ATP from Subplasmalemmal Mitochondria Controls Ca\(^{2+}\)–dependent Inactivation of CRAC Channels*

Gema B. Montalvo¹, Antonio R. Artalejo, and Juan A. Gilbert²

From the Department of Toxicology and Pharmacology, Instituto de Farmacología y Toxicología, Facultad de Veterinaria, Universidad Complutense de Madrid, Avenida Puerta de Hierro, s/n, 28040 Madrid, Spain

A sustained Ca\(^{2+}\) entry is the primary signal for T lymphocyte activation after antigen recognition. This Ca\(^{2+}\) entry mainly occurs through store-operated Ca\(^{2+}\) channels responsible for a highly selective Ca\(^{2+}\) current known as \(I_{\text{CRAC}}\). Ca\(^{2+}\) ions act as negative feedback regulators of \(I_{\text{CRAC}}\) promoting its inactivation. Mitochondria, which act as intracellular Ca\(^{2+}\) buffers, have been proposed to control all stages of CRAC current and, hence, intracellular Ca\(^{2+}\) signaling in several types of non-excitable cells. Using the whole-cell configuration of the patch clamp technique, which allows control of the intracellular environment, we report here that respiring mitochondria located close to CRAC channels can regulate slow Ca\(^{2+}\)–dependent inactivation of \(I_{\text{CRAC}}\) by increasing the Ca\(^{2+}\)-buffering capacity beneath the plasma membrane, mainly through the release of ATP.

A sustained elevation of cytosolic free Ca\(^{2+}\) \([\text{Ca}^{2+}]_c\)\(^3\) is the first cellular signal for T lymphocyte activation. This process starts with the specific recognition of an antigen by T cell receptors and the subsequent generation of diacylglycerol and 1,4,5-inositol trisphosphate (InsP\(_3\)). In terms of Ca\(^{2+}\) signaling, InsP\(_3\) produces a biphasic response, due to a brief initial release of Ca\(^{2+}\) from InsP\(_3\)-sensitive stores and a sustained Ca\(^{2+}\) entry through plasma membrane channels activated by the emptying of those stores. This latter phenomenon originally termed capacitative Ca\(^{2+}\) entry occurs through store-operated Ca\(^{2+}\) channels responsible for a highly selective Ca\(^{2+}\) current known as \(I_{\text{CRAC}}\) (Ca\(^{2+}\) release-activated Ca\(^{2+}\) current) (1). At present, \(I_{\text{CRAC}}\) has been found in multiple cell types including T cells where it serves as the principal Ca\(^{2+}\) entry pathway (2, 3).

Interestingly, Ca\(^{2+}\) ions themselves modulate the time course of \(I_{\text{CRAC}}\) by several mechanisms: a fast inactivation process occurring in milliseconds (4) and a slower inactivation process operating in the range of seconds, which comprises both a store-dependent component due to the refilling of stores and a Ca\(^{2+}\)-dependent but store-independent component (5).

Hence, any organelle and transport systems that regulate [Ca\(^{2+}\)], could, in principle, also modulate CRAC channel activity. In this context, the regulatory actions of mitochondria on Ca\(^{2+}\) entry were first proposed by Lewis and coworkers in the late 1990s (6). These authors proved that Jurkat T cell mitochondria were able to reduce Ca\(^{2+}\)-mediated inhibition of store-operated CRAC channels by sequestering Ca\(^{2+}\) ions entering through those channels (6, 7). Since then, these results have been extended to other cell types where mitochondria located close to the plasma membrane have been involved in such a Ca\(^{2+}\)-buffering effect (8). However, recent evidence supports the idea that mitochondria may also regulate \(I_{\text{CRAC}}\) by some mechanism distinct from direct buffering of Ca\(^{2+}\), such as the release of one or more factors (e.g. glutamate or ATP) (8–11), although there is no direct evidence yet.

CRAC current is generally measured in the presence of strong intracellular Ca\(^{2+}\) buffers (EGTA or BAPTA at mM concentrations) to decrease both store refilling and the other components of Ca\(^{2+}\)-dependent inactivation of the channels. Under these conditions, a supramaximal concentration of InsP\(_3\) activates \(I_{\text{CRAC}}\) to its maximal extent. However, in the presence of weak intracellular Ca\(^{2+}\) buffering (0.1 mM EGTA or BAPTA), InsP\(_3\) is largely ineffective for activating \(I_{\text{CRAC}}\) despite releasing Ca\(^{2+}\) from the stores. Only when store refilling is prevented by using inhibitors of the sarco/endoplasmic reticulum Ca\(^{2+}\)-ATPase (SERCA) pump (e.g. thapsigargin) (12) or mitochondria are metabolically potentiated with a mixture or cocktail containing respiratory substrates, \(I_{\text{CRAC}}\) can be measured in cells dialyzed with low concentration of Ca\(^{2+}\) buffers (13). On the other hand, in the presence of a high concentration of a Ca\(^{2+}\) buffer, InsP\(_3\), and thapsigargin, global [Ca\(^{2+}\)]\(_c\) changes are not expected to occur and Ca\(^{2+}\) regulatory actions would be restricted to zones in the proximity of sustained sources of Ca\(^{2+}\) ions, like the CRAC channels.

Our working hypothesis is that mitochondria may act as a complex Ca\(^{2+}\)-buffering system. The influx of Ca\(^{2+}\) ions into the mitochondrial matrix is dependent on the electrochemical gradient for Ca\(^{2+}\), which is maintained by the aerobic respiration. In turn, three rate-limiting dehydrogenases (pyruvate, NAD\(^+\)-isocitrate, and 2-oxoglutarate) of the tricarboxylic acid cycle are stimulated by the increase in mitochondrial [Ca\(^{2+}\)], so that in the presence of metabolic substrates an enhanced production of ATP takes place (14). ATP produced through oxidative phosphorylation may then serve not only to fuel ATP-de-

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¹FPI predoctoral fellow from the Spanish Education and Science Ministry.

²Researcher of the Ramón y Cajal Programme. To whom correspondence should be addressed. Tel.: 34-91-394-4036; Fax: 34-91-394-3851; E-mail: jagilabe@vet.ucm.es

The abbreviations used are: [Ca\(^{2+}\)], cytosolic free Ca\(^{2+}\); InsP\(_3\), 1,4,5-inositol trisphosphate; CRAC, Ca\(^{2+}\) release-activated Ca\(^{2+}\) current; A/O, antimycin plus oligomycin; RR, ruthenium red; Atr, atracyloside; BA, bongkrekic acid; PMCA, plasma membrane Ca\(^{2+}\)-ATPase; BAPTA, 1,2-bis(2-aminophenoxy)ethane-N,N,N’,N’-tetraacetic acid.
dependent processes but also as an effective Ca\(^{2+}\) buffer once transferred to the cytosol.

In this study we have evaluated the ability of Ca\(^{2+}\) microdomains generated beneath the plasma membrane by Ca\(^{2+}\) influx through CRAC channels to modulate \(I_{\text{CRAC}}\) in Jurkat T cells. We have also investigated the involvement of subplasmalemmal mitochondria in the regulation of such Ca\(^{2+}\) microdomains and, therefore, of \(I_{\text{CRAC}}\) characteristics. Our results indicate that energized mitochondria regulate slow Ca\(^{2+}\)-dependent inactivation of \(I_{\text{CRAC}}\) by increasing subplasmalemmal Ca\(^{2+}\)-buffering capacity mainly through a localized ATP production.

**EXPERIMENTAL PROCEDURES**

**Cell Culture**—Human leukemic Jurkat T cells were cultured in RPMI 1640 culture medium supplemented with 2 mM L-glutamine, 10% fetal bovine serum, 10 mM HEPES, and penicillin-streptomycin (100 units/ml and 0.1 mg/ml, respectively) in a humidified atmosphere of 5% CO\(_2\) at 37°C. The glucose concentration of the culture medium was 11.1 mM.

**Electrophysiology**—\(I_{\text{CRAC}}\) was measured using the whole-cell configuration of the patch clamp technique at room temperature. Wax-coated and fire-polished borosilicate Kimax-51© glass pipettes had DC resistances of 2.5–3.5 MΩ when filled with a standard internal solution containing (mM): 145 Cs\(^+\)-glutamate, 8 NaCl, 1 MgCl\(_2\), 2 Mg-ATP, 0.03 InsP\(_3\), 0.002 thapsigargin, and 10 HEPES, pH 7.2/CsOH. The Ca\(^{2+}\) chelators EGTA or BAPTA were included in the recording pipette at 10 mM each so that free ATP concentrations (calculated using the MaxChelator WEBMAXC Standard program, available at www.stanford.edu/~cpaton) were 0.23 and 0.22 mM, respectively at pH 7.2, 20°C, 0.16 M ionic strength and 3 mM total Mg\(^{2+}\). The supplement of mitochondrial metabolites referred to as mitochondrial mixture contained (mM): 2 pyruvic acid, 2 malic acid, and 1 NaH\(_2\)PO\(_4\). The extracellular solution contained (mM): 145 NaCl, 2.8 KCl, 10 CaCl\(_2\), 10 CsCl, 2 MgCl\(_2\), 10 D-glucose and 10 HEPES, pH 7.4/NaOH.

\(I_{\text{CRAC}}\) was measured during voltage ramps applied every 2 s. Currents were amplified and filtered with an EPC-9 patch clamp amplifier (HEKA Elektronik). A correction of +10 mV was applied to compensate for the liquid junction potential. Cell membrane capacitance was automatically canceled before each ramp. Series resistance values were usually <10 mΩ and were not compensated for. The first or second current responses to ramps were taken as a measure of leak current and subtracted from all subsequent traces. A cell was considered as an inactivating one when \(I_{\text{CRAC}}\) amplitude decayed by >10% at steady state (5 min after breaking-in) with respect to its maximum.

**Flow Cytometry Analysis**—Jurkat T cells (2.5–5 × 10\(^5\) cells/ml) were washed in fresh culture medium before being resuspended in the presence or absence of mitochondrial mixture and incubated for 15 min at 37°C. The cells were treated with antimycin plus oligomycin (A/O); both at 5 μg/ml) for 25 min at 37°C. Cells were subsequently washed and incubated with 1 μM rhodamine 123 for 15 min at 37°C before being analyzed with a FACSort\textsuperscript{TM} flow cytometer.

For JC-1 labeling, Jurkat T cells (5 × 10\(^5\) cells/ml) were washed in fresh culture medium before being resuspended and incubated in a solution containing mitochondrial mixture in the presence or absence of oligomycin (5 μg/ml) for 25 min at 37°C. The cells were treated with 2 μM JC-1 for 25 min at 37°C and washed with phosphate-buffered saline just before the analysis in a FACSort\textsuperscript{TM} flow cytometer.

**Statistical Analysis**—Data are presented as mean ± S.E. The statistical differences between means were assessed by unpaired or paired Student’s t tests using GraphPad Prism\textsuperscript{®} v. 4.00 software. ns, not significant; *, \(p \leq 0.05\); **, \(p \leq 0.01\); ***, \(p \leq 0.001\).

**RESULTS**

**Effects of High Concentrations of Two Different Ca\(^{2+}\) Chelators on \(I_{\text{CRAC}}\)**—The normal procedure to maximally activate \(I_{\text{CRAC}}\) is to provoke the depletion of InsP\(_3\)-sensitive Ca\(^{2+}\) stores at the endoplasmic reticulum. To achieve this, a supramaximal concentration (30 μM) of InsP\(_3\) and thapsigargin (2 μM) was included in the internal solution. Furthermore, we decided to add high concentrations (10 mM) of two different Ca\(^{2+}\) buffers...
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**FIGURE 2. Kinetic analysis of $I_{\text{CRAC}}$ in the presence of EGTA, BAPTA, or EGTA + mitochondrial mixture (Cktl).** The activation and inactivation characteristics of $I_{\text{CRAC}}$ were analyzed by estimating several kinetic parameters. Thus, the time constant of activation ($\tau_{\text{on}}$) was obtained by fitting a single exponential function to the $I_{\text{CRAC}}$ time course from the start of the current to its peak when the current reached its maximum ($t_{\text{max}}$). The time constant of inactivation ($\tau_{\text{off}}$) was similarly obtained by fitting a single exponential function from $t_{\text{max}}$ to the first point where a steady state level of current was reached. A panels, activation-related parameters: current amplitude ($A_1$), time constant for activation ($\tau_{\text{on}}$) ($A_2$), and time to peak ($t_{\text{max}}$) ($A_3$). B panels, analysis of inactivation: percentage of inactivating cells ($B_1$), extent of inactivation ($B_2$), and time constant for inactivation ($\tau_{\text{off}}$) ($B_3$) in the inactivating cells.

(EGTA or BAPTA) to this solution in an attempt to replace the buffering effect of mitochondria. EGTA and BAPTA have similar affinity for Ca$^{2+}$ ($K_D = 1.8 \times 10^{-7}$ and $2.2 \times 10^{-7}$ M, respectively) but differ in the kinetics of Ca$^{2+}$ binding in two orders of magnitude ($k_{\text{on}} = 2.5 \times 10^6$ and $4 \times 10^8$ M$^{-1}$ s$^{-1}$, respectively).

In the presence of either EGTA or BAPTA, $I_{\text{CRAC}}$ exhibited its characteristic features when probed with a voltage ramp: an inwardly rectifying current-voltage relation with a positive reversal potential and a voltage-independent gating (Fig. 1A). The time course of $I_{\text{CRAC}}$ during long-term recordings was obtained from a series of voltage ramps (see “Experimental Procedures”). Fig. 1B shows average values of $I_{\text{CRAC}}$ at $-80$ mV from cells dialyzed with EGTA ($n = 12$) or BAPTA ($n = 17$).

Fig. 2 shows several parameters derived from the analysis of individual time courses. In the presence of either EGTA or BAPTA, no differences were observed in the amplitude or the activation kinetic parameters analyzed (time constant of activation, $\tau_{\text{on}}$, and time to peak, $t_{\text{max}}$) (Fig. 2, panels A1–A3), making it unlikely that rapid changes in [Ca$^{2+}$]$_i$ play a significant role in this process. On the contrary, marked differences were seen regarding $I_{\text{CRAC}}$ inactivation.

First, the percentage of inactivating cells (45%; 8/17) was largely reduced in BAPTA as compared with that in EGTA (95%; 11/12) (Fig. 2, panel $B_1$). Second, BAPTA also reduced the extent of inactivation (36.85 ± 6.12%) compared with EGTA (62.45 ± 6.75%) (Fig. 2, panel $B_2$). On the contrary, no statistically significant differences were observed between EGTA and BAPTA data regarding the time constant of inactivation in the inactivating cells ($\tau_{\text{off}}$; see Fig. 2, panel $B_3$).

The present results suggest that the spatio-temporal profile of Ca$^{2+}$ near CRAC channels determines both the amplitude and extent of the slow inactivation of $I_{\text{CRAC}}$.

**Effects of Supported Mitochondrial Function on $I_{\text{CRAC}}$—**The mitochondria can be maintained in an energized metabolic state during whole-cell recordings by supplementing the internal solution with a mixture containing two respiratory substrates, pyruvate and malate, and NaH$_2$PO$_4$ (13, 15–17). Under these conditions and the presence of intracellular EGTA, the current-voltage profile and the amplitude as well as the activation kinetic parameters of $I_{\text{CRAC}}$ were similar to those observed in the presence of EGTA alone (Fig. 1 and Fig. 2, panels A1–A3). However, the percentage of inactivating cells and the extent of their inactivation were significantly reduced compared with EGTA alone, exhibiting similar values to those obtained with BAPTA (Fig. 2, panels $B_1$–$B_3$).

These results suggest that mitochondria act to reduce the inactivation of $I_{\text{CRAC}}$ by a mechanism similar to that employed by BAPTA. Furthermore, adding the mitochondrial mixture to a solution containing BAPTA resulted in 100% non-inactivating cells whose amplitude of current did not differ from that observed with BAPTA alone (data not shown).

**Modulatory Effects of Mixture on $I_{\text{CRAC}}$ Occurs at the Mitochondrial Level—**We set out to prove the participation of mitochondria in the regulation of $I_{\text{CRAC}}$ using inhibitory drugs of mitochondrial respiration. For this purpose, cells were treated...
dependent processes occurring at the submembrane level, such as $I_{\text{CRAC}}$ inactivation.

The ability of pyruvate and malate to keep mitochondria in an energized state was also directly evaluated by using flow cytometry techniques in cells incubated with rhodamine 123. Rhodamine 123 is a potentiometric dye that crosses the plasma membrane easily and emits fluorescence after accumulating into mitochondria with a negative $\Delta\Psi_m$. As it is shown in Fig. 3B, the fluorescent signal associated to homogeneous and viable populations of Jurkat T cells increased when the cells were bathed in a solution containing the mitochondrial mixture, whereas it decreased when the cells were incubated with the mixture but in the presence of mitochondrial inhibitors (A/O).

**ATP Released from Mitochondria Regulates $I_{\text{CRAC}}$ Inactivation**—The mitochondrial ability to regulate $[Ca^{2+}]_i$ can be due to either $Ca^{2+}$ uptake, to $Ca^{2+}$ buffering by ATP produced by respiring mitochondria or to both processes. To clarify this point, the hexavalent cation ruthenium red (RR) (100 $\mu$M), an inhibitor of the mitochondrial uniporter, was added to the pipette’s solution containing mixture also. RR caused a small increment in the extent of inactivation although it was not statistically significant (Fig. 4A). However, intracellular application of atracyloside (Atr) (20 $\mu$M), a potent inhibitor of the mitochondrial adenine nucleotide translocase that exports ATP from mitochondria to cytosol, canceled out the effects of the mixture on $I_{\text{CRAC}}$ inactivation (Fig. 4A). No significant changes were observed when atracyloside and RR were applied together compared with those obtained with atracyloside alone.

RR is a widely used inhibitor of the uniporter although it targets other membrane proteins (e.g. BK channels, rymodine receptor, etc.). For this reason, we sought to reproduce the former results by using RU360, a more selective and potent blocker of the mitochondrial uniporter (18). RU360 (1 $\mu$M) did not affect the extent of inactivation of $I_{\text{CRAC}}$ in cells dialyzed with an internal solution containing the mitochondrial mixture (Fig. 4B). On the other hand, besides its translocase function, mitochondrial adenine nucleotide translocase is a constituent of the inner membrane multimeric channel known as the mitochondrial permeability tran-

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![Image](https://example.com/image.png)

**FIGURE 3.** The modulatory effects of mixture on $I_{\text{CRAC}}$ occur at the mitochondrial level. A. percentage of inactivation of $I_{\text{CRAC}}$ in cells dialyzed with EGTA, EGTA + mitochondrial mixture (Cktl), or EGTA + Cktl + antimycin (0.05 $\mu$g/ml) and oligomycin (0.5 $\mu$g/ml) (A/O); cells were also incubated in the A/O mixture during 25 min before the recording. All the experiments were carried out in the same batch of cells. B, effect of mitochondrial mixture and A/O mixture on mitochondrial function determined by flow cytometry in cells loaded with rhodamine 123. Left, size-complexity dot plot showing the selected region (R1) of viable cells where the analysis of fluorescence was performed; right, distribution of fluorescence in cells bathed in extracellular solution (EGTA) and extracellular solution supplemented with mitochondrial mixture (EGTA + Cktl) or mitochondrial mixture plus A/O mixture (EGTA + Cktl + A/O). The fluorescent signal corresponding to 10,000 cells was analyzed.

with antimycin A (A) (0.05 $\mu$g/ml), an inhibitor of the complex III respiratory chain in combination with oligomycin (O) (0.5 $\mu$g/ml), an inhibitor of F$_1$F$_{\text{O}}$-ATP synthase. The use of both drugs ultimately causes the collapse of the mitochondrial membrane potential ($\Delta\Psi_m$) and loss of mitochondrial function.

As was expected, the effects of the mitochondrial mixture on $I_{\text{CRAC}}$ inactivation were abolished in cells treated with A/O. Thus, in paired experiments, both the extent of slow inactivation of $I_{\text{CRAC}}$ (Fig. 3A) and the percentage of inactivating cells (83.33%; 5/6) increased to values similar to those obtained in cells dialyzed with EGTA alone (100%; 8/8). On the contrary, no significant differences were observed in cells dialyzed with BAPTA + A/O compared with those with BAPTA alone with regard to $I_{\text{CRAC}}$ inactivation (data not shown). These results confirm the ability of functional mitochondria to regulate $Ca^{2+}$-dependent processes occurring at the submembrane level, such as $I_{\text{CRAC}}$ inactivation.

![Image](https://example.com/image.png)
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**FIGURE 4.** Slow inactivation of $I_{\text{CRAC}}$ is modulated by ATP released from mitochondria. A, percentage of inactivation in cells dialyzed with EGTA, EGTA + mitochondrial mixture (EGTA+Cktl), EGTA + Cktl + 100 μM ruthenium red (RR), EGTA + Cktl + RR, EGTA + Cktl + 20 μM atracyloside (EGTA+Cktl+Atr), or EGTA + Cktl + 20 μM atracyloside + 100 μM ruthenium red (EGTA+Cktl+Atr+RR). B, percentage of inactivation in cells dialyzed with EGTA + mitochondrial mixture (EGTA+Cktl), EGTA + Cktl + 1 μM RU360 (EGTA+Cktl+Ru360), EGTA + Cktl + 10 μM bongkrekic acid (EGTA+Cktl+BA).

**FIGURE 5.** Oligomycin cancels cocktail effect without changes in mitochondrial membrane potential. A, percentage of inactivation of $I_{\text{CRAC}}$ in cells dialyzed with EGTA + mitochondrial mixture (Cktl) or EGTA + Cktl + oligomycin (0.5 μg/ml, equivalent to 0.6 μM) (O); cells were also incubated in oligomycin during 30 min before the analysis. All the experiments were carried out in the same batch of cells. B, fluorescence intensities from cells loaded with the potentiometric probe JC-1 analyzed by flow cytometry incubated in the presence of mitochondrial mixture (Cktl) or Cktl + oligomycin. The fluorescent signal corresponding to 10,000 cells was analyzed.

Interestingly, mitochondrial adenine nucleotide translocase can be inhibited by atracyloside and bongkrekic acid (BA) acting through opposite mechanisms; whereas Atr prevents binding of adenine nucleotides to carrier sites, BA prevents their dissociation from mitochondrial adenine nucleotide translocase. Moreover, in contrast to BA, Atr has been reported to open the mitochondrial permeability transition pore (19), which could potentially cause a massive Ca$^{2+}$ release and, hence, inactivation of $I_{\text{CRAC}}$.

Despite these differences between the two drugs, their effects on $I_{\text{CRAC}}$ inactivation were coincident, thus supporting a common action on the translocase function (Fig. 4B). Altogether, the results obtained with the different inhibitors of mitochondrial transporters (RR, RU360, Atr, and BA) suggest a predominant role of ATP export over the mitochondrial Ca$^{2+}$ uptake to regulate Ca$^{2+}$-dependent inactivation of $I_{\text{CRAC}}$ under our experimental conditions.

To further support this suggestion we decided to explore the effects of oligomycin on $I_{\text{CRAC}}$ inactivation. As has already been mentioned, oligomycin acts to halt ATP production, an effect that is not readily associated to an inhibition of Ca$^{2+}$ uptake or even a change in ΔΨ$m$ (20). In accordance with the proposed role of mitochondrial ATP, the mixture of respiratory substrates was unable to reduce Ca$^{2+}$-dependent inactivation of CRAC in cells exposed to oligomycin (5 μg/ml) (Fig. 5A). However, ΔΨ$m$ was not affected by the presence of oligomycin in Jurkat T cells stained with the cationic dye JC-1, a more specific and reliable mitochondrial versus plasma membrane potential probe than rhodamine 123 or DiOC$6$ (21) (Fig. 5B).

To get direct evidence involving ATP in the regulation of $I_{\text{CRAC}}$, intracellular solutions containing high concentrations (10 mM) of ATP as disodium (ATP-2Na) or magnesium (ATP-Mg) salts were dialyzed in two groups of cells. The difference between these two salts lies in their affinity for Ca$^{2+}$ ions, ATP-2Na being a more effective Ca$^{2+}$ chelator than ATP-Mg. As is shown in Fig. 6A, the rate of inactivation of $I_{\text{CRAC}}$ was significantly reduced when 10 mM ATP-2Na ([ATP] free = 9.05 mM) was included in the pipette’s solution instead of ATP-Mg ([ATP] free = 0.92 mM). This result indicates that ATP-2Na can regulate the slow inactivation of...
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predicts the Ca\(^{2+}\) gradient generated in the presence of high concentrations of a barely saturated and highly mobile exogenous chelator from the opening of a single channel. The extent of the gradient is a function of Ca\(^{2+}\) flux through the channel, the diffusion coefficient for Ca\(^{2+}\), and the Ca\(^{2+}\) binding rate for the chelator used as shown in Equation 1

\[
[Ca^{2+}]_d(r) = [Ca^{2+}]_\infty + \frac{i_{CRAC}}{4\pi FD_{Ca}^\infty} \exp(-r/\lambda) \quad (Eq. 1)
\]

where [Ca\(^{2+}\)]\(d\)(r) is the steady state free Ca\(^{2+}\) concentration as a function of distance from the channel, [Ca\(^{2+}\)]\(\infty\) is the free Ca\(^{2+}\) in the bulk solution (i.e. 10\(^{-7}\) M), \(F\) is the Faraday’s constant, \(D_{Ca}\) is the diffusion coefficient for Ca\(^{2+}\) (2.2 \times 10\(^{-6}\) cm\(^2\)/s, (23)), \(r\) is the distance from the channel, and \(\lambda\) is the mean path length of free Ca\(^{2+}\). As shown in Equation 2, \(\lambda\) can be expressed as

\[
\lambda = \sqrt{D_{Ca}/(k_{on} \cdot [B])} \quad (Eq. 2)
\]

where \(k_{on}\) is the on rate constant for binding of Ca\(^{2+}\) by the chelator (\(k_{on} = 2.5 \times 10^{6} \text{ M}^{-1} \text{s}^{-1}\) for EGTA, 4 \times 10\(^{8}\) M\(^{-1}\) s\(^{-1}\) for BAPTA, and 1 \times 10\(^{8}\) M\(^{-1}\) s\(^{-1}\) for ATP (23)), and \([B]\) is the concentration of free buffer in the bulk solution. As shown in Equation 3, the free buffer concentration \([B]\) is calculated according to

\[
[B] = \frac{[B]_\infty \cdot K_D}{K_D + [Ca^{2+}]_\infty} \quad (Eq. 3)
\]

where \([B]_\infty\) is the concentration of total buffer (free plus bound; 10\(^{-7}\) M) and \(K_D\) is the dissociation constant for the Ca\(^{2+}\) buffer complex (\(K_D = 1.8 \times 10^{-7}\) M for EGTA, 2.2 \times 10\(^{-7}\) M for BAPTA, and 1.95 \times 10\(^{-9}\) M for ATP).

The current through CRAC channels is too small to be resolved at the single channel level under typical recording conditions. As a consequence, different indirect approaches like fluctuation analysis (3) or single channel recording in divalent-free external solutions (24) have been used to estimate the unitary conductance and also the number of CRAC channels/cell. Moreover, other cationic conductances can contaminate CRAC current recordings in the absence of intracellular Mg\(^{2+}\). Therefore, Prakriya and Lewis (25) studied the biophysical properties of CRAC channels isolated from the Mg\(^{2+}\)-sensitive component and estimated the unitary CRAC Ca\(^{2+}\) current (i\(_{CRAC}\)) to be \(-3.8\) fA in 20 mM external Ca\(^{2+}\) at \(-110\) mV. Therefore, to study only the contribution of the different buffers in the shaping of the Ca\(^{2+}\) microdomains, the same unitary current was considered for the different experimental conditions assuming that open probability of a single CRAC channel is not affected for the different intracellular buffers employed.

Our calculation assumes that standing gradients develop in microseconds after the opening of channels, provided that Ca\(^{2+}\) buffers do not saturate, a condition fulfilled in our experiments due to the small single unitary conductance of CRAC channels (a few fA) and the high concentration (10 mM) of intracellular exogenous buffers used. However, this model does not consider how channels are arranged in the plasma mem-

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**Figure 6. Effect of different ATP salts on the extent of i\(_{CRAC}\) inactivation.** A, 10 mM ATP-Mg or ATP-2Na were added to the standard intracellular solution containing 10 mM EGTA and the pH corrected to 7.2/CsOH. Free ATP concentrations were 0.92 mM for ATP-Mg and 9.05 mM for ATP-2Na. B, relationship between the free ATP concentrations and percentage of inactivation over a range of ATP-2Na concentrations. The cells were incubated in antimycin (0.05 μg/ml) and oligomycin (0.5 μg/ml) (A/O) during 25 min before the beginning of the recordings and dialyzed with 10 mM EGTA + A/O and different concentrations of ATP-2Na (0, 2, 5, and 10 mM). Free ATP concentrations were calculated using the MaxChelator WEBMAXC Standard program.

\(i_{CRAC}\) and suggests that endogenous ATP released from mitochondria may act in a similar way.

To establish the relationship between the ATP-2Na concentration and the degree of inactivation, the free ATP concentration corresponding to different amounts of ATP-2Na added to the intracellular solution was represented against the percentage of inactivation in cells dialyzed with EGTA + A/O. As it is shown in Fig. 6B, the increase of ATP-2Na, and hence of free ATP concentration, was associated with a reduction in the percentage of \(i_{CRAC}\) inactivation in a dose-dependent manner.

**Estimation of the Extent of Ca\(^{2+}\) Microdomains Generated by a Single CRAC Channel in the Presence of High Concentrations of Exogenous Ca\(^{2+}\) Chelators**—To estimate the amplitude and spatial extent of Ca\(^{2+}\) microdomains generated by Ca\(^{2+}\) entry through a single CRAC channel under our recording conditions, we used PORE, a freeware application developed by Dr. James Kenyon, University of Nevada. PORE is an Excel spreadsheet with an attached Visual Basic Program that utilizes the equation proposed by Dr. Erwin Neher (22). This equation
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FIGURE 7. Estimation of Ca\(^{2+}\) microdomains generated from a single CRAC channel in the presence of a high (10 mM) total concentration of three different exogenous Ca\(^{2+}\) chelators (EGTA, BAPTA, and ATP) as a function of the distance to the channel (see "Results" for details). The mean path length (\(\lambda\)) values for free EGTA (9.65 nm), BAPTA (9.73 nm), and ATP (9.03 nm) concentrations calculated using the MaxChelator WEBMAXC Standard program were 95.5, 7.5, and 4.9 nm, respectively.

Mitochondria and a high density and velocity of the Ca\(^{2+}\) uniporter does not transport Ca\(^{2+}\) below a concentration of ~200–300 nM, which we wanted to estimate how far a microdomain will expand beyond the mouth of a CRAC channel (Fig. 2, panels B1-B3).

The functional state of mitochondria also seems not to contribute to activation of \(I_{\text{CRAC}}\) as respiratory substrates did not cause any effect on \(\tau_{\text{inv}}\) (Fig. 2, A panels). This conclusion agrees with previous results from RBL-1 cells in which mitochondrial depolarization with A/O in the presence of strong cytosolic buffering did not affect the activation of \(I_{\text{CRAC}}\) (13).

In contrast, different results were observed when inactivation parameters were measured. BAPTA reduced both the rate and extent of inactivation and also the percentage of inactivating cells as compared with EGTA (Fig. 2, panels B1-B3). Interestingly, when the mitochondrial function was boosted with the mitochondrial mixture, respiring mitochondria could then regulate slow inactivation of \(I_{\text{CRAC}}\) in cells dialyzed with EGTA in a manner similar to that observed with BAPTA.

At variance with exogenous buffers, regulatory effects exerted by mitochondria on [Ca\(^{2+}\)]\(_c\) are very conditioned by their metabolic state and topological distribution with respect to sources of Ca\(^{2+}\) mobilization such as the plasma membrane or the endoplasmic reticulum (26). The question then arises as to whether mitochondria are homogenous organelles in terms of their location and/or functional properties. Indeed, functional heterogeneity has been described in several types of cells regarding the Ca\(^{2+}\) signaling abilities of particular subsets of mitochondria (16, 27). Collins \textit{et al.} (27) found that mitochondria located in the vicinity of the plasma membrane have larger \(\Delta\Psi_{\text{m}}\) values and sequester more Ca\(^{2+}\) than those located around the nucleus. Likewise, the ability of subplasmalemmal mitochondria to modulate the activity of ion channels like large conductance Ca\(^{2+}\)-dependent potassium channels (BK Ca channels) or store-operated Ca\(^{2+}\) channels has been demonstrated in different cell types (28, 29). In a recent study, mitochondria from HeLa cells were relocalized from the cell periphery to the perinuclear area by overexpression of the dynactin subunit dynamin-related protein (Drp-1). As a consequence, the number of endoplasmic reticulum-mitochondria contacts increased and Ca\(^{2+}\) influx through store-operated Ca\(^{2+}\) channels was severely reduced, thus indicating a requirement of peripheral mitochondria for optimal store-operated Ca\(^{2+}\) activity (28, 30).

On the other hand, the inclusion of metabolic substrates like pyruvate and malate into the intracellular solution during whole-cell recordings is essential to maintain mitochondrial respiration and ATP production (15, 17). In addition, oxidative phosphorylation in the mitochondria depends on the presence of micromolar levels of [Ca\(^{2+}\)]\(_m\) to induce the activation of three dehydrogenases of the tricarboxylic acid cycle. Because mitochondrial Ca\(^{2+}\) uniporter does not transport Ca\(^{2+}\) below a concentration of ~200–300 nM, we wanted to estimate how far a microdomain extends from a Ca\(^{2+}\) source (the CRAC channel) in the presence of high concentrations of a diversity of exogenous Ca\(^{2+}\) buffers.

A rough estimation of how long Ca\(^{2+}\) ions can diffuse into the cell from the mouth of a CRAC channel before they bind to an exogenous buffer is given by the mean path length parameter, \(\lambda\) (see Equation 2). The value of \(\lambda\) is 95.5 nm in the presence of 10 mM of EGTA and 7.5 nm in the presence of 10 mM of BAPTA, which may explain the differences in \(I_{\text{CRAC}}\) inactivation observed between the two chelators.

Considering the small \(\lambda\) value estimated for BAPTA and the effectiveness of this buffer to reduce \(I_{\text{CRAC}}\) inactivation, both a molecular colocalization between CRAC channels and mitochondria and a high density and velocity of the Ca\(^{2+}\) uniporter would be required to account for the observed mitochondrial modulation of \(I_{\text{CRAC}}\). From our experimental results, an alternative mechanistic explanation would consist of the release by
mitochondria of a highly mobile and effective \(Ca^{2+}\) chelator able to raise high concentrations (several millimolar) near CRAC channels.

Like BAPTA, ATP can act as a very effective \(Ca^{2+}\) chelator due to its rapid reaction with \(Ca^{2+}\) \(k_{	ext{on}} = 1 \times 10^7 \text{M}^{-1} \text{s}^{-1}\) (23). Thus, in the presence of 10 mM added ATP-2Na in the intracellular solution, the spatial extension of a \(Ca^{2+}\) microdomain generated by \(Ca^{2+}\) influx through a single CRAC channel would be even narrower than that occurring when BAPTA is used \(\lambda = 4.9 \text{ nm}\).

Furthermore, according to the results obtained using unipor and mitochondrial adenine nucleotide translocase blockers (Fig. 4A) a plausible scenario could then be that ATP originated from peripheral mitochondria would shape spatially the \(Ca^{2+}\) microdomains generated by CRAC channels. This interpretation implies that ATP concentrations must be in the order of several millimolar in the microdomain region. It is now worth recalling that ATP microdomains of this size have been reported in pancreatic \(\beta\)-cells where they regulate the activity of ATP-sensitive \(K^+\) channels at the plasma membrane (31).

Recent data indicate that other \(Ca^{2+}\) transport systems could also be considered. This is the case of plasma membrane \(Ca^{2+}\)-ATPase (PMCA), whose activity is regulated by the \(Ca^{2+}\) microdomains generated by CRAC channels, suggesting a close functional association between both \(Ca^{2+}\) transport systems in T cells (32).

Because specific PMCA inhibitors do not exist, vanadate, a nonspecific inhibitor of ATPases and phosphatases, has been used in studies requiring the inhibition of PMCA (33). To rule out a possible regulatory role of PMCA on \(I_{\text{CRAC}}\), we evaluated the effect of 0.1 mM vanadate (sodium orthovanadate) in cells dialyzed with 10 mM EGTA and mitochondrial mixture. No differences were observed in cells treated with vanadate compared with control cells at the amplitude or time course of \(I_{\text{CRAC}}\) (data not shown). Thus, PMCA does not appear to play a predominant role in controlling slow \(Ca^{2+}\)-dependent inactivation in Jurkat T cells under our particular experimental conditions.

A similar cross-talk has been proposed to exist between CRAC channels and the plasma membrane \(Na^+/Ca^{2+}\) exchanger (NCX) in mast cells (34). Nevertheless, the relative contribution of these systems to the control of local \([Ca^{2+}]\) varies significantly with the experimental conditions used, the time window analyzed and the cell type under study. So, NCX does not appear to contribute significantly to \(Ca^{2+}\) clearance in Jurkat T cells (35), whereas the regulatory role of PMCA was established under conditions of low buffer capacity and unsupported mitochondrial metabolism.

To sum up, our results show that ATP produced by subplasmalemmal mitochondria is a soluble messenger that regulates the \(Ca^{2+}\)-dependent inactivation of CRAC channels in Jurkat T cells, supporting and refining the already existing notion of a functional relationship between CRAC channels and peripheral mitochondria.

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