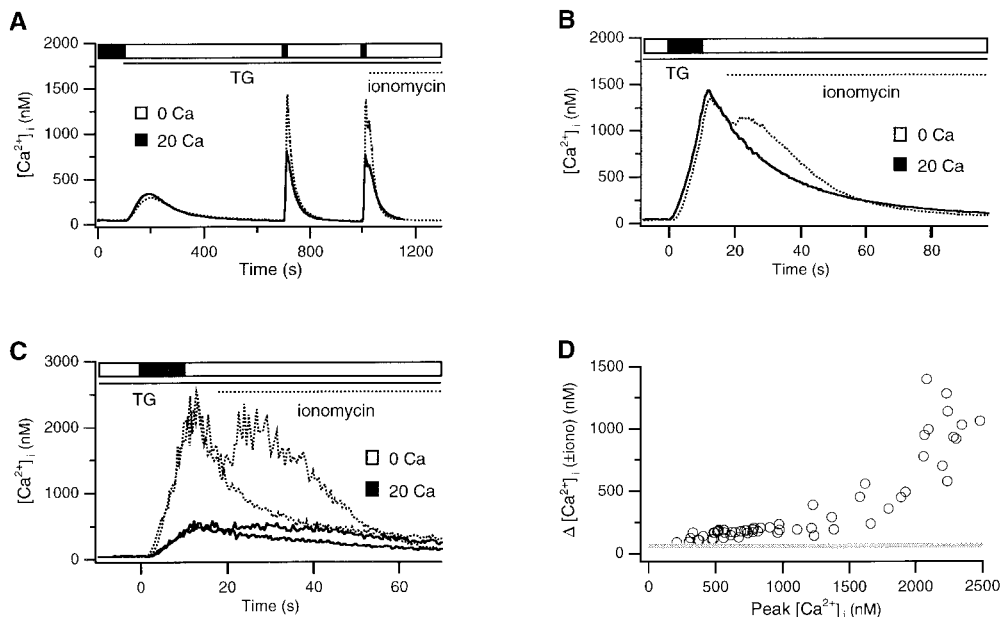


Figure 4. Rapid loading of the TG-insensitive store. (A) After depletion of the TG-insensitive store, 20 mM Ca^{2+} was added twice for 8 s (solid trace) or 10 s (dotted trace) in two separate experiments. 5 s after the second application of Ca^{2+} , 4 μ M ionomycin was added to assay the content of the TG-insensitive store. Each trace is the average of 40 cells. (B) The initial falling phases of the two 10-s transients from (A) are aligned on an expanded timescale. Ionomycin releases stored Ca^{2+} after the second transient (dotted trace). (C) Data from two cells displaying either a high (dotted trace) or a low (solid trace) response to ionomycin. For each cell, the initial falling phases of the

two transients are aligned as in B to enable detection of the Ca^{2+} released by ionomycin. (D) Ca^{2+} dependence of store loading in response to brief Ca^{2+} transients. Cells from four experiments were analyzed as described in C. The first pulse was subtracted from the second one, and the peak of the resulting “ionomycin release transient” was taken as a measure of store loading. This is plotted as a function of the peak $[Ca^{2+}]_i$ during the second transient before ionomycin. The shaded bar shows the range of ionomycin response (40–80 nM) observed in resting cells before store loading.

test whether the dye was specifically localized to mitochondria. In double-labeling experiments, rhod-2 and MitoTracker™ Green FM, a mitochondria-specific dye, were colocalized in most intracellular structures (Fig. 5 A). The only notable difference between the labeling patterns was the result of rhod-2 labeling of one or several round intranuclear structures, most likely representing the nucleolus. Nucleolar and mitochondrial stainings were easily distinguished by virtue of the much lower fluorescence of the nucleolus (Fig. 5 A, left).

Initiation of capacitative Ca^{2+} entry caused the brightness of mitochondrial rhod-2 to increase by $\sim 43\%$ within 3 min (Fig. 5, B and C), while areas of diffuse rhod-2 fluorescence adjacent to the “hot spots” increased only $\sim 10\%$ under the same conditions. Since the diffuse areas receive contributions from cytosolic dye and probably some diffusely localized mitochondria as well, their intensity places an upper limit on the contamination of the mitochondrial

signal by cytosolic dye. The increase in mitochondrial rhod-2 fluorescence was prevented by preincubation with $1 \mu\text{M}$ CCCP (Fig. 5, B and C), a membrane-permeant protonophore that rapidly depolarizes mitochondria and effectively inhibits their ability to take up Ca^{2+} (Thayer and Miller, 1990; Werth and Thayer, 1994; Budd and Nicholls, 1996; Herrington et al., 1996; Park et al., 1996; Babcock et al., 1997). These results directly support the conclusion that the TG-insensitive store represents mitochondria.

Effects of Mitochondrial Inhibitors

To test further whether the store represents mitochondria, we examined the ability of several inhibitors of mitochondrial Ca^{2+} transport to affect the content of the ionomycin-releasable pool. As described below, these inhibitors did interfere with store loading and therefore enabled us to test whether the store also plays an active role in modulat-

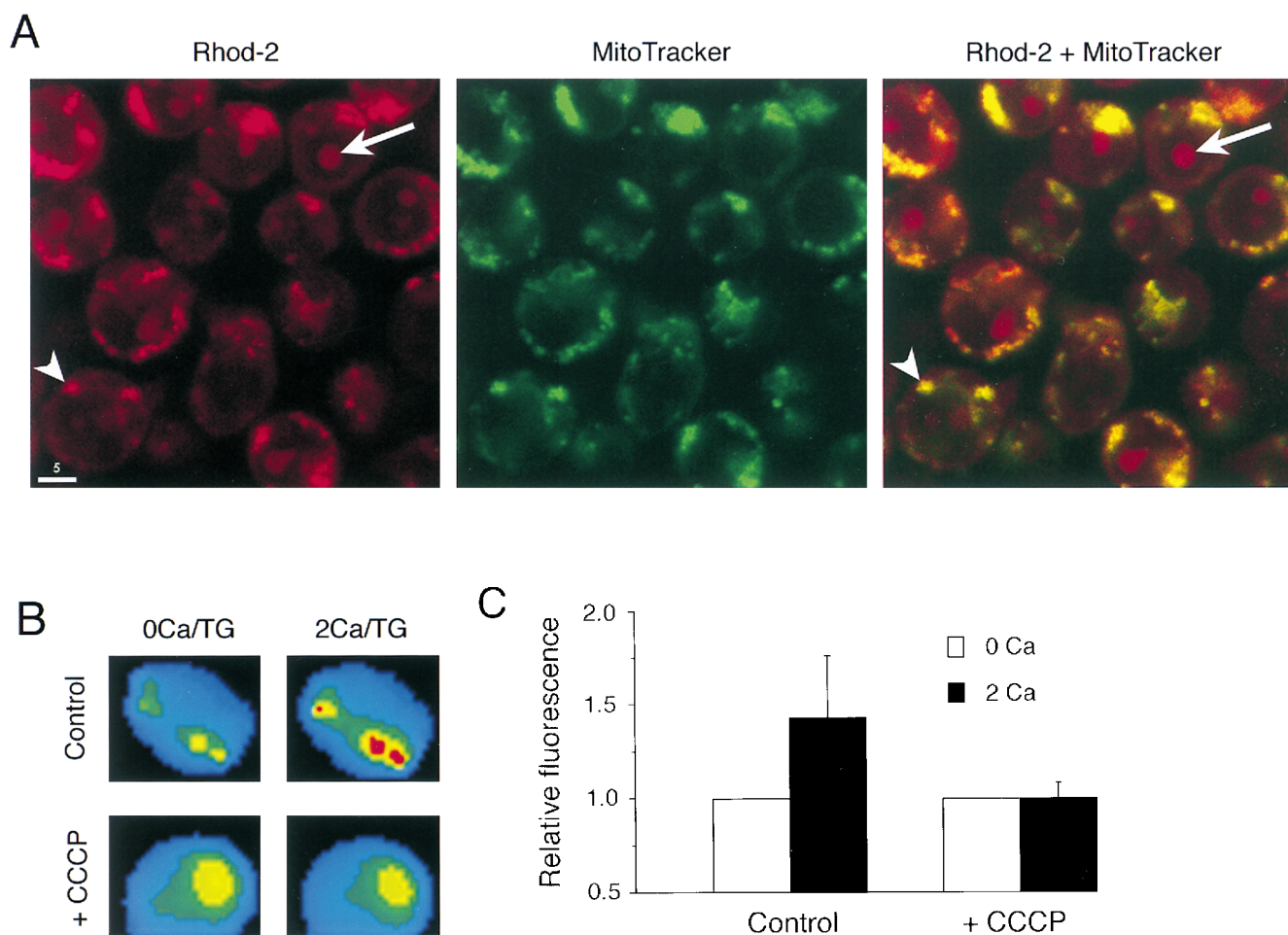


Figure 5. Rhod-2/AM labels mitochondria and reports changes in intramitochondrial $[\text{Ca}^{2+}]$. (A) Confocal micrographs of Jurkat cells after incubation with rhod-2/AM (left) or MitoTracker™ Green FM, a mitochondria-specific marker (center). Rhod-2 labeling of mitochondria (arrowhead) is easily distinguished from labeling of the nucleolus (arrow). An overlay of the two images demonstrates specific colocalization of the two dyes in the mitochondria (right). (B) Mitochondrial Ca^{2+} imaging with rhod-2. In these pseudocolored video microscopic images of a single TG-treated cell, rhod-2 fluorescence in the mitochondria increases within 3 min after readdition of 2 mM Ca^{2+} (top). $1 \mu\text{M}$ CCCP prevents the fluorescence increase in another cell (bottom). Warmer colors indicate higher rhod-2 fluorescence. (C) Quantification of rhod-2 fluorescence in “hot spots” in cells of the experiment shown in B. Hot spot fluorescence was measured before and 3 min after readdition of 2 mM Ca^{2+} . The fluorescence intensity before readdition was normalized to one. 35 cells (control) and 28 cells (+CCCP) were analyzed. Error bars reflect standard deviations. Bar, $5 \mu\text{m}$.

ing capacitative Ca^{2+} entry. In a first series of experiments, CCCP ($1 \mu\text{M}$) was applied shortly before the readdition of Ca^{2+} to TG-treated cells. CCCP had two prominent effects: it greatly attenuated the size of the Ca^{2+} plateau produced by Ca^{2+} readdition, and it effectively prevented loading of the TG-insensitive store, as indicated by the small amount of Ca^{2+} released by ionomycin (Fig. 6 A and Table I). The inhibition of loading could result from the fact that CCCP reduced the $[\text{Ca}^{2+}]_i$ plateau to a level near

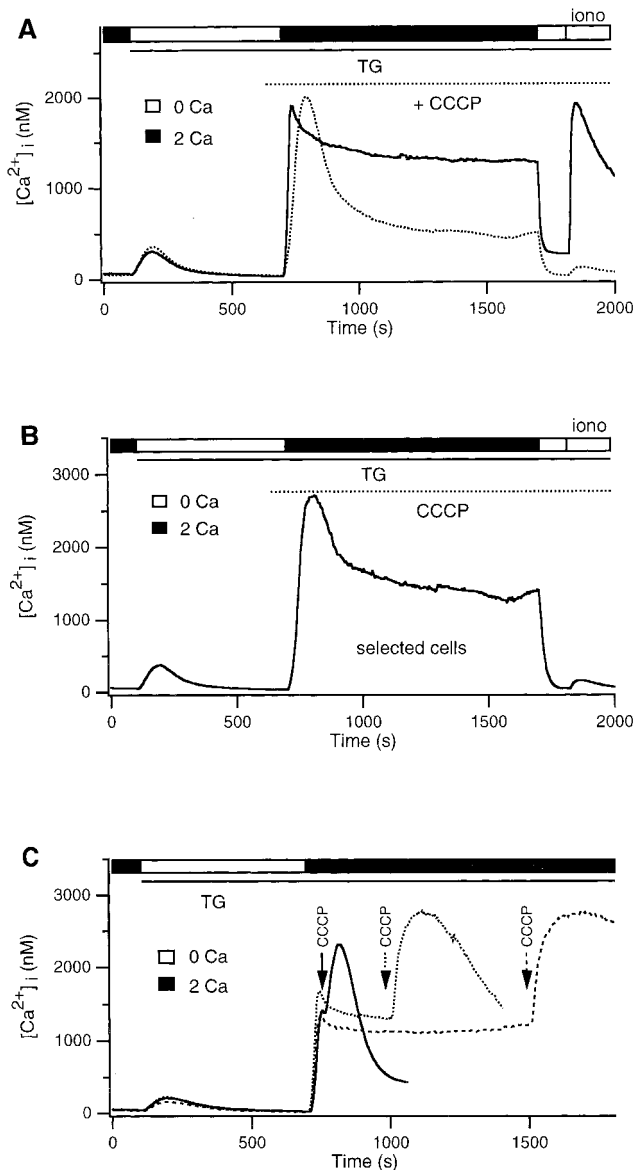


Figure 6. CCCP attenuates the Ca^{2+} plateau and inhibits loading of the TG-insensitive store. (A) 2 mM Ca^{2+} was readded to TG-treated cells in the presence (dotted trace) or absence (solid trace) of $1 \mu\text{M}$ CCCP. After termination of the store loading period, ionomycin was added to assay store content. (B) Average response of CCCP-treated cells selected for a final $[\text{Ca}^{2+}]_i$ plateau $>1 \mu\text{M}$ from the experiment shown in A (43/264 cells). CCCP prevents store loading even if $[\text{Ca}^{2+}]_i$ is high. (C) $1 \mu\text{M}$ CCCP was applied at different times during the plateau response in 2 mM Ca^{2+} . Each trace represents the average of 250–300 cells.

the threshold for Ca^{2+} uptake (see Fig. 2 B). However, the ionomycin response was similarly inhibited in the minority of cells that displayed a $[\text{Ca}^{2+}]_i$ plateau $>1 \mu\text{M}$ (Fig. 6 B), demonstrating that CCCP directly blocks uptake by the store. Similar results were obtained with a related protonophore, FCCP ($2 \mu\text{M}$). Application of CCCP had little or no effect on $[\text{Ca}^{2+}]_i$ in resting Jurkat cells (data not shown). However, CCCP added during the high Ca^{2+} plateau evoked an immediate transient rise in the average $[\text{Ca}^{2+}]_i$ (Fig. 6 C). The magnitude and the duration of the rise in $[\text{Ca}^{2+}]_i$ induced by CCCP increased with the duration of store loading, demonstrating that even at these supramicromolar levels of $[\text{Ca}^{2+}]_i$, uptake by the store continues for at least 15 min.

Qualitatively similar results were observed in cells treated with antimycin A1 ($2 \mu\text{M}$). Antimycin A1 inhibits electron transport through the respiratory chain at complex III, which by itself prevents oxidative reactions from creating a proton electrochemical gradient across the inner mitochondrial membrane (Mohr and Fewtrell, 1990; Nicholls and Ferguson, 1992; Ali et al., 1994). The Ca^{2+} plateau was diminished (Fig. 7 A), and uptake by the TG-insensitive store was significantly reduced (Table I), although both effects were not as extreme as those seen in the presence of protonophores. Inhibition of electron transport by itself may not depolarize mitochondria completely; if a supply of ATP is available through glycolysis, mitochondria uncoupled by antimycin can maintain their membrane potential by running the ATP synthase “in reverse,” i.e., by hydrolyzing ATP to pump protons out of the mitochondrial matrix (Mohr and Fewtrell, 1990; Budd and Nicholls, 1996). Oligomycin prevents this by inhibiting the ATP synthase and allows the mitochondria to depolarize (Mohr and Fewtrell, 1990; Budd and Nicholls, 1996). Simultaneous application of antimycin A1 and oligomycin inhibited the high Ca^{2+} plateau and completely blocked Ca^{2+} uptake by the TG-insensitive store (Fig. 7 B). As seen after CCCP treatment, ionomycin applied after antimycin A1 + oligomycin failed to release a significant amount of Ca^{2+} even in the cells that happened to have a $[\text{Ca}^{2+}]_i$ plateau $>1 \mu\text{M}$ (Fig. 7 C). Together with the effects of the protonophores, these results support the notion that the TG-insensitive store in T cells is mitochondrial. Furthermore, they suggest that Ca^{2+} uptake by mitochondria is required to maintain the high Ca^{2+} plateau.

Effects of Na^+ Depletion

A large proportion of Ca^{2+} export from mitochondria is carried out by a $\text{Na}^+/\text{Ca}^{2+}$ exchanger in the mitochondrial inner membrane (Crompton et al., 1976; Carafoli, 1987; Gunter and Pfeiffer, 1990; Cox and Matlib, 1993; Gunter et al., 1994). As a further test of whether the TG-insensitive store is mitochondrial, we asked whether the depletion of intracellular Na^+ , a condition that inhibits $\text{Na}^+/\text{Ca}^{2+}$ exchange in mitochondria (Crompton et al., 1976; Gunter and Pfeiffer, 1990; Thayer and Miller, 1990; Gunter et al., 1994), enhances the accumulation of Ca^{2+} in the TG-insensitive store. The removal of external Na^+ to deplete Na^+ diminished the Ca^{2+} plateau evoked by Ca^{2+} readdition (Fig. 8 A). Similar results were obtained when

Table 1. Effects of Mitochondrial Inhibitors on the Ca^{2+} Plateau and Loading of the TG-insensitive Store

	Plateau $[\text{Ca}^{2+}]_i$		Store content	
	Jurkat	Human T cells	Jurkat	Human T cells
	<i>nM</i>		<i>nM</i>	
Control (2 mM Ca^{2+}_o)	1,003 ± 249 (14)	1,100 (1)	1,412 ± 555 (14)	3,052 (1)
+ CCCP	399 ± 110 (6)	623 (1)	116 ± 15 (3)	76 (1)
+ Antimycin A1	395 ± 137 (6)	400 (1)	626 ± 43 (3)	1,033 (1)
+ Antimycin A1/oligomycin	298 (1)	ND	60 (1)	ND
- Na	334 ± 64 (5)	279 (1)	1,731 ± 699 (2)	1,929 (1)

Plateau $[\text{Ca}^{2+}]_i$ was measured 1,000 s after readdition of Ca^{2+} to TG-treated cells. The store content of the TG-insensitive store is expressed as the peak $[\text{Ca}^{2+}]_i$ after addition of ionomycin (see Fig. 2). All values are given as a mean ± SD (number of experiments), and 250–300 cells were analyzed for each experiment.

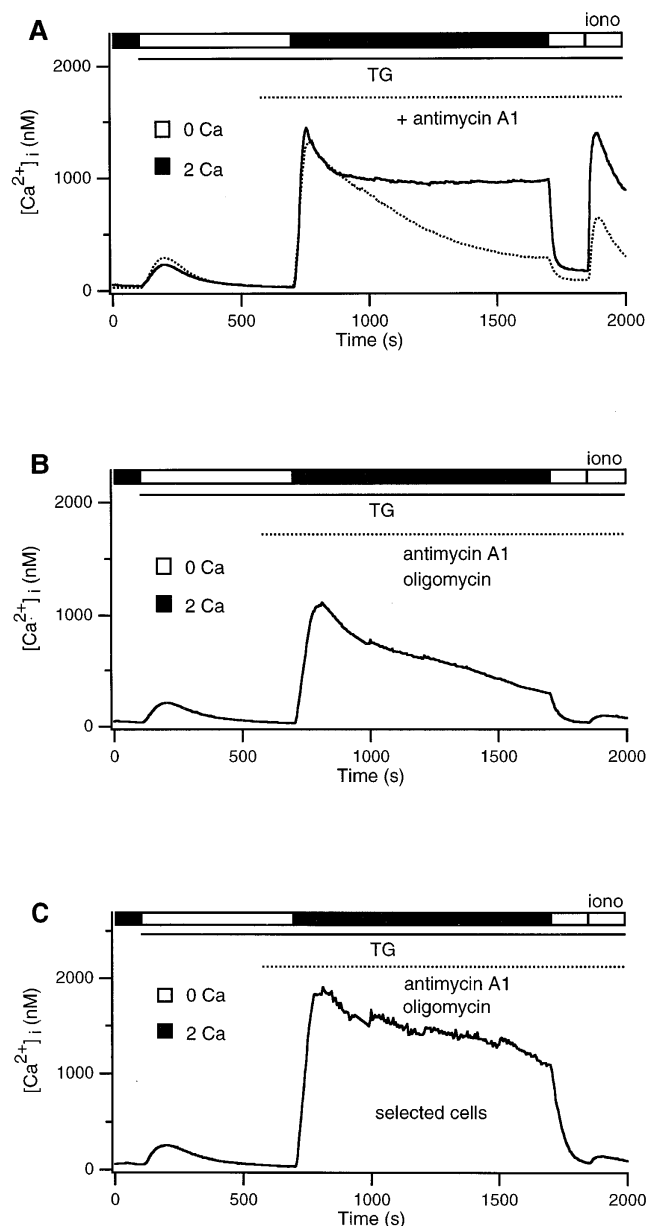


Figure 7. Antimycin A1 + oligomycin attenuates the Ca^{2+} plateau and inhibits loading of the TG-insensitive store. (A) 2 mM Ca^{2+}_o was readded to TG-treated cells in the presence (dotted trace) or absence (solid trace) of 2 μM antimycin A1. Antimycin alone inhibits the plateau and store loading, although part of the reduction in loading is due to the reduced plateau. (B) Antimycin

Na^+ was removed 600 s (Fig. 8 A) or 30 s before the readdition of Ca^{2+} . Na^+ depletion also enhanced store loading; this is most clearly evident from a comparison of ionomycin-induced release in cells bathed in 0 Na^+ (Fig. 8 B, solid trace) or in normal Na^+ selected for a comparably low plateau $[\text{Ca}^{2+}]_i$ (dotted trace). Readdition of Na^+ evoked the export of intracellular Ca^{2+} after a delay of ~ 30 s (Fig. 8 C), consistent with the reactivation of mitochondrial $\text{Na}^+/\text{Ca}^{2+}$ exchange. Na^+ readdition did not elicit a $[\text{Ca}^{2+}]_i$ rise in cells not previously exposed to Ca^{2+} , i.e., cells in which the TG-insensitive store was empty (data not shown). These results support the idea that mitochondria constitute the TG-insensitive store, and they suggest that Ca^{2+} export from mitochondria is essential for maintenance of the high Ca^{2+} plateau.

Potential Side Effects of Mitochondrial Inhibitors

Several potential nonspecific effects of the inhibitors we applied should be considered, including decreased Ca^{2+} influx resulting from CRAC channel blockade or depolarization, as well as changes in intracellular pH. Antimycin A1 exhibited neither of these effects (data not shown). As measured with BCECF, Ca^{2+} readdition caused a slight acidification of the cytoplasm, from a value of 7.52 to 7.44. CCCP did not affect pH_i beyond this range. CCCP did suppress the rate of Ca^{2+} influx by $\sim 50\%$, but restoration of the normal rate of entry by elevating $[\text{Ca}^{2+}]_o$ to 8 mM did not restore the high Ca^{2+} plateau. Thus, while the reduction in influx by CCCP might contribute to the observed kinetics of the Ca^{2+} response, it cannot by itself account for the inhibition of the high Ca^{2+} plateau. Na^+ depletion did not affect the Ca^{2+} influx rate but did increase the acidification observed after Ca^{2+} readdition to a value of 7.1. However, alkalization of the cytoplasm to pH 7.44 with 5 mM NH_4Cl (see Materials and Methods) did not restore the high Ca^{2+} plateau in the absence of Na^+ . This result is consistent with a report that intracellular acidification does not affect capacitative Ca^{2+} entry in thymocytes (Marriott and Mason, 1995). It should also be noted that even if $\text{Na}^+/\text{Ca}^{2+}$ exchange is functional in the

A1 applied together with 5 $\mu\text{g}/\text{ml}$ oligomycin completely inhibits store loading. (C) Average response of cells selected for a final $[\text{Ca}^{2+}]_i$ plateau $>1 \mu\text{M}$ from the experiment shown in B (15/278 cells). Antimycin A1 and oligomycin together completely abolish store loading even if $[\text{Ca}^{2+}]_i$ is high.

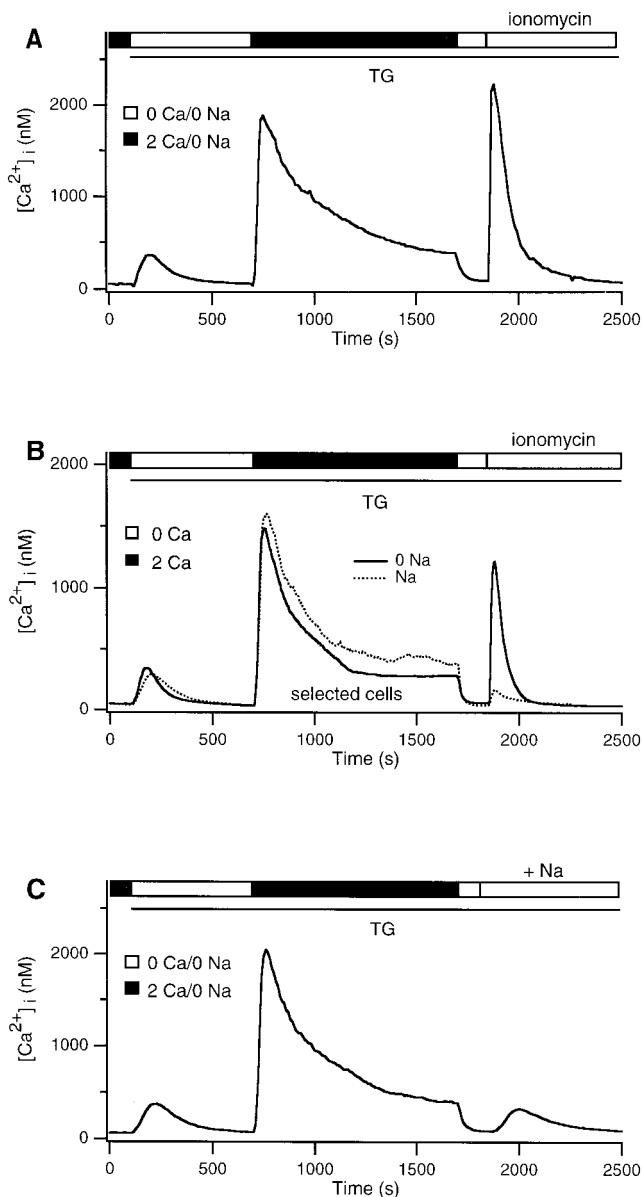


Figure 8. Na^+ depletion attenuates the Ca^{2+} plateau but enhances loading of the TG-insensitive store. (A) In the continuous absence of Na^+ , 2 mM Ca^{2+} was readded to TG-treated cells. The plateau was attenuated by Na^+ removal, yet ionomycin released a large amount of Ca^{2+} from the store. (B) Comparison of the average responses of cells in the presence (dotted trace; 17/191 cells) or absence (solid trace; 131/313 cells) of Na^+ , in which the mean $[\text{Ca}^{2+}]_i$ was selected to be <500 nM during the last half of the Ca^{2+} readdition period. Despite similar $[\text{Ca}^{2+}]_i$ profiles during the loading period, cells bathed in Na^+ -free solution retain significantly more Ca^{2+} in the TG-insensitive store. (C) 2 mM Ca^{2+} was readded to TG-treated cells in Na^+ -free Ringer's solution as depicted in A. Subsequent addition of Na^+ in Ca^{2+} -free Ringer's released Ca^{2+} from the TG-insensitive store.

plasma membrane of Jurkat cells (Balasubramanyam et al., 1993; but see Donnadiu and Trautmann, 1993), inhibition of its activity by Na^+ removal would be expected to impede Ca^{2+} efflux and thus elevate $[\text{Ca}^{2+}]_i$, opposite to the effect that was observed. Hence, side effects of the in-

hibitors are unlikely to account for the observed effects on the Ca^{2+} plateau. The only exception is oligomycin, which appears to inhibit the clearance of Ca^{2+} from the cytosol (compare the rates of clearance in 0 Ca^{2+}_o in Fig. 7, A and C). An inhibition of Ca^{2+} extrusion may account for the unexpected finding that oligomycin slows down the decay of the high Ca^{2+} plateau in antimycin A1-treated cells despite the fact that it helps to prevent Ca^{2+} uptake by the mitochondria (Fig. 7, A and B).

Mitochondrial Modulation of Ca^{2+} Signaling Is Similar in Human T Lymphocytes

The experiments described thus far were carried out using Jurkat cells, a human leukemic T cell line. Unlike normal human T cells that are quiescent before contact with antigen, Jurkat cells proliferate continuously, and it is possible that they express a relative overabundance of mitochondria to cope with their increased metabolic needs. Thus, to extend our results to a more physiological context, it is important to verify that mitochondria exert comparable effects on Ca^{2+} signaling in normal human T cells isolated from peripheral blood. As summarized in Table I, a TG-insensitive store is loaded by the sustained high Ca^{2+} plateau in human T cells as it is in Jurkat cells. The loading of the store is decreased or eliminated by antimycin A1 or CCCP, respectively, and is enhanced by Na^+ removal (Table I). Moreover, all three conditions preclude maintenance of the high Ca^{2+} plateau; therefore, mitochondria appear to function in normal quiescent T cells to modulate capacitative Ca^{2+} entry in a manner similar to that observed in Jurkat cells.

Abrupt Transitions of $[\text{Ca}^{2+}]_i$ in Single Cells

Examination of single-cell responses to Ca^{2+} readdition may shed light on the mechanism by which mitochondria help to maintain the high Ca^{2+} plateau. Under control conditions, most of the cells maintain a high intracellular Ca^{2+} concentration of >1 μM for the entire 1,000 s of exposure to 2 mM Ca^{2+} (Fig. 9 A). However, in a minority of cells, $[\text{Ca}^{2+}]_i$ declines rapidly to a much lower level around 400 nM, a phenomenon that we refer to as a "transition." In the experiment shown in Fig. 9 A, 14% of the cells showed transitions, which occurred at widely varying times after Ca^{2+} readdition. An amplitude histogram of $[\text{Ca}^{2+}]_i$ compiled from 272 cells during the exposure to Ca^{2+} is depicted in Fig. 9 B. The bimodal shape of the histogram indicates that the cells spend most of their time in one of two states (a high and a low amplitude plateau), with relatively little time spent at intermediate levels. The rate of capacitative Ca^{2+} entry can be estimated during the high and low Ca^{2+} states by the rate of $[\text{Ca}^{2+}]_i$ decline ($\Delta[\text{Ca}^{2+}]_i/\Delta t$) immediately after removal of extracellular Ca^{2+} . For the experiment in Fig. 9 A, these values were 56 nM/s and 15 nM/s for the high and low Ca^{2+} states, respectively. Thus, the transition from high to low Ca^{2+} plateau reflects a sudden decrease in the rate of Ca^{2+} influx.

Interestingly, mitochondrial uncouplers (CCCP or antimycin A1) or Na^+ depletion increase the likelihood and reduce the latency of these abrupt transitions. The variable latencies of the transitions among single cells play the dominant role in determining the smooth decline of the

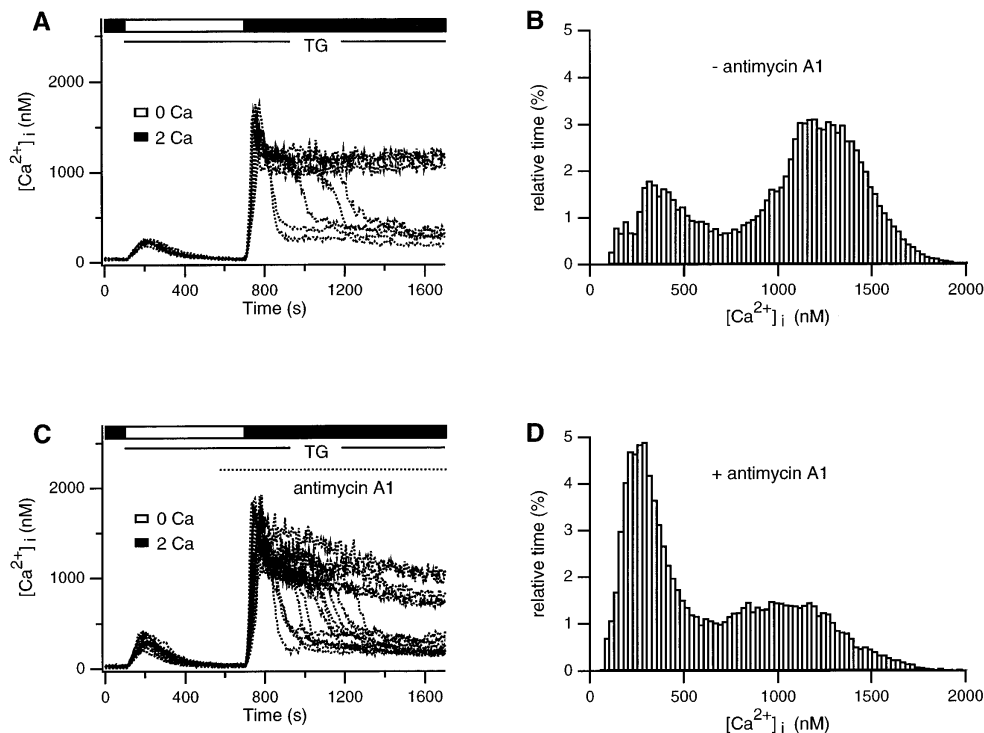


Figure 9. Abrupt transitions of $[Ca^{2+}]_i$ in single cells. Data were obtained from the experiments shown in Fig. 7 A. (A) Superimposition of control responses from eight cells shows rapid transitions from a high Ca^{2+} to a low Ca^{2+} plateau. (B) An amplitude histogram from period after Ca^{2+} readdition in the experiment shown in A is bimodal, indicating that cells spend most of their time in one of two well-defined states. (C) 2 μ M antimycin A1 increases the frequency of transitions from the high to low Ca^{2+} states. (D) Amplitude histogram from period after Ca^{2+} readdition in the experiment shown in C. The high Ca^{2+} peak is somewhat lower than in the control cells, probably due to the slow decline in the high Ca^{2+} plateau illustrated in C.

averaged population Ca^{2+} response (e.g., Figs. 6 A and 7 A). A number of single-cell responses in the presence of antimycin A1 are superimposed in Fig. 9 C. While the amplitudes of the high and low Ca^{2+} plateaus (Fig. 9 D) are similar to those seen in control cells, transitions to the low Ca^{2+} state are much more common, occurring in 63% of the cells (125/200). Similar results were observed with 1 μ M CCCP and Na^+ depletion (not shown). The implication of these results is that functional mitochondria maintain a high rate of capacitative Ca^{2+} influx by delaying or preventing abrupt decreases in the influx rate in individual cells.

Discussion

In this study we report that a TG-insensitive store most likely representing mitochondria regulates store-operated Ca^{2+} signaling in T cells. The store appears to be nearly empty of Ca^{2+} in resting cells, but after depletion of TG-sensitive stores, it senses the local Ca^{2+} elevation near activated CRAC channels and takes up incoming Ca^{2+} rapidly and without signs of saturation. The TG-insensitive store plays an active role in maintaining a high rate of Ca^{2+} entry, and hence a high Ca^{2+} plateau after the depletion of TG-sensitive stores. These results provide new evidence that mitochondria not only shape Ca^{2+} signals by acting as high capacity Ca^{2+} buffers, but that they can dynamically regulate the generation of Ca^{2+} signals as well.

Evidence for a Mitochondrial Ca^{2+} Store in T Cells

A mitochondrial Ca^{2+} store has not been previously described in T cells; therefore, several independent approaches were used to test the idea that the TG-insensitive store is mitochondrial. First, FCCP and CCCP were found

to prevent store loading. These protonophores are known to inhibit Ca^{2+} uptake through the mitochondrial uniporter by collapsing the mitochondrial proton gradient and dissipating the membrane potential across the inner membrane that provides the driving force for Ca^{2+} import (Thayer and Miller, 1990; Friel and Tsien, 1994; Werth and Thayer, 1994; Budd and Nicholls, 1996; Herrington et al., 1996; Park et al., 1996; Babcock et al., 1997). In addition, CCCP added to cells during the Ca^{2+} plateau transiently elevates $[Ca^{2+}]_i$. A similar effect has been reported in neurons (Werth and Thayer, 1994) and chromaffin cells (Herrington et al., 1996), and it may be explained by the sudden inhibition of Ca^{2+} uptake by the mitochondria, which would be expected to increase transiently the net Ca^{2+} flux into the cytosol from both extracellular and intramitochondrial sources. It is important to note that in some cells protonophores such as FCCP are known to interfere with nonmitochondrial Ca^{2+} stores (Jensen and Rehder, 1991; Marrion and Adams, 1992). For this reason, we also tested the ability of antimycin and oligomycin to prevent store loading. These agents act differently from the protonophores to depolarize mitochondria and to prevent Ca^{2+} uptake by inhibiting electron transport and the mitochondrial ATP synthase (Mohr and Fewtrell, 1990; Nicholls and Ferguson, 1992; Ali et al., 1994). Antimycin + oligomycin effectively prevented uptake of Ca^{2+} by the TG-insensitive store in T cells, consistent with the view that the TG-insensitive store is mitochondrial.

The effects of Na^+ removal further support this conclusion. Export of Ca^{2+} from mitochondria occurs through Na^+ -dependent and well as Na^+ -independent mechanisms (Crompton et al., 1976; Carafoli, 1987; Gunter and Pfeiffer, 1990; Cox and Matlib, 1993; Gunter et al., 1994). While the contribution of each appears to vary according to cell type, a significant part of mitochondrial Ca^{2+} export in many

cells is due to $\text{Na}^+/\text{Ca}^{2+}$ exchange across the inner membrane. Na^+ depletion enhances net Ca^{2+} uptake by mitochondria in vivo and in vitro, while readdition of Na^+ triggers its release (Crompton et al., 1976; Thayer and Miller, 1990). Likewise, we found that removal of extracellular Na^+ , which is expected to deplete intracellular Na^+ , enhanced the loading of the TG-insensitive store in T cells, and Na^+ readdition evoked Ca^{2+} release. Thus, while none of the several treatments used is absolutely specific for mitochondria, the ability of protonophores, an unrelated electron transport inhibitor, and Na^+ depletion to affect loading of the store argues strongly that it represents mitochondria.

Finally, experiments with rhod-2 offer independent evidence for a mitochondrial Ca^{2+} store. Rhod-2 has been used to detect changes in mitochondrial $[\text{Ca}^{2+}]$ in several cells, including hepatocytes (Hajnóczky et al., 1995), CHO cells (Rutter et al., 1996), and chromaffin cells (Babcock et al., 1997). The precise location of rhod-2 within mitochondria has not to our knowledge been demonstrated in any cell. However, several factors argue that the dye reports primarily $[\text{Ca}^{2+}]$ in the matrix rather than in the space between mitochondrial inner and outer membranes: (a) the intermembrane volume is small compared to the matrix volume; (b) porins and voltage-dependent anion-selective channels in the outer membrane would be expected to conduct rhod-2 out of the intermembrane space (Zalman et al., 1980); and (c) the highly negative potential that exists across the inner but not the outer membrane (Benz, 1994) would be expected to promote accumulation of positively charged rhod-2 in the matrix. We found that rhod-2 labeled the mitochondria in Jurkat cells, as judged from its colocalization with a selective mitochondrial stain, MitoTracker™ Green FM. The compartmentalized rhod-2 increased its fluorescence in response to increases in $[\text{Ca}^{2+}]_i$, and this response was inhibited by CCCP. These studies provide the most direct evidence that mitochondria do in fact constitute the TG-insensitive store in T cells.

Interestingly, cyclic ADP ribose has recently been shown to release Ca^{2+} from a TG-insensitive store in permeabilized Jurkat T cells (Guse et al., 1995). It is not clear how this store is related to the mitochondrial store we describe. One possibility is that cyclic ADP ribose releases Ca^{2+} from mitochondria; alternatively, a separate cyclic ADP ribose-sensitive store may be responsible for the small amount of Ca^{2+} released by ionomycin in resting cells (Fig. 1) or in cells treated with mitochondrial inhibitors (Figs. 6 and 7).

Properties of the Mitochondrial Ca^{2+} Store

Several characteristics of the TG-insensitive store are consistent with the known behavior of mitochondria in other cells and in vitro (Carafoli, 1987; Gunter and Pfeiffer, 1990; Gunter et al., 1994). These features include the modest threshold of ~ 400 nM for Ca^{2+} uptake, and high capacity that shows no signs of saturation even at high $[\text{Ca}^{2+}]_i$ (>1.8 μM) sustained over long periods (10–20 min). Occasionally, we observed significant uptake at $[\text{Ca}^{2+}]_i$ of 100–400 nM, implying that the threshold for uptake may be quite close to resting $[\text{Ca}^{2+}]_i$ in some cells. Alternatively,

some mitochondria in these “hypersensitive” cells may simply lie close to the plasma membrane or near a region of high CRAC channel density, where they are exposed to high local $[\text{Ca}^{2+}]_i$ (see below). Interestingly, the Ca^{2+} dependence of loading appears to be biphasic, consisting of a low rate of uptake at low $[\text{Ca}^{2+}]_i$ and a faster nonlinear rate at higher $[\text{Ca}^{2+}]_i$. The nonlinear increase in uptake with $[\text{Ca}^{2+}]_i$ is consistent with reports that the activity of the uniporter in vitro increases with the second power of $[\text{Ca}^{2+}]$ (Gunter and Pfeiffer, 1990).

Calcium uptake by mitochondria in T cells is rapid, occurring within 8–10 s at physiological levels of $[\text{Ca}^{2+}]_i$ (Fig. 4). Comparably rapid uptake has been detected with rhod-2 or mitochondrially targeted aequorin in other cells after Ca^{2+} release from IP_3 -sensitive stores (Rizzuto et al., 1993, 1994; Hajnóczky et al., 1995). A recent report of hepatocyte mitochondria in vitro has resolved two modes of Ca^{2+} uptake: a rapid mode that is active at $[\text{Ca}^{2+}]_i$ as low as 171 nM and inactivates within 1 s, and a slower mode that is activated in a sustained fashion by $[\text{Ca}^{2+}]_i > \sim 400$ nM (Sparagna et al., 1995). Unfortunately, it is difficult to assess the contributions of each mode to the Ca^{2+} loading we have observed, due to the relatively slow rise time of the $[\text{Ca}^{2+}]_i$ increase in our experiments.

The Mitochondrial Store Can Sense Local Ca^{2+} Gradients near CRAC Channels

We find that mitochondrial uptake at any given level of global cytosolic $[\text{Ca}^{2+}]_i$ is enhanced by increasing the driving force for Ca^{2+} entry. This result implies that the store responds not merely to the global $[\text{Ca}^{2+}]_i$ averaged over the entire cell, but to localized Ca^{2+} gradients that exist in the vicinity of open CRAC channels as well. Previous studies in hepatocytes and HeLa cells have shown that Ca^{2+} released from IP_3 -sensitive stores is taken up by mitochondria more efficiently than comparable amounts of Ca^{2+} entering through plasma membrane channels, suggesting that in those cells mitochondria can sense the Ca^{2+} microdomains near open IP_3 receptors (Rizzuto et al., 1993; Hajnóczky et al., 1995). This result does not apply to all cells, however; in endothelial cells, for example, mitochondrial Ca^{2+} is more efficiently raised by Ca^{2+} influx across the plasma membrane than by ER release (Lawrie et al., 1996). Interestingly, EM measurements indicate that more of the mitochondria in HeLa cells are close to the ER than to the plasma membrane, whereas the opposite is true for endothelial cells. In Jurkat and peripheral blood T cells, the nucleus occupies most of the cell volume, thus confining the mitochondria to a region close to the plasma membrane. Although a detailed ultrastructural analysis remains to be done, a substantial proportion of mitochondrial profiles can be seen within 100–500 nm of the plasma membrane in electron micrographs of T cells (Veerman and van Rooijen, 1975; Huhn et al., 1976; Fachetti et al., 1988). Thus, it appears reasonable that mitochondria are able to sense local gradients in the proximity of CRAC channels.

How much of the Ca^{2+} that enters the T cell through CRAC channels is taken up by mitochondria? A rough estimate can be made as follows. During the high Ca^{2+} plateau, influx and efflux of Ca^{2+} from the cytosol are equal.

Thus, influx through CRAC channels is balanced by net uptake into mitochondria plus extrusion across the plasma membrane. At a plateau level of 1 μM , the extrusion rate (measured in 0 Ca^{2+} + CCCP) is 50 nM s^{-1} . The total mitochondrial content as estimated by the integral of ionomycin-released Ca^{2+} is $\sim 5 \mu\text{M}$ (Fig. 2 B), which upon dividing by the loading time (1,000 s) implies an average rate of 5 nM s^{-1} for net uptake. Thus, during the high Ca^{2+} plateau, the rate of capacitative Ca^{2+} entry is equal to 50 nM s^{-1} (the extrusion rate) plus 5 nM s^{-1} (the net mitochondrial uptake rate), or 55 nM s^{-1} . By this estimate, mitochondria may accumulate $\sim 10\%$ (5/55) of the incoming Ca^{2+} . Since mitochondria are also continuously exporting Ca^{2+} , the true uptake rate is likely to exceed 5 nM s^{-1} , as explained by the following argument. After Ca^{2+} removal, the low Ca^{2+} plateau declines slowly, implying that Ca^{2+} extrusion across the plasma membrane is nearly balanced by the release of Ca^{2+} from mitochondria (the only significant source of Ca^{2+} in these TG-treated cells). The extrusion rate was measured to be $\sim 15 \text{nM s}^{-1}$ at cytosolic Ca^{2+} values corresponding to the low Ca^{2+} plateau (see Materials and Methods). Therefore, the implied unidirectional uptake rate is 20 nM s^{-1} (5 nM s^{-1} net uptake plus 15 nM s^{-1} export), suggesting that $\sim 36\%$ (20/55) of the incoming Ca^{2+} enters the mitochondria.

Mitochondria Help Maintain a High Rate of Capacitative Calcium Entry

Functional mitochondria appear to help maintain a high rate of capacitative Ca^{2+} entry after the continued depletion of IP_3/TG -sensitive stores. This conclusion follows from the ability of several types of mitochondrial inhibitors to dramatically reduce the level of the Ca^{2+} plateau. The gradual decline in the population average $[\text{Ca}^{2+}]_i$ in the presence of mitochondrial inhibitors results primarily from abrupt stochastic transitions between states of high and low Ca^{2+} influx occurring asynchronously in each cell. While these transitions take place even in control cells, the inhibitors increase their frequency and decrease their latency. Thus, functional mitochondria appear to maintain the high Ca^{2+} plateau by preventing or delaying an abrupt decrease in the Ca^{2+} influx rate.

The reduction of capacitative Ca^{2+} entry during the transitions is extremely rapid. In single cells, the rate of $[\text{Ca}^{2+}]_i$ decline during a transition reaches a maximal value that can be as much as two-thirds of the Ca^{2+} clearance rate measured in the absence of Ca^{2+}_o , and this can be reached within 5 s of the onset of the transition. These results imply that capacitative Ca^{2+} entry is reduced by about two-thirds in <5 s. The mechanism that governs these rapid flux-rate transitions is unknown. The rapid time course is reminiscent of the permeability transition of mitochondria, a catastrophic increase in permeability to small solutes and ions that can be triggered by excessive loading of mitochondria with Ca^{2+} (Gunter and Pfeiffer, 1990; Gunter et al., 1994). However, this process would release the entire mitochondrial Ca^{2+} content in an explosive fashion, and an increase in $[\text{Ca}^{2+}]_i$ was never observed immediately preceding the transitions. For this reason, we believe it is unlikely that the $[\text{Ca}^{2+}]_i$ transitions we have observed involve the permeability transition of mitochon-

dria. Alternatively, the flux-rate transitions may result from Ca^{2+} accumulation and the Ca^{2+} -dependent inactivation of CRAC channels (see below) or from membrane depolarization, which is known to profoundly inhibit Ca^{2+} entry through CRAC channels (for review see Lewis and Cahalan, 1995). Tests of these possible mechanisms await further study.

In what capacity do the mitochondria act to maintain capacitative Ca^{2+} entry? Given the dominant role of mitochondria in producing most of the cell's ATP, one obvious possibility is that mitochondria are required to maintain a high $[\text{ATP}]/[\text{ADP}]$ ratio in the cell. Gamberucci et al. (1994) reported inhibitory effects of mitochondrial poisons on the TG-induced $[\text{Ca}^{2+}]_i$ rise in Jurkat cells and several other cell lines, an effect they attributed to a decreased $[\text{ATP}]/[\text{ADP}]$ ratio or most likely to increased $[\text{ADP}]$. Later patch-clamp studies in rat basophilic leukemia cells demonstrated that increased $[\text{ADP}]$ can inhibit I_{CRAC} (Innocenti et al., 1996). ATP depletion also inhibits capacitative Ca^{2+} entry in rat thymocytes at 37°C (Marriott and Mason, 1995). However, even though changes in $[\text{ATP}]/[\text{ADP}]$ could certainly contribute to the effects of mitochondrial inhibitors we have seen, a number of arguments suggest that they are not primarily responsible for these effects. First, ADP inhibits I_{CRAC} at 37°C but not at 25°C, and the effects of mitochondrial inhibitors on the Ca^{2+} response at 37°C are reduced in the presence of glucose (Innocenti et al., 1996). We conducted all experiments below 25°C and included 10 mM extracellular glucose to provide a substrate for glycolytic ATP production that has been shown to be sufficient to keep ATP levels high (Mohr and Fewtrell, 1990; Budd and Nicholls, 1996). Second, antimycin A1 (Fig. 6) and CCCP (data not shown) inhibited the high Ca^{2+} plateau even when oligomycin was included to prevent reverse-mode hydrolysis of ATP by the ATP synthase. Third, CCCP, antimycin, or Na^+ depletion did not alter the rate of Ca^{2+} clearance after prolonged Ca^{2+} elevation (data not shown), suggesting that none of these treatments caused a gross depletion of intracellular ATP. Finally, the initial Ca^{2+} influx rate in CCCP-treated cells was constant (38 nM/s) in response to multiple readditions of Ca^{2+} separated by a 200-s recovery period in 0 Ca^{2+}_o . These results argue against a long-lasting depletion of intracellular ATP as the basis for the observed effects on the Ca^{2+} plateau.

Hypothesis for the Mitochondrial Regulation of Capacitative Calcium Entry

Inhibition of either mitochondrial Ca^{2+} uptake (by CCCP or antimycin A1) or export (by Na^+ depletion) attenuates the high Ca^{2+} plateau, suggesting that both uptake and export of Ca^{2+} are necessary to maintain a high rate of capacitative Ca^{2+} entry. A model based on Ca^{2+} -dependent inhibition of CRAC channels (Hoth and Penner, 1993; Zweifach and Lewis, 1995a,b) may provide a way to reconcile these results. As shown in Fig. 10 A, in the absence of functional mitochondria (e.g., with CCCP or antimycin + oligomycin present), Ca^{2+} entering the cell through CRAC channels accumulates near the plasma membrane and inhibits Ca^{2+} entry, leading eventually to a steady-state level of I_{CRAC} and $[\text{Ca}^{2+}]_i$. Intracellular Ca^{2+} inhibits I_{CRAC} in several ways. Fast inactivation is thought to in-

volve binding of Ca^{2+} to sites located within several nanometers of the intracellular mouth of the channels (Zweifach and Lewis, 1995a), while slow inactivation occurs via store refilling as well as by a store-independent process that persists in the presence of TG (Zweifach and Lewis, 1995b). Slow inactivation can be prevented by intracellular EGTA, implying that the Ca^{2+} binding sites underlying this process are located further than ~ 100 nm from the channels (Zweifach and Lewis, 1995a,b). Considering the evidence that mitochondria can sense $[\text{Ca}^{2+}]_i$ gradients near CRAC channels (Fig. 3), it seems reasonable that they may reduce $[\text{Ca}^{2+}]_i$ at locations that influence slow inactivation. Effects on fast inactivation are less likely given the extreme constraints that the inactivation site would place on mitochondrial location and uptake rates. Thus, when mitochondria are allowed to take up but not export Ca^{2+} (e.g., in Na^+ -depleted cells; Fig. 10 B), they may act as a sink to reduce the local $[\text{Ca}^{2+}]_i$. The reduction of local $[\text{Ca}^{2+}]_i$ would lessen CRAC channel inactivation until a new steady state is reached, in which I_{CRAC} is somewhat greater than it was before and the local $[\text{Ca}^{2+}]_i$ is somewhat less than it was before. Thus, the paradoxical result would be that, without export, mitochondrial uptake would enhance I_{CRAC} but not necessarily global $[\text{Ca}^{2+}]_i$. Only if Ca^{2+} export from mitochondria is also permitted (e.g., in untreated cells; Fig. 10 C) can the increased Ca^{2+} influx contribute effectively to the global $[\text{Ca}^{2+}]_i$. Thus, mitochondria may serve two functions; as Ca^{2+} buffers, they remove Ca^{2+} from sites where it inhibits CRAC channels, and through Ca^{2+} export, they redistribute it to distant sites where it does not inhibit I_{CRAC} . To explain why transitions are uncommon in normal cells, we would speculate that the redistribution of Ca^{2+} by mitochondria helps to prevent transitions. As discussed above, further study will be required to test this hypothesis.

Possible Physiological Roles of Mitochondria during T Cell Activation

The properties described here for the mitochondrial Ca^{2+} store are consistent with a role of mitochondria in Ca^{2+} signaling during T cell activation by antigen. Stimulation of the T cell receptor by antigen or polyclonal mitogens is known to elevate $[\text{Ca}^{2+}]_i$ to levels of 0.5 to >1 μM in hu-

man T cells and Jurkat cells, largely by triggering Ca^{2+} influx through CRAC channels (Lewis and Cahalan, 1995). The $[\text{Ca}^{2+}]_i$ rise can take a variety of forms, including sustained Ca^{2+} plateaus, periodic oscillations, and irregular Ca^{2+} transients (Lewis and Cahalan, 1989; Donnadieu et al., 1992a,b; Negulescu et al., 1996). The modest threshold and rapid kinetics of Ca^{2+} uptake by mitochondria in T cells, as well as their ability to sense Ca^{2+} in the vicinity of CRAC channels, should enable them to respond to and modulate these Ca^{2+} signals. Ca^{2+} -dependent activation of the T cell transcription factor NFAT is rapidly reversible, and this characteristic is believed to underlie the requirement for sustained $[\text{Ca}^{2+}]_i$ elevation during T cell activation (Shibasaki et al., 1996; Timmerman et al., 1996). Mitochondria may therefore help to enhance NFAT-dependent transcription and T cell activation by prolonging Ca^{2+} plateaus, or by slowly releasing sequestered Ca^{2+} to lengthen Ca^{2+} transients.

One of the most commonly ascribed functions of mitochondria is to buffer pathophysiological rises of $[\text{Ca}^{2+}]_i$. Such a role may be an especially important safeguard in T cells considering the prolonged period of $[\text{Ca}^{2+}]_i$ elevation needed to commit cells to the activation pathway. In addition, increases in $[\text{Ca}^{2+}]_m$ are known to activate several dehydrogenases that supply substrates to the Krebs cycle, an action that has been interpreted as a means of upregulating ATP generation in response to increased metabolic demand in the heart and liver (Gunter et al., 1994; Hansford, 1994). In an analogous fashion, Ca^{2+} influx through CRAC channels may signal mitochondria to prepare quiescent T cells for the increased metabolic commitments associated with entry into the cell cycle and cell proliferation. Thus, mitochondria may fill multiple roles in the T cell, acting within a feedback loop to sense and regulate Ca^{2+} entry via store-operated Ca^{2+} channels, while preparing the cell for the increased demands of cell activation.

M. Hoth would like to dedicate this paper to his late friend Kai Stockhusen, with whom he learned in school about the importance of the mitochondrial Ca^{2+} store.

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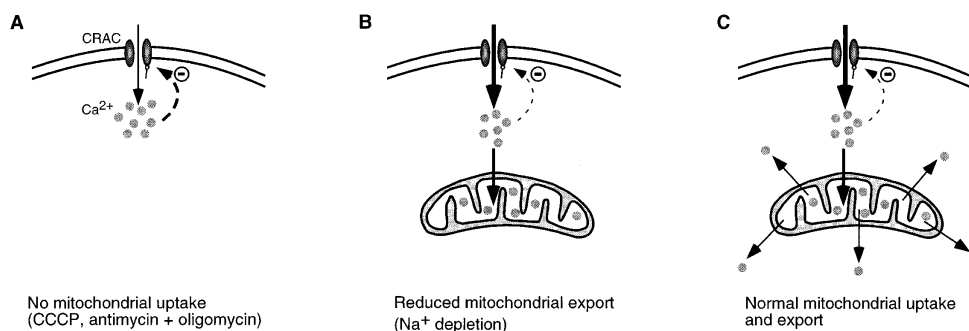


Figure 10. A hypothesis for mitochondrial modulation of capacitative Ca^{2+} entry. (A) When Ca^{2+} uptake by mitochondria is blocked, Ca^{2+} accumulates near CRAC channels and inhibits them (**bold dashed line**). A steady-state $[\text{Ca}^{2+}]_i$ near the channels is reached as a result of this negative feedback. (B) Sequestration of Ca^{2+} by mitochondria in the absence of export reduces the local $[\text{Ca}^{2+}]_i$, thereby

partially relieving inactivation of I_{CRAC} (**thin dashed line**). At steady state, I_{CRAC} is larger than in A, but much of the Ca^{2+} from this increased influx is sequestered and hence is not available to enhance global $[\text{Ca}^{2+}]_i$. (C) In the presence of normal mitochondrial uptake and release of Ca^{2+} , mitochondria redistribute the Ca^{2+} derived from enhanced I_{CRAC} to distant sites where it can elevate global $[\text{Ca}^{2+}]_i$.

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References

- Ali, H., K. Maeyama, R. Sagi-Eisenberg, and M.A. Beaven. 1994. Antigen and thapsigargin promote influx of Ca^{2+} in rat basophilic RBL-2H3 cells by ostensibly similar mechanisms that allow filling of inositol 1,4,5-trisphosphate-sensitive and mitochondrial Ca^{2+} stores. *Biochem. J.* 304:431–440.
- Ankarcrona, M., J.M. Dypbukt, E. Bonfoco, B. Zhivotovsky, S. Orrenius, S.A. Lipton, and P. Nicotera. 1995. Glutamate-induced neuronal death: a succession of necrosis or apoptosis depending on mitochondrial function. *Neuron*. 15:961–973.
- Babcock, D.F., J. Herrington, P.C. Goodwin, Y.B. Park, and B. Hille. 1997. Mitochondrial participation in the intracellular Ca^{2+} network. *J. Cell Biol.* 136: 833–844.
- Balasubramanyam, M., M. Kimura, A. Aviv, and J.P. Gardner. 1993. Kinetics of calcium transport across the lymphocyte plasma membrane. *Am. J. Physiol.* 265:C321–C327.
- Benz, R. 1994. Permeation of hydrophilic solutes through mitochondrial outer membranes: review on mitochondrial porins. *Biochim. Biophys. Acta.* 1197: 167–196.
- Berridge, M.J. 1995. Capacitative calcium entry. *Biochem. J.* 312:1–11.
- Budd, S.L., and D.G. Nicholls. 1996. A reevaluation of the role of mitochondria in neuronal Ca^{2+} homeostasis. *J. Neurochem.* 66:403–411.
- Carafoli, E. 1987. Intracellular calcium homeostasis. *Annu. Rev. Biochem.* 56: 395–433.
- Cox, D.A., and M.A. Matlib. 1993. Modulation of intramitochondrial free Ca^{2+} concentration by antagonists of Na^{+} - Ca^{2+} exchange. *Trends Pharmacol. Sci.* 14:408–413.
- Crompton, M., M. Capano, and E. Carafoli. 1976. The sodium-induced efflux of calcium from heart mitochondria. *Eur. J. Biochem.* 69:453–462.
- Dolmetsch, R., and R.S. Lewis. 1994. Signaling between intracellular Ca^{2+} stores and depletion-activated Ca^{2+} channels generates $[\text{Ca}^{2+}]_i$ oscillations in T lymphocytes. *J. Gen. Physiol.* 103:365–388.
- Donnadieu, E., and A. Trautmann. 1993. Is there a $\text{Na}^{+}/\text{Ca}^{2+}$ exchanger in macrophages and in lymphocytes? *Pflügers Arch.* 424:448–455.
- Donnadieu, E., G. Bismuth, and A. Trautmann. 1992a. Calcium fluxes in T lymphocytes. *J. Biol. Chem.* 267:25864–25872.
- Donnadieu, E., D. Cefai, Y.P. Tan, G. Paresys, G. Bismuth, and A. Trautmann. 1992b. Imaging early steps of human T cell activation by antigen-presenting cells. *J. Immunol.* 148:2643–2653.
- Facchetti, F., C. De Wolf-Peeters, J.J. van den Oord, R. De Vos, and V.J. Desmet. 1988. Plasmacytoid T cells: a cell population normally present in the reactive lymph node. *Hum. Pathol.* 19:1085–1092.
- Fanger, C.M., M. Hoth, G.R. Crabtree, and R.S. Lewis. 1995. Characterization of T cell mutants with defects in capacitative calcium entry: genetic evidence for the physiological roles of CRAC channels. *J. Cell Biol.* 131:655–667.
- Fasolato, C., B. Innocenti, and T. Pozzan. 1994. Receptor-activated Ca^{2+} influx: how many mechanisms for how many channels? *Trends Pharmacol. Sci.* 15: 77–83.
- Friel, D.D., and R.W. Tsien. 1994. An FCCP-sensitive Ca^{2+} store in bullfrog sympathetic neurons and its participation in stimulus-evoked changes in $[\text{Ca}^{2+}]_i$. *J. Neurosci.* 14:4007–4024.
- Gamberucci, A., B. Innocenti, R. Fulceri, G. Bãnhgyi, R. Giunti, T. Pozzan, and A. Benedetti. 1994. Modulation of Ca^{2+} influx dependent on store depletion by intracellular adenine-guanine nucleotide levels. *J. Biol. Chem.* 269:23597–23602.
- Goldsmith, M.A., and A. Weiss. 1988. Early signal transduction by the antigen receptor without commitment to T cell activation. *Science (Wash. DC)*. 240: 1029–1031.
- Gunter, T.E., and D.R. Pfeiffer. 1990. Mechanisms by which mitochondria transport calcium. *Am. J. Physiol.* 258:C755–C786.
- Gunter, T.E., K.K. Gunter, S.-S. Sheu, and C.E. Gavin. 1994. Mitochondrial calcium transport: physiological and pathological relevance. *Am. J. Physiol.* 267:C313–C339.
- Guse, A.H., C.P. da Silva, F. Emmrich, G.A. Ashamu, B.V.L. Potter, and G.W. Mayr. 1995. Characterization of cyclic adenosine diphosphate-ribose-induced Ca^{2+} release in T lymphocyte cell lines. *J. Immunol.* 155:3353–3359.
- Hajnoczky, G., L.D. Robb-Gaspers, M.B. Seitz, and A.P. Thomas. 1995. Decoding of cytosolic calcium oscillations in the mitochondria. *Cell.* 82:415–424.
- Hansford, R.G. 1994. Physiological role of mitochondrial Ca^{2+} transport. *J. Bioenerg. Biomembr.* 26:495–508.
- Herrington, J., Y.B. Park, D.F. Babcock, and B. Hille. 1996. Dominant role of mitochondria in clearance of large Ca^{2+} loads from rat adrenal chromaffin cells. *Neuron*. 16:219–228.
- Hoth, M., and R. Penner. 1993. Calcium release-activated calcium current in rat mast cells. *J. Physiol. (Lond.)*. 465:359–386.
- Huhn, D., H. Rodt, E. Thiel, U. Fink, and W. Ruppelt. 1976. Elektronenmikroskopische und immunhistochemische Untersuchungen an menschlichen Lymphozyten. *Blut*. 32:87–102.
- Innocenti, B., T. Pozzan, and C. Fasolato. 1996. Intracellular ADP modulates the Ca^{2+} release-activated Ca^{2+} current in a temperature- and Ca^{2+} -dependent way. *J. Biol. Chem.* 271:8582–8587.
- Jensen, J.R., and V. Rehder. 1991. FCCP releases Ca^{2+} from a non-mitochondrial store in an identified *Helisoma* neuron. *Brain Res.* 551:311–314.
- Jouaville, L.S., F. Ichas, E.L. Holmuhamedov, P. Camacho, and J.D. Lechleiter. 1995. Synchronization of calcium waves by mitochondrial substrates in *Xenopus laevis* oocytes. *Nature (Lond.)*. 377:438–441.
- Lawrie, A.M., R. Rizzuto, T. Pozzan, and A.W.M. Simpson. 1996. A role for calcium influx in the regulation of mitochondrial calcium in endothelial cells. *J. Biol. Chem.* 271:10753–10759.
- Lewis, R.S., and M.D. Cahalan. 1989. Mitogen-induced oscillations of cytosolic Ca^{2+} and transmembrane Ca^{2+} current in human leukemic T cells. *Cell Regul.* 1:99–112.
- Lewis, R.S., and M.D. Cahalan. 1995. Potassium and calcium channels in lymphocytes. *Annu. Rev. Immunol.* 13:623–653.
- Marrion, N.V., and P.R. Adams. 1992. Release of intracellular calcium and modulation of membrane currents by caffeine in bull-frog sympathetic neurones. *J. Physiol. (Lond.)*. 445:515–535.
- Marriott, I., and M.J. Mason. 1995. ATP depletion inhibits capacitative Ca^{2+} entry in rat thymic lymphocytes. *Am. J. Physiol.* 269:C766–C774.
- Minta, A., J.P.Y. Kao, and R.Y. Tsien. 1989. Fluorescent indicators for cytosolic calcium based on rhodamine and fluorescein chromophores. *J. Biol. Chem.* 264:8171–8178.
- Mohr, F.C., and C. Fewtrell. 1990. The effect of mitochondrial inhibitors on calcium homeostasis in tumor mast cells. *Am. J. Physiol.* 258:C217–C226.
- Negulescu, P.A., N. Shastri, and M.D. Cahalan. 1994. Intracellular calcium dependence of gene expression in single T lymphocytes. *Proc. Natl. Acad. Sci. USA.* 91:2873–2877.
- Negulescu, P.A., A. Khan, T.B. Krasieva, H.H. Kerschbaum, and M.D. Cahalan. 1996. Polarity of T cell shape, motility, and sensitivity to antigen. *Immunity*. 4:421–430.
- Nicholls, D.G., and S.J. Ferguson. 1992. *Bioenergetics 2*. Academic Press, London. 255 pp.
- Nichols, B.J., and R.M. Denton. 1995. Towards the molecular basis for the regulation of mitochondrial dehydrogenases by calcium ions. *Mol. Cell. Biochem.* 149/150:203–212.
- Park, Y.B., J. Herrington, D.F. Babcock, and B. Hille. 1996. Ca^{2+} clearance mechanisms in isolated rat adrenal chromaffin cells. *J. Physiol. (Lond.)*. 492: 329–346.
- Partiseti, M., F. Le Diest, C. Hivroz, A. Fischer, H. Korn, and D. Choquet. 1994. The calcium current activated by T cell receptor and store depletion in human lymphocytes is absent in a primary immunodeficiency. *J. Biol. Chem.* 269:32327–32335.
- Penner, R., C. Fasolato, and M. Hoth. 1993. Calcium influx and its control by calcium release. *Curr. Opin. Neurobiol.* 3:368–374.
- Putney, J.W., Jr., and G.S.J. Bird. 1993. The inositol phosphate-calcium signaling system in nonexcitable cells. *Endocr. Rev.* 14:610–631.
- Rizzuto, R., A.W.M. Simpson, M. Brini, and T. Pozzan. 1992. Rapid changes of mitochondrial Ca^{2+} revealed by specifically targeted recombinant aequorin. *Nature (Lond.)*. 358:325–327.
- Rizzuto, R., M. Brini, M. Murgia, and T. Pozzan. 1993. Microdomains with high Ca^{2+} close to IP_3 -sensitive channels that are sensed by neighboring mitochondria. *Science (Wash. DC)*. 262:744–747.
- Rizzuto, R., C. Bastianutto, M. Brini, M. Murgia, and T. Pozzan. 1994. Mitochondrial Ca^{2+} homeostasis in intact cells. *J. Cell Biol.* 126:1183–1194.
- Roos, A., and W.F. Boron. 1981. Intracellular pH. *Physiol. Rev.* 61:296–434.
- Rutter, G.A., P. Burnett, R. Rizzuto, M. Brini, M. Murgia, T. Pozzan, J.M. Tavaré, and R.M. Denton. 1996. Subcellular imaging of intramitochondrial Ca^{2+} with recombinant targeted aequorin: significance for the regulation of pyruvate dehydrogenase activity. *Proc. Natl. Acad. Sci. USA.* 93:5489–5494.
- Sheu, S.-S., and M.-L. Jou. 1994. Mitochondrial free Ca^{2+} concentration in living cells. *J. Bioenerg. Biomembr.* 26:487–493.
- Shibasaki, F., E.R. Price, D. Milan, and F. McKeon. 1996. Role of kinases and the phosphatase calcineurin in the nuclear shuttling of transcription factor NF-AT4. *Nature (Lond.)*. 382:370–373.
- Sparagna, G.C., K.K. Gunter, S.-S. Sheu, and T.E. Gunter. 1995. Mitochondrial calcium uptake from physiological-type pulses of calcium. A description of the rapid uptake mode. *J. Biol. Chem.* 270:27510–27515.
- Thastrup, O., P.J. Cullen, B.K. Drobak, M.R. Hanley, and A.P. Dawson. 1990. Thapsigargin, a tumor promoter, discharges intracellular Ca^{2+} stores by specific inhibition of the endoplasmic reticulum Ca^{2+} -ATPase. *Proc. Natl. Acad. Sci. USA.* 87:2466–2470.
- Thayer, S.A., and R.J. Miller. 1990. Regulation of the intracellular free calcium concentration in single rat dorsal root ganglion neurones *in vitro*. *J. Physiol. (Lond.)*. 425:85–115.
- Thomas, J.A., R.N. Buchsbaum, A. Zimniak, and E. Racke. 1979. Intracellular pH measurements in Ehrlich ascites tumor cells utilizing spectroscopic probes generated *in situ*. *Biochemistry*. 18:2210–2218.
- Timmerman, L.A., N.A. Clipstone, S.N. Ho, J.P. Northrop, and G.R. Crabtree. 1996. Rapid shuttling of NF-AT in discrimination of Ca^{2+} signals and immunosuppression. *Nature (Lond.)*. 383:837–840.

- Uto, A., H. Arai, and Y. Ogawa. 1991. Reassessment of fura-2 and the ratio method for determination of intracellular Ca^{2+} concentrations. *Cell Calcium*. 12:29–37.
- Veerman, A.J.P., and N. van Rooijen. 1975. Lymphocyte capping and lymphocyte migration as associated events in the *in vivo* antigen trapping process. An electron-microscopic autoradiographic study in the spleen of mice. *Cell Tissue Res*. 161:211–217.
- Werth, J.L., and S.A. Thayer. 1994. Mitochondria buffer physiological calcium loads in cultured rat dorsal root ganglion neurons. *J. Neurosci*. 14:348–356.
- Zalman, L.S., H. Nikaido, and Y. Kagawa. 1980. Mitochondrial outer membrane contains a protein producing nonspecific diffusion channels. *J. Biol. Chem.* 255:1771–1774.
- Zweifach, A., and R.S. Lewis. 1993. Mitogen-regulated Ca^{2+} current of T lymphocytes is activated by depletion of intracellular Ca^{2+} stores. *Proc. Natl. Acad. Sci. USA*. 90:6295–6299.
- Zweifach, A., and R.S. Lewis. 1995a. Rapid inactivation of depletion-activated calcium current (I_{CRAC}) due to local calcium feedback. *J. Gen. Physiol.* 105:209–226.
- Zweifach, A., and R.S. Lewis. 1995b. Slow calcium-dependent inactivation of depletion-activated calcium current. Store-dependent and -independent mechanisms. *J. Biol. Chem.* 270:14445–14451.