Separation and Characterization of Currents through Store-operated CRAC Channels and Mg$^{2+}$-inhibited Cation (MIC) Channels

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**ABSTRACT** Although store-operated calcium release–activated Ca$^{2+}$ (CRAC) channels are highly Ca$^{2+}$-selective under physiological ionic conditions, removal of extracellular divalent cations makes them freely permeable to monovalent cations. Several past studies have concluded that under these conditions CRAC channels conduct Na$^+$ and Cs$^+$ with a unitary conductance of ~40 pS, and that intracellular Mg$^{2+}$ modulates their activity and selectivity. These results have important implications for understanding ion permeation through CRAC channels and for screening potential CRAC channel genes. We find that the observed 40-pS channels are not CRAC channels, but are instead Mg$^{2+}$-inhibited cation (MIC) channels that open as Mg$^{2+}$ is washed out of the cytosol. MIC channels differ from CRAC channels in several critical respects. Store depletion does not activate MIC channels, nor does store refilling deactivate them. Unlike CRAC channels, MIC channels are not blocked by SKF 96365, are not potentiated by low doses of 2-APB, and are less sensitive to block by high doses of the drug. By applying 8–10 mM intracellular Mg$^{2+}$ to inhibit MIC channels, we examined monovalent permeation through CRAC channels in isolation. A rapid switch from 20 mM Ca$^{2+}$ to divalent-free extracellular solution evokes Na$^+$ current through open CRAC channels (Na$^+$-I$_{CRAC}$) that is initially eightfold larger than the preceding Ca$^{2+}$ current and declines by ~80% over 20 s. Unlike MIC channels, CRAC channels are largely impermeable to Cs$^+$ (P$_{Cs}$/P$_{Na}$ = 0.13 vs. 1.2 for MIC). Neither the decline in Na$^+$-I$_{CRAC}$ nor its low Cs$^+$ permeability are affected by intracellular Mg$^{2+}$ (90 μM to 10 mM). Single openings of monovalent CRAC channels were not detectable in whole-cell recordings, but a unitary conductance of 0.2 pS was estimated from noise analysis. This new information about the selectivity, conductance, and regulation of CRAC channels forces a revision of the biophysical fingerprint of CRAC channels, and reveals intriguing similarities and differences in permeation mechanisms of voltage-gated and store-operated Ca$^{2+}$ channels.

**KEY WORDS:** calcium channel • calcium signaling • ion/membrane channel • TRP-PLIK • LTRPC7

**INTRODUCTION**

The depletion of Ca$^{2+}$ from the ER in nonexcitable cells triggers Ca$^{2+}$ influx across the plasma membrane, a process termed store-operated Ca$^{2+}$ entry. Store-operated channels (SOCs)* are widely, if not ubiquitously, expressed in nonexcitable cells, where they provide a major route for Ca$^{2+}$ entry (for reviews see Parekh and Penner, 1997; Lewis, 1999; Putney and McKay, 1999). One of the best described SOCs, the calcium release–activated Ca$^{2+}$ (CRAC) channel, is present in mast cells, T lymphocytes, and several related cell lines. CRAC channels influence a variety of important physiological processes, including the release of inflammatory mediators from mast cells during allergic reactions, and the generation of [Ca$^{2+}$], oscillations leading to gene expression and the differentiation and activation of T cells (for reviews see Parekh and Penner, 1997; Lewis, 2001). The critical role of CRAC channels in human physiology is shown clearly by the devastating immunodeficiencies that arise from the absence of CRAC channel activity in T cells from human patients (Parti et al., 1994; Feske et al., 2001).

In view of their critical physiological functions, considerable effort has been focused on isolating the gene(s) encoding the CRAC channel and on understanding how the channel’s gating is regulated. Although several genes of the TRP family have been proposed to encode the CRAC channel, it is unclear as to whether the currents resulting from heterologous expression of these genes are identical to native I$_{CRAC}$ (Putney and McKay, 1999; Clapham et al., 2001; Prakriya and Lewis, 2002). Several classes of activation mechanisms are currently being studied, including a diffusible messenger released from the ER, depletion-triggered insertion of CRAC channels into the plasma membrane, and physical coupling between the channels and IP$_{3}$ receptors in the ER. However, there is as yet no consensus on which if any of these mechanisms may be correct (Putney et al., 2001). Progress in resolving these issues has been hampered by several factors that are unique to this class of channels, including a lack of selective inhibitors, very small whole cell cur-
rents (on the order of a few pA per cell), and an extremely small single-channel Ca\(^{2+}\) conductance (2–26 fS estimated from noise analysis) (Zweifach and Lewis, 1993), which has precluded single-channel recording in membrane patches.

The study of currents carried by monovalent cations through CRAC channels could, in principle, bypass some of these problems. Like voltage-gated Ca\(^{2+}\) channels (Almers and McCleskey, 1984; Hess et al., 1986), CRAC channels under physiological conditions are exquisitely selective for Ca\(^{2+}\), conducting Ca\(^{2+}\) ~1,000 times better than the more prevalent Na\(^{+}\) (Hoth and Penner, 1993; Hoth, 1995). However, as with voltage-gated Ca\(^{2+}\) channels (Almers and McCleskey, 1984), the removal of extracellular divalent cations renders CRAC channels permeable to Na\(^{+}\) (Hoth and Penner, 1993; Lepple-Wienhues and Cahalan, 1996). Initially, upon removal of extracellular divalents, this Na\(^{+}\) current is approximately six times larger than the preceding Ca\(^{2+}\) current, but it declines by >90% over tens of seconds. The slow loss of channel activity is reversed following readdition of extracellular Ca\(^{2+}\), a process referred to as Ca\(^{2+}\)-dependent potentiation (CDP) (Christian et al., 1996b; Zweifach and Lewis, 1996). Kerschbaum and Cahalan (1998) found that removal of intracellular Mg\(^{2+}\) made the Na\(^{+}\) current larger and sustained, leading to the idea that intracellular Mg\(^{2+}\) is required for CRAC channel depotentiation. Furthermore, in the absence of intracellular Mg\(^{2+}\), the whole-cell current was seen to arise from the progressive, all-or-none activation of single 40-pS channels having a high open probability (P\(_o\) > 0.9) (Kerschbaum and Cahalan, 1999). Similar results were later found in human T cells (Fomina et al., 2000) and RBL cells (Braun et al., 2001). The Na\(^{+}\) currents were considered to arise from CRAC channels based on their slow time course of activation and inhibition by extracellular Ca\(^{2+}\), Mg\(^{2+}\), Ni\(^{2+}\), and Gd\(^{3+}\) (Kerschbaum and Cahalan, 1999; Fomina et al., 2000; Braun et al., 2001). These results are significant because resolution of CRAC currents at the single-channel level is expected to greatly facilitate studies of the molecular mechanism of store-operated Ca\(^{2+}\) entry. In fact, the conductance, selectivity, and high open probability of the Na\(^{+}\)-conducting channels was exploited in studies of the CRAC channel’s activation mechanism (Braun et al., 2001; Rychkov et al., 2001), changes in CRAC channel expression during T cell activation (Fomina et al., 2000), and for the identification of genes that may encode the CRAC channel pore region (Yue et al., 2001).

Although the discovery of the large, sustained monovalent current seen with Mg\(^{2+}\)-free intracellular solutions arises from a store-independent channel that differs from I\(_{\text{CRAC}}\) in its ion selectivity, pharmacology, and regulation. Because its activity is suppressed by intracellular Mg\(^{2+}\), we refer to this channel as the Mg\(^{2+}\)-inhibited cation (MIC) channel. By using conditions that prevent the activation of I\(_{\text{MIC}}\), we have been able to characterize monovalent fluxes through CRAC channels. Several key properties of monovalent CRAC channels, including ion selectivity, unitary conductance, and regulation by intracellular Mg\(^{2+}\), differ significantly from those described previously. These new results reveal similarities and differences in the ionic selectivity mechanisms of store-operated and voltage-gated Ca\(^{2+}\) channels, and force a revision of the biophysical fingerprint of CRAC channels that will have important implications for the identification of CRAC channel genes.

MATERIALS AND METHODS

Cells

Jurkat E6-1 human leukemic T cells (American Type Culture Collection) were grown in a medium consisting of RPMI 1640 supplemented with 10% fetal calf serum, 2 mM glutamine, 50 U/ml penicillin and 50 µg/ml streptomycin. The cells were maintained in log-phase growth at 37°C in 6% CO\(_2\).

Solutions and Chemicals

The standard extracellular Ringer’s solution contained (in mM): 155 NaCl, 4.5 KCl, 2 or 20 CaCl\(_2\), 1 MgCl\(_2\), 10 D-glucose, and 5 Na-HEPES (pH 7.4). Ca\(^{2+}\)-free Ringer’s was prepared by substituting 1 mM EGTA + 2 mM MgCl\(_2\) for CaCl\(_2\). The divalent-free (DVF) Ringer’s solutions contained (in mM): 155 NaCl, Cs, or NMDG-methanesulfonate, and 10 EDTA, 1 EDTA and 10 Hepes (pH 7.4 with NaOH, CsOH, or HCl, respectively). The standard internal solution contained (in mM): 150 Cs methanesulfonate, 3–10 mM MgCl\(_2\), 10 BAPTA, and 10 Cs-Hepes (pH 7.2). The Mg\(^{2+}\)-free (MGF) intracellular solution contained (in mM): 150 Cs methanesulfonate, 10 EDTA, 0.5 CaCl\(_2\) (calculated [Ca\(^{2+}\)] = 10 mM) and 10 Cs-Hepes (pH 7.2). Where noted, 10 mM BAPTA was substituted for EDTA. In the experiments on I\(_{\text{CRAC}}\) deactivation, the intracellular solution contained (in mM): 150 Cs methanesulfonate, 2 mM CsCl, 1.2 EGTA or 1 BAPTA, and 10 Cs-HEPES (pH 7.2). In the excised patch experiments, the pipette solution contained an Na-based DVF solution (composition listed above), and the cytoplasmic face of the patch was exposed to the Mg\(^{2+}\) solution (listed above, but with 0 CaCl\(_2\)) to which an appropriate quantity of MgCl\(_2\) was added to yield the free [Mg\(^{2+}\)] indicated in the figure legends. MgATP and Na\(_4\)ATP (Sigma-Aldrich) were added to the intracellular solution in some experiments, and free [Mg\(^{2+}\)], and [Ca\(^{2+}\)], were calculated using MaxChelator software (WEBMAXC 2.10, available at http://www.stanford.edu/~cpatton/webmaxc2.html).

2-aminoethylidiphenyl borate (2-APB) was provided by Dr. K. Mikoshiba (Tokyo University, Japan). In some experiments, 2-APB obtained from Sigma-Aldrich was used; no difference was found between the drugs from the different sources. Stock solutions of 2-APB and thapsigargin (Sigma-Aldrich) were prepared in DMSO at concentrations of 20 mM and 1 mM, respectively; SKF 96365 (Sigma-Aldrich) was dissolved in deionized water at a concentration of 10 mM. The drugs were diluted to the concen-
tations indicated in the legends and applied to the cells using a multi-barrel local perfusion pipette with a common delivery port. The time for 90% solution exchange was measured to be <1 s, based on the rate at which the K+ current reversal potential changed when the external [K+] was switched from 2 to 150 mM.

**Patch-Clamp Measurements**

Patch-clamp experiments were conducted in the standard whole-cell recording configuration at 22–25°C using an Axopatch 200 amplifier (Axon Instruments, Inc.), an ITC-16 interface (Instrutech) and a Macintosh G3 computer. Recording electrodes were pulled from 100-µl pipettes coated with Sylgard and fire-polished to a final resistance of 2–5 MΩ. Stimulation and data acquisition and analysis were performed using in-house routines developed on the Igor Pro platform (Wavemetrics). The holding potential was 20 mV unless otherwise indicated. Voltage stimuli usually consisted of a 100-ms step to −110 mV, immediately followed by a 100-ms ramp from −110 to 90 mV applied every 1–2 s. Currents were filtered at 1 kHz with a 4-pole Bessel filter and sampled at 5 kHz without series resistance compensation. Data are corrected for the liquid junction potential of the pipette solution relative to Ringer’s in the bath (−10 mV) and of the bath DVF solution relative to Ringer’s in the bath-ground agar bridge (5 mV). The averaged results are presented as the mean value ± SEM. Curve fitting was done by least-squares methods using built-in functions in Igor Pro 4.0. For the analysis of MIC channel kinetics, excised patches showing the activity of only one MIC channel were selected and processed using TAC (Bruxton Corporation). Channel transitions were idealized by setting a discriminator at 50% of the current between the open and closed levels, and the channel kinetics were obtained from the idealized traces.

**Leak Current Subtraction**

The activity of MIC channels when [Mg2+] is ≤3 mM (conditions of the majority of published papers on I_{CRAC} can pose special problems for the isolation of I_{CRAC} in Jurkat cells. In our experience, MIC activity often changes significantly during the course of whole-cell recording (depending, among other things, on the free [Mg2+] in the pipette). Thus, the commonly employed practice of subtracting the current present at the start of whole-cell recording (before I_{CRAC} induction) from later currents will not necessarily isolate I_{CRAC} cleanly. A more consistent leak subtraction method is to expose the cell to a Ca2+-free extracellular solution (0 Ca2+/3 mM Mg2+) shortly before or after measurement of the Ca2+ current, and to use this current as the “leak,” because these ionic conditions eliminate I_{CRAC} (Zweifach and Lewis, 1993), but not I_{MIC} (see Fig. 7 C). Therefore, we used the zero-Ca2+/leak subtraction method to isolate I_{CRAC} in the experiments reported here. For measurements of I_{MIC}, leak current was collected in 20 mM Ca2+ immediately after whole-cell break-in.

**Current Fluctuation Analysis**

Current noise data were recorded using two methods. In the first, current at a holding potential of −110 mV was recorded continuously at 32 kHz bandwidth onto digital audio tape (Sony DTC-700 modified for DC coupling). For mean-variance analysis, data from tape were then low-pass-filtered at 2 kHz (8-pole Bessel filter; Frequency Devices) and digitized at 5 kHz for analysis. In the second method, current during 500-ms steps to −110 mV applied once per second was low-pass-filtered at 1 kHz, digitized at 5 kHz, and recorded directly to hard disk. The series resistance was typically ∼5 MΩ, which in conjunction with the average membrane capacitance of 10 pF creates a 1-pole rolloff characteristic at 3.2 kHz; this filtering was ignored as it occurred well above the 1–2 kHz 8-pole Bessel cutoff we applied. The mean and variance were calculated from 200-ms segments of the digitized current. This short duration was chosen to minimize changes due to de-epitentiation of Na+ I_{CRAC} during each sweep (<2% change in current). For spectral analysis, data from tape were replayed through a 20-Hz high-pass filter (Krohn-Hite) and a 24 kHz low-pass Bessel filter (Frequency Devices) in series and were digitized at 5 kHz. Power spectra were computed from 512-point sweeps using a Hanning window (Igor Pro; Wavemetrics) and were averaged.

**RESULTS**

**Induction of a Large Monovalent Conductance by Washout of Intracellular Mg2+**

As described in previous studies of Jurkat and human T cells (Kerschbaum and Cahalan, 1998, 1999; Fomina et al., 2000) and RBL cells (Braun et al., 2001), intracellular dialysis with Mg2+-free internal solution in the absence of extracellular divalent cations triggers the slow development of a large monovalent conductance. In the experiment shown in Fig. 1, a Jurkat cell was dialysed with a Mg2+-free intracellular solution containing HEDTA, and continuous exposure to a DVF Ringer’s solution revealed the induction of a large inward current over a period of ∼400 s (Fig. 1 A). With equimolar extracellular Na+ and intracellular Cs+, the current reversed at −5 mV (Fig. 1 B), indicating approximately equal permeabilities for Na+ and Cs+. Substitution of NMDG for Na+ selectively suppressed the inward current, demonstrating that the larger cation cannot permeate (Fig. 1, A and B). Early in the induction phase of the whole-cell current, the progressive opening of single channels could be resolved (Fig. 1 C). These channels generally opened in an all-or-none fashion to a very high open probability (P_o = 0.97 at −110 mV; n = 4 cells), such that openings of more than five channels could be clearly resolved under whole-cell conditions as described previously (Kerschbaum and Cahalan, 1999; Fomina et al., 2000). Based on the amplitude and reversal potential of the single-channel current, the average chord conductance was 44 ± 3 pS. We observed similar single-channel and whole-cell currents in human T cells freshly isolated from blood, although their activation following break-in was slower (unpublished data).

With DVF Ringer’s in the recording pipette, these channels could also be observed in cell-attached patches and after patch excision into a Mg2+-free intracellular solution (Fig. 1 D and E). These channels were similar to those seen in whole-cell recordings (Fig. 1 C) in terms of their conductance, lack of selectivity, high open probability, and brief closures. The channels were weakly voltage-dependent, with the average P_o changing from 0.97 at −135 mV to 0.84 at −55 mV (n = 4 patches). Kinetic analysis indicated that the mean closed time (τ_c) varied from 1.1 ± 0.1 ms at −135 mV to 7.9 ± 1.5 ms at −55 mV (n = 4 patches), whereas the
The mean open time was relatively constant (32.2 ± 3.5 ms at −135 mV and 36.3 ± 4.8 ms at −55 mV). In terms of unitary conductance, kinetics, open probability and reversal potential, these channels closely resemble the 40-pS channels described previously in Jurkat, human T cells, and RBL cells (Kerschbaum and Cahalan, 1998, 1999; Fomina et al., 2000; Braun et al., 2001).

In previous studies, the large, sustained monovalent current and the underlying 40-pS single-channel currents were ascribed to the activity of CRAC channels (Kerschbaum and Cahalan, 1998, 1999; Fomina et al., 2000; Braun et al., 2001). However, we noted several discrepancies in our own experiments that led us to question this conclusion. First, monovalent inward current under Mg²⁺-free conditions activated much more slowly than the Ca²⁺ current in the presence of 20 mM extracellular Ca²⁺, with half times of 210 ± 27 s (n = 5) and 109 ± 16 s (n = 6), respectively. Second, the ratio of I_{Ca} to I_{Na} varied widely among cells (from 13 in Fig. 1 A to more than 100), even to the point where monovalent currents in the nA range occurred in some cells lacking a measurable Ca²⁺ current. Finally, using a DVF pipette solution we observed 40-pS channels in the majority of cell-attached patches from resting cells in which stores were not deliberately depleted, suggesting that the large monovalent current may be store-independent. To determine whether it does in fact reflect CRAC channel activity, we compared its dependence on store depletion and its pharmacological profile with that of I_{CRAC}.

The Large Monovalent Current Does Not Activate and Deactivate in Parallel With I_{CRAC}

If the large monovalent current flows through CRAC channels, then it should be active immediately upon establishing the whole-cell recording configuration in cells with empty Ca²⁺ stores. To test this, 1 μM thapsigargin (TG) was applied for 5–10 min before seal formation to fully deplete stores and maximally activate CRAC channels. Soon after break-in to the whole-cell
configuration, application of 20 mM Ca\(^{2+}\) to the cell causes an inward current to develop over \(~10\) s (Fig. 2A). This inward current is \(I_{\text{CRAC}}\) as judged from its current-voltage relationship (Fig. 2D, top graph), \(Ca^{2+}\) and store-dependence, sensitivity to various pharmacological agents (unpublished data), and characteristic delayed appearance following each application of \(Ca^{2+}\). This latter process, termed CDP, has been described previously (Zweifach and Lewis, 1996). Following \(Ca^{2+}\) readdition, the first application of DVF solution revealed only a small transient \(Na^{+}\) current at \(~110\) mV that declined to \(~60\%) within 20 s (arrow, Fig. 2A). Subsequent short applications of DVF solution revealed the slow development of a large \(Na^{+}\) current over the next 400 s, shown more clearly at lower gain (Fig. 2A, right graph). Two observations suggest that the large \(Na^{+}\) current and \(I_{\text{CRAC}}\) are not related. First, the amplitudes of the inward \(Ca^{2+}\) and \(Na^{+}\) currents did not increase in parallel (Fig. 2B), causing the ratio of the \(Na^{+}\) to the \(Ca^{2+}\) current to change from \(~2:1\) at the beginning of the experiment (\(t = 37\) s) to \(~50:1\) at later times (\(t = 570\) s). Thus, the amplitudes of the \(Ca^{2+}\) current and the large \(Na^{+}\) current are not well correlated in time. Second, during each application of DVF, the large \(Na^{+}\) current was roughly constant, even though CRAC channels appeared to be closing. This is shown by the small initial \(Ca^{2+}\) current seen immediately after each \(Ca^{2+}\) readdition, followed by a prominent increase due to CDP. Similar results were obtained in five cells.

Importantly, several key properties of the monovalent currents changed during the experiment. Whereas the \(Na^{+}\) current seen at early times in DVF solution decayed
rapidly, at later times ($t > 200$ s) it was more sustained (Fig. 2, A and B). Second, the shape of the current-voltage relationship changed dramatically. At early times the monovalent current showed no clear reversal potential, even up to 90 mV, suggesting that intracellular Cs$^+$ permeates poorly if at all compared with Na$^+$ (Fig. 2 C, top), whereas at later times the current did not rectify and reversed near 0 mV, consistent with equal permeabilities for the two ions (Fig. 2 C, bottom). Time-dependent changes in current selectivity and rectification were also observed in the presence of extracellular Ca$^{2+}$. At the beginning of the experiment ($t < 50$ s), the whole-cell current in 20 mM Ca$^{2+}$ displayed prominent inward rectification, characteristic of $I_{\text{CRAC}}$ (Fig. 2 D, top). However, at later time points ($>300$ s) the whole-cell current displayed strong outward rectification, generating a large outward current at potentials above 50 mV (Fig. 2 D, bottom). A likely explanation for these results is that an additional type of channel, unrelated to CRAC, activates slowly and produces a large, sustained and nonselective monovalent current that eventually dominates the whole-cell current under DVF conditions, and produces the outwardly rectifying current seen in the presence of Ca$^{2+}$. An alternative explanation (Kerschbaum and Cahalan, 1998) is that gradual removal of intracellular Mg$^{2+}$ changes the properties of the CRAC channel, keeping it from inactivating and allowing it to conduct Cs$^+$ more efficiently.

To distinguish between these possibilities, we asked whether the large monovalent conductance deactivates in parallel with $I_{\text{CRAC}}$ in response to store refilling. We have shown previously that in the presence of weak intracellular Ca$^{2+}$ buffering (1 mM EGTA in the pipette solution), prolonged Ca$^{2+}$ influx through CRAC channels can overwhelm the buffer, causing a global rise of [Ca$^{2+}$], store refilling, and deactivation of $I_{\text{CRAC}}$ (Zweifach and Lewis, 1995). This behavior is illustrated in the experiment of Fig. 3 A (left graph), in which $I_{\text{CRAC}}$ measured in the presence of 20 mM Ca$^{2+}$ activated slowly in response to passive store depletion, rose to a peak, then declined over $\sim 150$ s back to baseline. DVF solution was applied periodically in this experiment to
monitor the large monovalent current. The monovalent current did not decline in parallel with $I_{\text{CRAC}}$, but instead grew progressively larger over 400 s (Fig. 3 A, right). A plot of the time courses of the $\text{Ca}^{2+}$ current and the $\text{Na}^+$ current in this cell shows clearly that the amplitude of the large $\text{Na}^+$ current increases even as CRAC channels close (Fig. 3 B). Similar results were observed in 5/5 cells. Together with the changes in ion selectivity and kinetic behavior noted above, these results argue strongly that the large monovalent current arises from store-independent channels distinct from the CRAC channel.

**The Large Monovalent Current and $I_{\text{CRAC}}$ Have Different Pharmacological Profiles**

To further establish differences between the large monovalent current and CRAC channels and find conditions that could be used to isolate each current, we compared their sensitivities to SKF 96365 and 2-APB. SKF 96365 is an imidazole antimycotic compound that inhibits CRAC channels and several other SOCs with IC$_{50}$ values of 0.6–16 μM (Franzius et al., 1994; Christian et al., 1996a). Application of 20 μM SKF 96365 caused robust and partially reversible inhibition of $I_{\text{CRAC}}$ (Fig. 4 A). On average, the peak current amplitude was diminished by 87 ± 4% with a time constant of 17 ± 7 s ($n = 6$). In contrast, the same concentration of SKF 96365 had very little effect on the large monovalent current (Fig. 4 B), inhibiting by only 10 ± 2% after 120 s of exposure ($n = 4$). Two results argue that the resistance of the monovalent current to block by SKF 96365 is not due to DVF conditions per se. First, with 20 mM $\text{Ca}^{2+}$ present, the compound also failed to inhibit the outwardly rectifying current seen in Fig. 2 D, which we believe is mediated by the same channels that conduct the large monovalent current under DVF conditions (see below). Furthermore, complete inhibition of $I_{\text{CRAC}}$ by drug application in the presence of $\text{Ca}^{2+}$ did not affect or delay the appearance of the large monovalent current upon removal of extracellular divalents (Fig. 4 C). Thus, CRAC channels and the large monovalent current are distinctly different in their sensitivities to inhibition by SKF 96365.

A comparison of the effects of 2-APB provides further evidence that the two currents are distinct. 2-APB, originally described as a noncompetitive antagonist of IP$_3$ receptors, also inhibits store-operated $\text{Ca}^{2+}$ entry in several cell types (Ma et al., 2000; Broad et al., 2001; Prakriya and Lewis, 2001). We have shown previously that $I_{\text{CRAC}}$ in Jurkat cells is enhanced several-fold by low concentrations of 2-APB (<5 μM) and that this potentiation is followed by nearly irreversible inhibition at higher concentrations (IC$_{50}$ of ~10 μM) (Prakriya and Lewis, 2001). The dual effects of 2-APB are illustrated in Fig. 5, which shows potentiation of $I_{\text{CRAC}}$ by 5 μM 2-APB (Fig. 5 A), and potentiation and subsequent inhibition by 50 μM 2-APB (Fig. 5 B). The large monovalent

![Figure 4](image-url)  
**Figure 4.** $I_{\text{CRAC}}$ and the large monovalent current exhibit different sensitivities to SKF 96365. All cells were pretreated with 1 μM TG. In B and C, the recordings began after the monovalent current had activated to a steady-state level, as described in Fig. 2. Both currents were measured during steps to ~110 mV. (A) Inhibition of $I_{\text{CRAC}}$ by SKF 96365 (20 μM). Inhibition and recovery followed exponential time courses with time constants of 17.5 and 19 s, respectively. Internal solution: Cs methanesulfonate/10 BAPTA/8 $\text{Mg}^{2+}$. External: 20 mM $\text{Ca}^{2+}$. (B) The large monovalent current is relatively insensitive to 20 μM SKF 96365. Internal solution: MGF. (C) Even after nearly complete inhibition of $I_{\text{CRAC}}$ by SKF 96365 (20 μM), removal of extracellular divalent ions causes the monovalent current to rise rapidly to control levels. Thus, the insensitivity of the monovalent current to inhibition by SKF 96365 is not explained by a failure to block CRAC channels under DVF conditions. Internal solution: MGF.
lent current differed dramatically in its response to 2-APB. First, low concentrations of 2-APB (≤5 μM) failed to enhance the large monovalent current. Second, 50 μM 2-APB inhibited the large monovalent current by <50% (Fig. 5 C). In six cells, 40 μM 2-APB inhibited I_{CRAC} by 97 ± 2% (Prakriya and Lewis, 2001), but inhibited the large Na\(^+\) current by only 48 ± 6%. Inhibition by 50 μM 2-APB of the large monovalent current was also much slower (τ = 90 ± 8 s; n = 6) than inhibition of I_{CRAC} (τ = 10 ± 1 s, n = 6; Prakriya and Lewis, 2001). Finally, inhibition of the large Na\(^+\) current was rapidly and completely reversible, whereas block of I_{CRAC} was essentially irreversible (see Fig. 5 B; Prakriya and Lewis, 2001). Thus, the large monovalent current and I_{CRAC} exhibit clearly different sensitivities and responses to 2-APB.

The Large Monovalent Current Is Normal in Mutant Jurkat Cells Lacking I_{CRAC}

We also tested for the presence of the large monovalent current in Jurkat cell mutants that express low levels of I_{CRAC}. One of these lines, CJ-2, expresses ~14% of the normal level of I_{CRAC} (Fanger et al., 1995). Despite this defect in store-operated Ca\(^{2+}\) entry, CJ-2 cells express normal levels of I_{IMIC} (~114 ± 8 pA/pF [n = 5] in CJ-2 vs. ~127 ± 23 pA/pF [n = 7] in wild-type cells; measured at −110 mV). These results provide genetic evidence that CRAC channels and the large monovalent current represent distinct conduction pathways.

In sum, multiple independent lines of evidence demonstrate that the large monovalent current evoked in the absence of extracellular divalent ions and intracellular Mg\(^{2+}\) does not flow through CRAC channels. The large monovalent current is not store-dependent, its time course is independent of changes in CRAC channel activity, it is pharmacologically distinct from I_{CRAC} and its expression is regulated independently of CRAC in mutant cells. In the following section we examine the properties of the large monovalent conductance and describe methods for inhibiting it so that monovalent currents through CRAC channels can be measured in isolation.

The Large Monovalent Current Is Inhibited by Intracellular Mg\(^{2+}\)

The induction of the large monovalent current by intracellular pipette solutions lacking Mg\(^{2+}\) suggests that it may be activated as intracellular Mg\(^{2+}\) is dialysed out of the cell. We obtained direct support for this idea at both the whole-cell and single-channel levels. Addition of 8 mM MgCl\(_2\) to the whole-cell pipette solution completely suppressed the activation of the large monovalent current under DVF conditions (Fig. 6 A) with an IC\(_{50}\) of ~0.6 mM (Fig. 6 B). Intracellular Mg\(^{2+}\) also inhibited the development of the outwardly rectifying current seen in 20 mM Ca\(^{2+}\) (Fig. 2 D) with about the

**Figure 5.** I_{CRAC} and the large monovalent current have differing sensitivities to 2-APB. (A) A low concentration of 2-APB (5 μM) enhances I_{CRAC} after full store depletion by TG. I_{CRAC} is shown at −110 mV. Internal solution: Cs methanesulfonate/10 BAPTA/8 Mg\(^{2+}\). Holding potential: −40 mV (B) A high concentration of 2-APB (50 μM) initially enhances, then produces nearly complete and irreversible inhibition of I_{CRAC}. Experimental conditions as described in A. (C) Effects of 2-APB on the large monovalent current. A high concentration (50 μM) inhibits the current partially and reversibly. In contrast, the irreversible inhibition of I_{CRAC} in the same cell is shown by comparing currents during the first and second applications of Ca\(^{2+}\). 5 μM 2-APB fails to enhance the large monovalent current, although it enhances I_{CRAC} as shown in A. Internal solution: MGF.
same efficacy (Fig. 6 B), suggesting that both currents arise from the same channel.

Mg$^{2+}$ applied to the cytoplasmic side also inhibited single-channel monovalent currents in excised inside-out patches. An intriguing feature of Mg$^{2+}$-inhibition of these channels in excised patches was that full inhibition was observed even at Mg$^{2+}$ concentrations as low as 100 μM (Fig. 6 D; 3/3 patches). At present, we do not understand why the sensitivity to Mg$^{2+}$ increases after patch excision. Unlike the effect of 100 μM Mg$^{2+}$, application of 2 mM Mg$^{2+}$ to the cytoplasmic face of the membrane caused irreversible channel closure (Fig. 6 E; 8/8 patches). Similar effects have been reported for the 40-pS channels in RBL cells (Braun et al., 2001). The slow time course and variable reversibility of inhibition by Mg$^{2+}$ suggests that Mg$^{2+}$ causes channel closure rather than acting as a pore blocker. To denote their Mg$^{2+}$ sensitivity and nonselective cation permeability, we refer to these channels as Mg$^{2+}$-inhibited cation channels (MICs), and to the whole-cell current as IMIC. We apply this name for simplicity, and not to imply that intracellular Mg$^{2+}$ is a physiological regulator of channel activity (see discussion).

To isolate IMIC for further characterization, I_{CRAC} was blocked irreversibly by pretreatment with 100 μM 2-APB for 15 min. Following break-in with a Mg$^{2+}$-free internal solution, ramp currents were recorded while switching between 20 mM Ca$^{2+}$ and DVF Ringer’s solutions. Under these conditions, IMIC (observed during brief applications of DVF Ringer’s) developed in paral-
Monovalent Currents through CRAC Channels

lel with an outwardly rectifying current (observed during applications of 20 mM Ca\(^{2+}\) Ringer’s) (Fig. 7, A and B). Similar behavior was seen in 5/5 cells. As with \(I_{\text{MIC}}\), the outwardly rectifying current was not observed when 8–10 mM Mg\(^{2+}\) was included in the pipette solution (unpublished data), supporting the idea that this current is conducted by MIC channels.

Under DVF conditions, MIC currents reverse at \(-6 \pm 2\) mV (\(n = 5\)) cells. As with \(I_{\text{MIC}}\), the outwardly rectifying current was not observed when 8–10 mM Mg\(^{2+}\) was included in the pipette solution (unpublished data), supporting the idea that this current is conducted by MIC channels.

Isolating the Monovalent Current through CRAC Channels

Inhibition of \(I_{\text{MIC}}\) by intracellular Mg\(^{2+}\) offers a convenient strategy for isolating the monovalent current through CRAC channels for characterization. With 8 mM intracellular Mg\(^{2+}\) to suppress \(I_{\text{MIC}}\), passive depletion of Ca\(^{2+}\) stores by intracellular BAPTA (10 mM) slowly evoked \(I_{\text{CRAC}}\) in the presence of 20 mM Ca\(^{2+}\) (Fig. 8 A). Periodic exposure to DVF solution revealed a monovalent current which developed with the same time course as \(I_{\text{CRAC}}\) (Fig. 8 B). Similar results were seen
in 7/7 cells. The monovalent current was transient during each DVF episode, peaking immediately after solution exchange and subsequently declining with a time constant of $\sim 10$ s. The ratio of the peak amplitude of the Na$^+$ current to ICRAC measured just before solution exchange was 7.8 ± 0.4 ($n = 17$).

The parallel activation kinetics of ICRAC and the transient monovalent current suggests that the latter current represents flux through CRAC channels. As a further test, we examined the transient monovalent current’s dependence on store refilling under conditions that inhibit $I_{\text{MIC}}$ activation (8–10 mM Mg$^{2+}$). In the presence of 1 mM intracellular BAPTA and 20 mM Ca$^{2+}$, activation of ICRAC was followed by characteristic slow deactivation, presumably as Ca$^{2+}$ entering the cell overwhelmed the buffer and refilled the stores (Zweifach and Lewis, 1995) (Fig. 8 C). Periodic removal of external divalent ions showed that the transient Na$^+$ current decreased in parallel with ICRAC (Fig. 8, C and D). Similar behavior was seen in 6/6 cells. Together, these results show that the transient Na$^+$ current is store-dependent, similar to ICRAC but unlike $I_{\text{MIC}}$.

The pharmacological profile of the transient Na$^+$ current also closely matched that of ICRAC. As shown in Fig. 9 A, 20 mM SKF 96365 inhibited both currents to similar extents. On average, ICRAC was reduced by 84 ± 3% ($n = 4$), whereas the inactivating Na$^+$ current was reduced by 74 ± 3% in the same cells. Moreover, 5 mM 2-APB potentiated both currents, enhancing ICRAC by 241 ± 41% ($n = 7$) and the peak amplitude of the transient Na$^+$ current in the same cells by 185 ± 31% (Fig. 9 B). 2-APB also enhanced the steady-state component of the Na$^+$ current by a similar amount (224 ± 26%; $n = 6$). These results suggest that the residual current remaining after the Na$^+$ current has declined is also due to CRAC channels, a conclusion that is consistent with the observation that the peak and the residual currents reverse at the same potential (see below).

High concentrations of 2-APB inhibited the transient Na$^+$ current as they did ICRAC. As illustrated in Fig. 9 C, 40 mM 2-APB was applied to irreversibly inhibit ICRAC, and the transient Na$^+$ current was measured under DVF conditions following washout of the drug. In three cells, 50 mM 2-APB reduced ICRAC in the presence of 20

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**Figure 8.** Activation and deactivation of Ca$^{2+}$ and Na$^+$ currents through CRAC channels. Currents were measured during steps to −110 mV. (A) Activation of ICRAC and the transient Na$^+$ current during passive store depletion with a pipette solution containing 10 mM BAPTA + 8 mM Mg$^{2+}$. Periodic removal of external divalent ions reveals a transient monovalent current that increases in parallel with ICRAC. (B) The Ca$^{2+}$ current (●) and the peak Na$^+$ current (○) measured immediately before and during each application of DVF solution activate with similar kinetics. (C) Deactivation of ICRAC and the transient monovalent current during intracellular dialysis with 1 mM BAPTA + 8 mM Mg$^{2+}$. As described in Fig. 5 A, ICRAC deactivates in the presence of low intracellular Ca$^{2+}$ buffering, presumably due to store refilling. (D) The Ca$^{2+}$ current (●) and the peak Na$^+$ current (○) decline in parallel in the experiment in C. The similar activation and deactivation kinetics for the Na$^+$ and Ca$^{2+}$ currents support the idea that the transient monovalent current represents Na$^+$ flux through CRAC channels.
mM Ca\(^{2+}\) by 83 ± 3% and the monovalent current by 80 ± 6%. These results also show that block of the transient monovalent current by 2-APB outlasts bath application of the drug, as is the case for I\(_{\text{CRAC}}\) but not I\(_{\text{MIC}}\) (Fig. 5). Thus, the pharmacological properties of the transient Na\(^+\) current are consistent with those of I\(_{\text{CRAC}}\).

Based on these results as well as the evidence given above that I\(_{\text{CRAC}}\) and the transient Na\(^+\) current activate and deactivate in parallel and are store-dependent, we conclude that 8–10 mM intracellular Mg\(^{2+}\) suffices to isolate the monovalent current through CRAC channels in the absence of extracellular divalent ions. For convenience, the inward Na\(^+\) and Ca\(^{2+}\) currents through CRAC channels will hereafter be referred to as Na\(^+\)-ICRAC and Ca\(^{2+}\)-ICRAC, respectively.

**The Monovalent Selectivity of CRAC Channels Is Not Influenced by Intracellular Mg\(^{2+}\)**

The identification of a Mg\(^{2+}\)-sensitive monovalent current raises new questions about the possible role of Mg\(^{2+}\) in shaping the properties of CRAC channels. Previous studies have concluded that intracellular Mg\(^{2+}\) blocks permeation of Cs\(^+\) through CRAC channels under DVF conditions (Kerschbaum and Cahalan, 1998). Using the methods described above for separating I\(_{\text{MIC}}\) from I\(_{\text{CRAC}}\), we reassessed the role of Mg\(^{2+}\) in CRAC channel selectivity.

Fig. 10 A shows the transient monovalent CRAC current with 8 mM Mg\(^{2+}\) and with equimolar Cs\(^+\) and Na\(^+\) as the principal intracellular and extracellular cations, respectively. The I-V relations recorded as the current depotentiated show pronounced inward rectification and intersect at a single potential. This supports the idea that the whole-cell current decreases due to the closure of a single channel type, i.e., CRAC channels, and that the residual steady-state current is also due to CRAC channels as discussed above. The crossover point in eight cells was 52 ± 2 mV, implying that CRAC channels under DVF conditions are only weakly permeable to Cs\(^+\) (P\(_{\text{Cs}}\)/P\(_{\text{Na}}\) = 0.13 based on the Goldman-Hodgkin-Katz equation). A similar reversal potential was obtained from single ramp currents after subtraction of leak current recorded in 0-Ca Ringer’s (unpublished data). Thus, unlike MIC channels, CRAC channels in the absence of divalent cations select strongly against Cs\(^+\) (compare Fig. 7 B). Consistent with this conclusion, replacement of extracellular Na\(^+\) with Cs\(^+\) eliminates nearly all of the inward monovalent current through CRAC channels (Fig. 10 B).

These experiments do not address the role of Mg\(^{2+}\) in determining the selectivity against Cs\(^+\), because 8–10 mM intracellular Mg\(^{2+}\) was present to block I\(_{\text{MIC}}\). Unfortunately, if intracellular Mg\(^{2+}\) is removed, I\(_{\text{MIC}}\) develops to such an extent that it readily swamps the small monovalent current through CRAC channels. There-
fore, to measure Na\(^+\)-I\(_{\text{CRAC}}\) in the absence of Mg\(^{2+}\), we exploited the ability of 2-APB to enhance I\(_{\text{CRAC}}\) selectively. After the full development of I\(_{\text{MIC}}\) with 0 Mg\(^{2+}\), 5 \(\mu\)M 2-APB was applied to elevate I\(_{\text{CRAC}}\). The current immediately before 2-APB application was subtracted from the enhanced current to remove the contribution of I\(_{\text{MIC}}\) (Fig. 10 C). The I-V relation of the additional monovalent current is similar to that of the current recorded in the presence of 8 mM Mg\(^{2+}\) (Fig. 10 A). The 2-APB-enhanced current reverses at potentials >40 mV and shows inward rectification, though to a slightly lesser extent than in the presence of Mg\(^{2+}\) (compare Fig. 10 C with 10 A). Thus, the low permeability of CRAC channels to Cs\(^+\) does not require intracellular Mg\(^{2+}\), but is instead likely to be an intrinsic property of the CRAC channel pore.

Depotentiation of CRAC Channels Is Independent of Intracellular Mg\(^{2+}\)

The activity of CRAC channels slowly declines by up to 80% after removal of extracellular Ca\(^{2+}\), and recovers over 10–20 s after its reapplication by a process referred to as Ca\(^{2+}\)-dependent potentiation, or CDP (Christian et al., 1996b; Zweifach and Lewis, 1996). Thus, the current’s decline following the removal of extracellular divalents reflects depotentiation. Previous findings that transient monovalent currents (thought to be through CRAC channels) became large and sustained in the absence of intracellular Mg\(^{2+}\) led to the suggestion that internal Mg\(^{2+}\) is required for the depotentiation process, and that CDP may arise from the expulsion of Mg\(^{2+}\) ions from the pore of CRAC channels by permeant Ca\(^{2+}\) ions (Kerschbaum and Cahalan, 1998). Because I\(_{\text{MIC}}\) was likely mistaken for I\(_{\text{CRAC}}\) in the absence of intracellular Mg\(^{2+}\), we reexamined the role of Mg\(^{2+}\) in CRAC channel depotentiation under conditions that prevent activation of I\(_{\text{MIC}}\).

To effectively inhibit I\(_{\text{MIC}}\) under conditions of low intracellular Mg\(^{2+}\), we applied MgATP in combination with Na\(_2\)ATP to reduce free [Mg\(^{2+}\)]\(_i\) to 6–8 mM MgATP + 2–6 mM Na\(_2\)ATP in the pipette solution inhibited I\(_{\text{MIC}}\) while reducing free [Mg\(^{2+}\)]\(_i\) to calculated values of
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~92–185 μM (see materials and methods). Under these conditions, Na⁺-ICRAC still depotentiated after removal of extracellular divalents (Fig. 11 A), and the I-V relationship of the current was similar to that recorded with 8 mM Mg²⁺. Neither the time course (Fig. 11 C) nor the amplitude of depotentiation were altered relative to cells with 8 mM Mg²⁺; the fraction of current remaining after 50 s of DVF application was 20 ± 4% (n = 7) with 131 μM MgATP, vs. 18 ± 2% (n = 8) with 8 mM Mg²⁺. Finally, if depotentiation were to arise from Mg²⁺ binding in the pore of the CRAC channel and within the membrane field, hyperpolarization would be expected to inhibit it by reducing Mg²⁺ binding. However, shifting the holding potential from 40 mV to −110 mV did not significantly alter the rate of depotentiation at either high (6–10 mM) or low (131 μM) levels of Mg²⁺. (Fig. 11 B and C), even though the maximum amplitude of Na⁺-ICRAC was increased, consistent with the voltage dependence of CDP (Zweifach and Lewis, 1996). Together, these results argue against a necessary role for intracellular Mg²⁺ in the depotentiation of CRAC channels.

However, an alternative explanation could be that the putative Mg²⁺ site for depotentiation has a high affinity and does not lie within the membrane field. We examined this possibility by exploring Mg²⁺ effects on Ca²⁺-ICRAC. Full removal of intracellular Mg²⁺ in the presence of extracellular Ca²⁺ evokes only a small inward I_{MIC}, and the current is not affected by removal of Ca²⁺, alone (Fig. 7 C), providing a way of isolating it from I_{CRAC} through leak subtraction. Therefore, we used these conditions to ask whether Ca²⁺-ICRAC depotentiates in the absence of intracellular Mg²⁺. Depotentiation of I_{CRAC} in the absence of Ca²⁺ can be detected
indirectly by the slow recovery of full current amplitude after readdition of Ca\(^{2+}\) (Fig. 11 D). Under control conditions (8 mM Mg\(^{2+}\)), Ca\(^{2+}\) readdiction causes Ca\(^{2+}\)-I\(_{\text{CRAC}}\) to appear in two kinetically distinct stages: a small fraction of the current appears immediately (i.e., within the 2-s exchange time of the perfusion system) that represents the current through active CRAC channels, followed by a several-fold increase in current amplitude over the next 10–20 s due to CDP. Thus, the size of the slowly relaxing current component reflects the extent of depotentiation that occurred before the readdition of Ca\(^{2+}\). Significantly, neither the amplitude nor the time course of CDP was affected by intracellular Mg\(^{2+}\). At a holding potential of 20 mV, CDP caused a 104 ± 5% increase in Ca\(^{2+}\)-I\(_{\text{CRAC}}\) with an average τ of 2.9 ± 0.3 s (n = 6) in cells with 8 mM Mg\(^{2+}\), and a 114 ± 7% increase with τ = 3.1 ± 0.4 s (n = 4) in cells in which Mg\(^{2+}\) was chelated by 10 mM HEDTA. These results are consistent with those of Figs. 2 and 3, where I\(_{\text{CRAC}}\) depotentiates during applications of DVF solution in the absence of Mg\(^{2+}\). Thus, intracellular Mg\(^{2+}\) appears to have no influence on CRAC-channel depotentiation triggered by removal of extracellular Ca\(^{2+}\).

**Estimating the Unitary Na\(^{+}\) Current through CRAC Channels by Noise Analysis**

The finding that the 40-pS monovalent channels seen under DVF conditions are MIC channels raises new questions about the unitary monovalent conductance of CRAC channels. Under DVF conditions with 10 mM intracellular Mg\(^{2+}\) to inhibit I\(_{\text{M IC}}\), we were unable to detect any clear single-channel transitions of amplitude >0.4 pA during the induction of Na\(^{+}\)-I\(_{\text{CRAC}}\) by ionomycin in 5/5 cells and during the or depotentiation of Na\(^{+}\)-I\(_{\text{CRAC}}\). This contrasts with the obvious appearance of single-channel MIC currents when intracellular Mg\(^{2+}\) is washed out during whole-cell recording (Fig. 1). Therefore, we applied stationary fluctuation analysis to estimate the single-channel monovalent CRAC conductance.

Cells were treated with 1 μM TG to activate CRAC channels, and after establishing whole-cell recording the mean macroscopic current (I) and corresponding variance (σ\(^2\)) were measured at a holding potential of −110 mV. Application of 5 μM 2-APB enhanced I\(_{\text{CRAC}}\) in the presence of 20 mM Ca\(^{2+}\) and caused a barely detectable increase in current noise (Fig. 12 A). Subsequent removal of divalent cations (DVF conditions) evoked a transient Na\(^{+}\) current through CRAC channels in parallel with a more robust increase in noise. Several 200-ms current sweeps used to compute the current mean and variance for the DVF condition are shown in Fig. 12 B, and indicate the lack of any clear single-channel transitions as the current declines. Plots of the current variance against mean current amplitude were well fitted by straight lines (Fig. 12, C and D). The average slope (σ\(^2\)/I) was −3.8 ± 0.6 fA (n = 5) in 20 mM Ca\(^{2+}\), and −31 ± 2 fA (n = 10) under DVF conditions.

The relation of the variance to the mean current can be used to estimate the single-channel current given certain assumptions. For a homogeneous population of N\(_i\) independent channels with a single conducting level, i, stochastic gating predicts a parabolic relation between the current variance and the mean current as the open probability (P\(_i\)) varies between zero and one (Sigmworth, 1980):

\[
\sigma_i^2 = iI - \frac{I^2}{N} + \sigma_0^2,
\]

where σ\(_0^2\) is the variance at the zero-current level. Since I = N\(_i\)P\(_i\), it follows that

\[
\frac{\sigma_i^2 - \sigma_0^2}{I} = i(1 - P_0).
\]

Given these assumptions, the linear shape of the σ\(_i^2\)/I relation implies that the open probability of the channel is very low (<0.5) and that the unitary current amplitude is given by the slope (Eq. 1). Thus, the results would suggest that the unitary amplitude of monovalent I\(_{\text{CRAC}}\) at −110 mV is −31 fA; given the reversal potential of 52 mV, this corresponds to a single-channel chord conductance of ~0.2 pS.

There are several ways in which fluctuation analysis may seriously underestimate the unitary conductance. First, the bandwidth of the recording may not extend to high enough frequencies to capture the fluctuations due to very brief gating events. To test this, we analyzed the power spectrum of the current noise under DVF conditions. Fig. 12 E shows the background spectrum obtained in 0 Ca\(^{2+}\) + 2 mM Ni\(^{2+}\), together with the spectrum of Na\(^{+}\)-I\(_{\text{CRAC}}\) at its peak and after it depotentiated to a steady level. In both cases, the noise approaches the background level at frequencies above ~1 kHz, suggesting that no large component of high-frequency noise was missed under the 1–2 kHz filtering conditions of the current variance experiments.

Second, noise measurements may underestimate the size of i if P\(_i\) is high and the macroscopic current amplitude reflects changes in N\(_i\), the number of activatable channels, rather than P\(_i\) (Jackson and Strange, 1995). In this case, σ\(_i^2\) will increase linearly with N:\n
\[
\sigma_i^2 = Ni^2 P_0 (1 - P_0) + \sigma_0^2
\]

and the σ\(_i^2\)/I ratio underestimates i by a factor of (1 − P\(_i\)) (Eq. 1). If this situation applies, a small reduction in the P\(_i\) resulting from extracellular blockade of the channels would be expected to produce an increase in channel noise due to an increased number of gating events occurring within each 200-ms sampling period. To examine this possibility, we added 1 μM free Ca\(^{2+}\) to
the extracellular DVF solution to partially block CRAC channels and reduce channel $P_o$. The peak $I_{Na}/I_{Ca}$ ratio was reduced to $3.1 \pm 0.3$ ($n = 4$; versus a ratio of $7.8 \pm 0.4$ without the blocker), indicating $>50\%$ inhibition of the Na$^+$-ICRAC. Under these conditions, the $\sigma_{f}^2/I$ ratio was $-19 \pm 3$ fA ($n = 4$). This ratio is even smaller than the $\sigma_{f}^2/I$ ratios obtained in the absence of the blocker. Given these results, it seems unlikely that very high $P_o$ of CRAC channels is causing a gross underestimation of the single channel current. Thus, from the results of noise analysis, we estimate that the monovalent conductance of the CRAC channel is $\sim 0.2$ pS.

**DISCUSSION**

In the absence of extracellular divalent cations, removal of intracellular Mg$^{2+}$ during whole-cell recording activates a large monovalent current in T cells and RBL cells. This large Mg$^{2+}$-sensitive current has previously been attributed to monovalent permeation of CRAC channels, and its signature conductance and selectivity have been employed as a biophysical fingerprint to screen candidate CRAC channel genes. In this paper, we show that this large monovalent current, $I_{MIC}$, and the 40-pS channels that underlie it, differ in fundamental ways from $I_{CRAC}$ and therefore represent a distinct ion channel type. These findings necessitate a re-
vision of the biophysical fingerprint of CRAC channels, and have important implications for studies of CRAC channel activation, permeation, and cellular function.

**MIC and CRAC Currents Arise from Distinct Channels**

A number of characteristics distinguish MIC channels from CRAC channels. These include the mode of activation, inhibition by Mg$^{2+}$ or MgATP, kinetic properties, selectivity, pharmacology, and unitary conductance. These differences are summarized in Table I and are discussed below.

Several observations indicate that unlike I$_{CRAC}$, I$_{MIC}$ is not store-operated (Figs. 2 and 3). For example, I$_{MIC}$ slowly activates in cells in which I$_{CRAC}$ is already preactivated by store depletion, and it activates even as I$_{CRAC}$ deactivates due to store refilling. I$_{MIC}$ is effectively suppressed by intracellular Mg$^{2+}$ or MgATP, whereas CRAC channel gating is not noticeably affected. I$_{MIC}$ activates about twice as slowly as I$_{CRAC}$ in Jurkat cells; thus, the activation kinetics clearly do not reflect the time course of store depletion. Moreover, because introduction of 10 mM HEDTA into the cell would be expected to quickly buffer Mg$^{2+}$, it is unlikely that the time course of I$_{MIC}$ is limited by the rate at which Mg$^{2+}$ or MgATP are removed from the cell. Activation of single 40-pS channels after patch excision from RBL cells into a Mg$^{2+}$- and ATP-free solution can also be slow (Braun et al., 2001), suggesting that the rate-limiting step lies downstream of Mg$^{2+}$ or MgATP removal. Interestingly, Mg$^{2+}$ inhibits MIC channels more effectively in excised patches than in whole-cell recordings (Fig. 6), suggesting that the inhibition mechanism is more complex than simple channel blockade and may involve a diffusible molecule that is lost after patch excision.

Following removal of extracellular divalents, Na$^{+}$-I$_{CRAC}$ is transient (Fig. 10 A), whereas I$_{MIC}$ is relatively sustained (Fig. 5 C). The peak phase of the Na$^{+}$ current is linked to CRAC channels, based on its close correlation with Ca$^{2+}$-I$_{CRAC}$ during store depletion and refilling (Fig. 8), and its sensitivity to SKF 96365 and 2-APB (Fig. 9). Na$^{+}$-I$_{CRAC}$ is depotentiated in DVF solutions to an average level of ~20%. We believe the steady-state activity is also from CRAC channels because its amplitude varies in parallel with the peak Na$^{+}$-I$_{CRAC}$ and it has the same reversal potential, relative Cs$^{+}$ permeability and sensitivity to SKF 96365 and 2-APB, as the peak current. Although I$_{MIC}$ is generally sustained under Mg$^{2+}$-free conditions application of DVF solutions, it declines slowly in the presence of low levels of Mg$^{2+}$ (0.5–1 mM; unpublished data). Thus, current decline under DVF conditions cannot be used as a specific indicator for I$_{CRAC}$.

Finally, the pharmacological signatures of I$_{CRAC}$ and I$_{MIC}$ also differ significantly (Figs. 4 and 9). SKF 96365 at micromolar levels is an effective blocker of I$_{CRAC}$ (Franzius et al., 1994; Christian et al., 1996a), but not of I$_{MIC}$. High concentrations of 2-APB (20–40 μM) inhibit I$_{CRAC}$ completely and irreversibly (Prakriya and Lewis, 2001), whereas they cause modest and rapidly reversible inhibition of I$_{MIC}$. Low concentrations of 2-APB (≤5 μM) which strongly enhance I$_{CRAC}$ (Prakriya and Lewis, 2001) fail to affect I$_{MIC}$.

The rather large number of properties that distinguish the two conductances strongly suggest that I$_{CRAC}$ and I$_{MIC}$ arise from two different channels. However, an alternative explanation might be that removal of intracellular Mg$^{2+}$ merely alters the properties of CRAC channels, making them acquire the characteristics of what we now call MIC channels (Kirschbaum and Cahalan, 1998). We do not believe that the MIC channel is an altered state of the CRAC channel for several key reasons. First, it seems unlikely that removal of Mg$^{2+}$ alone would be able to alter such a diverse set of properties, including store-dependence, depotentiation, ion selectivity, pharmacological profile, and unitary conductance. Second, we find that removal of intracellular Mg$^{2+}$ does not in fact reduce the selectivity of CRAC for Na$^{+}$ over Cs$^{+}$ (Fig. 10 C), nor does it prevent depotentiation of CRAC channels (i.e., make CRAC activity sustained) under 0-Ca$^{2+}$o conditions (Figs. 2 A, 3 A, and 11 D). Finally, MIC channel activity is normal in mutant Jurkat cells that have very low levels of I$_{CRAC}$. Thus, the most parsimonious explanation for these data is that MIC and CRAC channels are separate and distinct proteins.2

In light of our results, various conclusions made in previous reports regarding the regulation of CRAC

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**TABLE I**

| Property                  | CRAC channels | MIC channels |
|---------------------------|---------------|--------------|
| Store-dependent activation| yes           | no           |
| Inhibition by Mg$^{2+}$, or MgATP | no            | yes          |
| Duration in DVF conditions| transient     | sustained    |
| (20 μM for 120 s)         |               |              |
| Selectivity ($P_{Na}$/$P_{Cs}$) | 0.13          | 1.2          |
| Inhibition by SKF 96365   | 87 ± 4%       | 10 ± 2%      |
| (20 μM for 120 s)         |               |              |
| Inhibition by 2-APB (IC$_{50}$) | ~10 μM, irreversible  | ~50 μM, quickly reversible |
| Enhancement by 2-APB (EC$_{50}$) | ~3 μM  | none |
| Unitary current           | ~31 ± 2 fA   | ~4.5 ± 0.4 pA |
| (Na$^{+}$, −110 mV)       | (n = 10)      | (n = 4)      |
| Unitary chord conductance | ~0.2 pS      | 44 ± 3 pS    |

1Results from Prakriya and Lewis (2001).

2While this manuscript was under review, Hermosura et al. (2002) published evidence showing that the large sustained monovalent current in RBL cells perfused with Mg$^{2+}$-free intracellular solutions (termed MagNuM) can be separated from I$_{CRAC}$. This report and another (Bakowski and Parekh, 2002) also showed that intracellular Mg$^{2+}$ does not significantly affect CRAC channel permeation in RBL cells, in agreement with our results.
channels probably apply more to MIC channels than to CRAC channels. For example, the voltage-dependent block of monovalent CRAC currents by extracellular Ca\(^{2+}\) (Kerschbaum and Cahalan, 1998; Fomina et al., 2000), the upregulation of CRAC channels in activated T cells (Fomina et al., 2000), and regulation by cytoplasmic Mg\(^{2+}\) and Ca\(^{2+}\) (Braun et al., 2001) were all measured under conditions optimized for activation of \(I_{\text{MIC}}\). These issues may have to be revisited using recording conditions explicitly optimized for isolating the monovalent CRAC current. No specific inhibitors of \(I_{\text{MIC}}\) are yet known, but high intracellular Mg\(^{2+}\) (8–10 mM) or MgATP (>4 mM) effectively blocks \(I_{\text{MIC}}\) without affecting \(I_{\text{CRAC}}\). It is important to note that moderate levels of Mg\(^{2+}\) (2–3 mM) commonly used in studies of \(I_{\text{CRAC}}\) may not be sufficient to completely eliminate MIC activity, as we often found 3–5 active MIC channels in Jurkat cells under these conditions. Although this represents a small fraction of the total MIC conductance in the cell, the unitary current of MIC channels is so much larger than that of CRAC channels that it can seriously contaminate noise measurements under DVF conditions (see below). Activity of MIC channels can also lead to inadvertent contamination of \(I_{\text{CRAC}}\) in the presence of divalent ions, although appropriate subtraction of the “leak” current can alleviate this problem (see materials and methods). 

\(I_{\text{MIC}}\) May Arise from TRP-PLIK/LTRPC7 Channels

Given that MIC channels are distinct from CRAC, what is their molecular identity? Recently, a novel member of the TRP family of ion channels has been cloned which has been named TRP-PLIK (Runnels et al., 2001) or LTRPC7 (Nadler et al., 2001). Several characteristics of TRP-PLIK/LTRPC7 are similar to MIC in Jurkat cells, LTRPC7 is expressed in cell lines commonly used for studies of \(I_{\text{CRAC}}\), such as Jurkat T cells and RBL cells. Activation of LTRPC7 in transfected cells is elicited by whole-cell dialysis with chelators of Mg\(^{2+}\), and introduction of Mg\(^{2+}\) and/or Mg-ATP inhibits channel activity (Nadler et al., 2001). TRP-PLIK/LTRPC7 produces a nonselective cation channel with strong outward rectification in the presence of extracellular divalent ions (Nadler et al., 2001; Runnels et al., 2001), much like the outward rectification of \(I_{\text{MIC}}\) we observe under similar conditions (Fig. 7). Elimination of extracellular Ca\(^{2+}\) or Na\(^{+}\) alone does not affect the inward current through LTRPC7 (Nadler et al., 2001) or MIC channels (Fig. 7 C and unpublished data). Together, these similarities to \(I_{\text{MIC}}\) in Jurkat cells suggest that TRP-PLIK/LTRPC7 encodes the MIC channel.

The activation mechanism and physiological roles of MIC channels are not well understood at this point. A small number of MIC channels appear to be active in resting Jurkat cells, based on the current seen in DVF conditions immediately after break-in with 0-Mg\(^{2+}\) pipette solution (Fig. 6 A). Although depletion of cytoplasmic Mg\(^{2+}\) can further activate MIC channels, it seems unlikely that this is the physiological stimulus (see Nadler et al., 2001), as cytosolic Mg\(^{2+}\) in many cells is held relatively constant between 0.5 and 1 mM (Roman and Scarpa, 2000). Its sensitivity to inhibition by Mg\(^{2+}\)-nucleotides has led to the hypothesis that it opens in response to declining ATP levels (Nadler et al., 2001), but there is also evidence that channel activation requires kinase activity of the COOH-terminal PLIK domain (Runnels et al., 2001). The physiological consequences of MIC activation are also unclear. Nadler et al. (2001) have proposed that LTRPC7 provides a conduit for Ca\(^{2+}\) entry that regulates mitochondrial activity and ATP homeostasis (Nadler et al., 2001). However, the precise Ca\(^{2+}\) permeability of this channel has not been measured. Although it has been stated that the block of Na\(^{+}\) flux by external divalent ions renders LTRPC7 solely permeable to divalent ions at negative potentials (Nadler et al., 2001), our preliminary experiments on MIC channels in Jurkat cells suggest that Na\(^{+}\) can carry inward current in the presence of Mg\(^{2+}\) (unpublished data). Thus, while MIC channels may provide a pathway for Ca\(^{2+}\) entry, they may also depolarize the cell by conducting monovalent ions. From the relative amplitudes of the single-channel and whole-cell currents we estimate that Jurkat cells express 250–500 MIC channels per cell.

Intracellular Mg\(^{2+}\) and CRAC Channel Function

In previous studies, the removal of intracellular Mg\(^{2+}\) under DVF conditions appeared to change a small transient and Na\(^{+}\)-selective current through CRAC channels into a much larger, sustained, and nonselective current with equal permeability to Cs\(^{+}\) and Na\(^{+}\) (Lepple-Wienhues and Cahalan, 1996; Kerschbaum and Cahalan, 1998). These observations were interpreted to mean that Mg\(^{2+}\) removal prevents depotentiation of CRAC and alters their selectivity. We have found that intracellular Mg\(^{2+}\) does not affect Ca\(^{2+}\)-dependent potentiation of CRAC channels, or the reverse process of depotentiation (Fig. 11), nor is it required to achieve selectivity for Na\(^{+}\) over Cs\(^{+}\) (Fig. 10 C). This discrepancy with the earlier results can now be explained by the fact that removal of Mg\(^{2+}\) activates \(I_{\text{MIC}}\), which is much larger, more sustained, and less selective than \(I_{\text{CRAC}}\).

The Unitary Conductance of CRAC Channels

Our results indicate that the monovalent conductance of CRAC channels is too small to be resolvable in whole-cell recordings. Noise analysis of whole-cell Na\(^{+}\)-\(I_{\text{CRAC}}\) suggests a conductance in the range of 0.2 pS. This is dramatically different from recent reports in T cells and RBL cells, where a conductance of 40 pS was
reported for monovalent currents through CRAC channels (Kerschbaum and Cahalan, 1999; Fomina et al., 2000; Braun et al., 2001). This discrepancy is explained by the fact that the earlier experiments were performed in the absence of cytoplasmic Mg2+, which as we have shown here activates store-independent MIC channels. Our estimate of the unitary conductance is also smaller than an earlier estimate of 2.6 pS based on noise analysis (Lepple-Wienhues and Cahalan, 1996). Using the ionic conditions of this earlier study (3 mM intracellular Mg2+ + 1 μM extracellular Mg2+), we also detect a significantly larger noise, which appears to arise from flickery block of several active MIC channels in the cell. Thus, even slight contamination of Na+-I_{CRAC} with IMIC may lead to large differences in the estimated unitary currents.

We considered several possible factors that could lead us to underestimate the unitary currents. Current fluctuations could be missed if they occur at frequencies outside the recording bandwidth. However, spectral analysis showed that most of the power of the current noise occurred at frequencies <1 kHz, well within the recording bandwidth (Fig. 12 E). A more difficult problem could arise if activation of I_{CRAC} occurs through an increase in N, the number of activatable channels, each of which has a high open probability and therefore contributes little noise. However, we were unable to detect discrete jumps in current during the activation or depolarization of monovalent I_{CRAC}. In addition, partial inhibition of monovalent I_{CRAC} with 1 μM extracellular Ca2+ did not increase the noise, indicating that whatever the mechanism of activation, the P_o of active CRAC channels conducting Na+ is quite small. Together with our estimates of the single channel conductance, these observations set limits on the maximum size of the single-channel conductance to <1 pS.

Our estimate of −3.8 fA at −110 mV for the unitary Ca2+-current compares well with a previous estimate in 110 mM Ca2+o at −80 mV (−3.7 fA) (Zweifach and Lewis, 1993). Note that this estimate is roughly eight-fold smaller than the single-channel estimates of the Na+ current given above (−31 fA). The fact that the whole-cell Na+-I_{CRAC} is also about eightfold larger than Ca2+-I_{CRAC} suggests that the increase in whole-cell current seen upon exchanging the Ca2+-o solution for the DVF solution can entirely be accounted for by the increase in conductance of the CRAC channels without a significant change in channel P_o. From the ratio of the unitary and whole-cell peak Na+ currents, we estimate that the number of CRAC channels per cell is at least 5,000–10,000. More would be expected if the open probability is low, as is suggested by the linear relation of variance to mean current. Finally, it should be noted that our estimate for the unitary conductance of Na+-I_{CRAC} (0.2 pS) is significantly smaller than the 42-pS conductance reported for the CaT1 channel under similar DVF conditions (Yue et al., 2001), consistent with recent suggestions that CaT1 may not comprise the complete CRAC-channel pore (Voets et al., 2001).

**Selectivity of CRAC Channels**

Similarities and differences between the permeation properties of CRAC channels and voltage-gated Ca2+ (CaV) channels have interesting implications for mechanisms of ion selectivity in CRAC channels. Under physiological conditions, CaV channels have an extremely high selectivity for Ca2+ over monovalent ions (P_{Ca}/P_{Na} > 1,000). The high Ca2+ selectivity is thought to arise from the high-affinity binding of Ca2+ within the pore, which prevents Na+ from conducting. In fact, the removal of extracellular divalent ions allows Na+ to conduct freely (Almers and McCleskey, 1984; Hess and Tsien, 1984). CRAC channels show a comparably high selectivity for Ca2+ over monovalent ions (Hoth and Penner, 1993), and similarly become freely permeable to Na+ in the absence of extracellular divalents. Thus, it is likely that the Ca2+ selectivity of CRAC channels also arises from high affinity Ca2+ binding within the pore, and this is consistent with the ability of 1 μM Ca2+ to block Na+-I_{CRAC} by ~50% (see results).

The most obvious differences between the two channels relates to their unitary conductances and permeability to Cs+. CaV channels have single-channel conductances for Ca2+ in the range of 5–20 pS and ~85 pS for Na+ under DVF conditions (Hess et al., 1986). By contrast, the unitary conductances of CRAC channels is estimated to be ~21 pS for Ca2+ and 0.2 pS for Na+, or ~500-fold smaller than CaV channels. In addition, under DVF conditions CaV channels readily pass Cs+ (P_{Ca}/P_{Na} ~0.6; Hess et al., 1986), whereas CRAC channels do not (P_{Ca}/P_{Na} = 0.13; Fig. 10; see also Lepple-Wienhues and Cahalan, 1996). These observations suggest that although CRAC channels and voltage-gated Ca2+ channels achieve selectivity for Ca2+ over other ions by high affinity Ca2+ binding within the pore, many structural features may be significantly different between these two channel types.

What unique structural features of CRAC channels could give rise to such a low conductance? A popular model proposes that the high throughput of CaV channels arises from ion–ion interactions as ions move single-file through the pore (Tsien et al., 1987). Thus, one possibility is that weak ion–ion interactions underlie the low flux rate of Ca2+ through CRAC channels. However, this idea cannot adequately explain why CRAC channels would have an equally low conductance for monovalent ions. We hypothesize that CRAC channels possess a nonselective rate-limiting barrier to ion permeation in series with the selectivity filter. Such a barrier may explain not only the uniformly low per-
meability to various ions, but also the lack of permeability to Ca$^2+$. Identification of CRAC channel genes will provide much needed tools for elucidating the mechanism of CRAC channel permeation. In the interim, systematic examination of permeation of various ions through native CRAC channels will improve our understanding of the properties of the channel pore.

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