Monovalent Permeability, Rectification, and Ionic Block of Store-operated Calcium Channels in Jurkat T Lymphocytes

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ABSTRACT We used whole-cell recording to characterize ion permeation, rectification, and block of monovalent current through calcium release-activated calcium (CRAC) channels in Jurkat T lymphocytes. Under physiological conditions, CRAC channels exhibit a high degree of selectivity for Ca$^{2+}$, but can be induced to carry a slowly declining Na$^+$ current when external divalent ions are reduced to micromolar levels. Using a series of organic cations as probes of varying size, we measured reversal potentials and calculated permeability ratios relative to Na$^+$. Ammonium (NH$_4^+$) exhibited the highest relative permeability ($P_{\text{NH}_4^+}/P_{\text{Na}^+} = 1.37$). The largest permeant ion, tetramethylammonium with a diameter of 0.55 nm, had $P_{\text{TMA}^+}/P_{\text{Na}^+}$ of 0.09. N-methyl-D-glucamine (0.50 × 0.64 × 1.20 nm) was not measurably permeant. In addition to carrying monovalent current, NH$_4^+$ reduced the slow decline of monovalent current (“inactivation”) upon lowering [Ca$^{2+}$]. This kinetic effect of extracellular NH$_4^+$ can be accounted for by an increase in intracellular pH (pH$_i$), since raising intracellular pH above 8 reduced the extent of inactivation. In addition, decreasing pH$_i$ reduced monovalent and divalent current amplitudes through CRAC channels with a pK$_a$ of 6.8. In several channel types, Mg$^{2+}$ has been shown to produce rectification by a voltage-dependent block mechanism. Mg$^{2+}$ removal from the pipette solution permitted large outward monovalent currents to flow through CRAC channels while also increasing the channel’s relative Ca$^{2+}$ conductance and eliminating the inactivation of monovalent current. Boltzmann fits indicate that intracellular Mg$^{2+}$ contributes to inward rectification by blocking in a voltage-dependent manner, with a z$_B$ product of 1.88. Ca$^{2+}$ block from the outside was also found to be voltage dependent with z$_B$ of 1.62. These experiments indicate that the CRAC channel, like voltage-gated Ca$^{2+}$ channels, achieves selectivity for Ca$^{2+}$ by selective binding in a large pore with current–voltage characteristics shaped by internal Mg$^{2+}$.

KEY WORDS: calcium channel • CRAC channel • $I_{\text{CRAC}}$ • ion selectivity • inward rectification

INTRODUCTION Stimulation of T lymphocytes by antigen-presenting cells initiates a cascade of events, including tyrosine phosphorylation and activation of phospholipase C, resulting in the release of Ca$^{2+}$ from intracellular IP$_3$-sensitive stores and an increase of cytoplasmic Ca$^{2+}$ concentration [Ca$^{2+}$]. (Crabtree and Clipstone, 1994). The increase in [Ca$^{2+}$]$_i$ is sustained by influx of Ca$^{2+}$ through Ca$^{2+}$ channels located in the plasma membrane. Sustained elevations or long-lasting oscillatory changes in [Ca$^{2+}$] are essential for proliferation and gene expression in T cells (Negulescu et al., 1994; Fanger et al., 1995; reviewed by Lewis and Cahalan, 1995). The most extensively investigated mechanism for Ca$^{2+}$ influx in T cells is a stores-operated Ca$^{2+}$ channel, known as the Ca$^{2+}$ release-activated Ca$^{2+}$ (CRAC)$^1$ channel. CRAC channels with a high degree of selectivity for Ca$^{2+}$ are observed in lymphocytes and mast cells (Lewis and Cahalan, 1989; Hoth and Penner, 1992; Zweifach and Lewis, 1993), while numerous other cell types possess similar Ca$^{2+}$-permeable channels activated by Ca$^{2+}$ store depletion. Regardless of the initiating stimulus (surface receptor engagement, passive di- alysis of the cytoplasm with Ca$^{2+}$ buffer, direct addition of IP$_3$ to empty IP$_3$-sensitive stores, addition of the Ca$^{2+}$ ionophore ionomycin, or inhibition of the Ca$^{2+}$-ATPase uptake pump with thapsigargin), depletion of Ca$^{2+}$ from intracellular stores activates CRAC channels through an unknown mechanism (Lewis and Cahalan, 1989; Hoth and Penner, 1992; Zweifach and Lewis, 1993; Premack et al., 1994; Zhang andMcCloskey, 1995). The resulting Ca$^{2+}$ current ($I_{\text{CRAC}}$) is not voltage dependent in its gating, but exhibits inward rectification and a very positive reversal potential. The primary mechanism of activation after depletion of intracellular Ca$^{2+}$ stores is not well defined, but channel gating is known to be regulated by [Ca$^{2+}$]$_i$, and by kinases and nucleotides (reviewed in Parekh and Penner, 1996; Lewis et al., 1996). The focus of this paper is ion permeation through CRAC channels.

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1Abbreviations used in this paper: CRAC, calcium release-activated calcium; IV, current–voltage; NMDG$^+$, N-methyl-D-glucamine; TMA$^+$, tetramethylammonium.
Under physiological conditions, CRAC channels are highly selective for Ca\(^{2+}\), enabling a very small current (~1 pA/pF at ~80 mV in Jurkat T cells) to support the [Ca\(^{2+}\)]\(_o\) signal (Lewis and Cahalan, 1989; Hoth and Penner, 1992; Zweifach and Lewis, 1993; Hoth, 1995). Reducing external divalent to the micromolar range reveals a much larger monovalent current through CRAC channels, carried by Na\(^+\) in low divalent Ringer or by other alkali cations in test solutions (Hoth and Penner, 1993; Premack et al., 1994; Lepple-Wienhues and Cahalan, 1996). In the absence of external Mg\(^{2+}\), the Na\(^+\) current through CRAC channels immediately after lowering [Ca\(^{2+}\)]\(_o\) peaks at a value ~5–10-fold larger than the preceding Ca\(^{2+}\)-selective current, and then declines by an unknown mechanism. Although differing fundamentally in gating (store depletion vs. depolarization to open the channel), CRAC channels and voltage-gated Ca\(^{2+}\) channels exhibit a similar loss of selectivity upon lowering [Ca\(^{2+}\)]\(_o\), and in both channel types selection against monovalents can be ascribed to the binding of Ca\(^{2+}\) ions with micromolar affinity to sites within the channel conduction pathway (Hess and Tsien, 1984; Almers and McCleskey, 1984; Lepple-Wienhues and Cahalan, 1996). Based on analysis of conductance fluctuations, CRAC channels have an extremely small unitary conductance of 24 fS in high [Ca\(^{2+}\)]\(_o\) (Zweifach and Lewis, 1993), but the conductance of CRAC channels carrying Na\(^+\) is ~100 times larger, compatible with a channel mechanism of ion permeation (Lepple-Wienhues and Cahalan, 1996). Under similar ionic conditions, the single-channel conductance of L-type voltage-gated Ca\(^{2+}\) channels is ~300× larger than that of CRAC channels (Zweifach and Lewis, 1993; Hess et al., 1986).

Although CRAC channels and voltage-gated Ca\(^{2+}\) channels differ in their gating behavior and unitary conductance, they share a high degree of divalent selectivity and exhibit similar affinities for Ca\(^{2+}\). This could indicate that both channels share similar structural features necessary for Ca\(^{2+}\) selectivity. Recently, the *Drosophila* trp (transient receptor potential) gene and its mammalian homologs have been proposed to mediate stores-dependent Ca\(^{2+}\) entry, although trp genes expressed in cell lines exhibit different selectivity properties, including rather low selectivity for Ca\(^{2+}\) over monovalent ions compared with the CRAC channel with normal levels of extracellular Ca\(^{2+}\) (Vaca et al., 1994; Zhu et al., 1996; Zitt et al., 1996; reviewed by Clapham, 1996). One motivation for further characterizing the selectivity properties of the CRAC channel is to facilitate identification of the gene encoding this physiologically important channel by comparison with the selectivity properties of expressed candidate genes.

This paper addresses properties of CRAC channels that are related to ion selectivity, rectification, and block: the physical size of the pore, its conduction properties as a function of intracellular pH, the Mg\(^{2+}\) dependence of inward rectification, and the Ca\(^{2+}\) dependence of current. In addition, we show that the decline of monovalent current through CRAC channels can be prevented by reducing the concentration of cytoplasmic protons or Mg\(^{2+}\) ions. We conclude that the CRAC channel is a large pore that achieves selectivity for Ca\(^{2+}\) by selective binding of external Ca\(^{2+}\), with current-voltage (I-V) rectification influenced by internal Mg\(^{2+}\).

**METHODOLOGY**

**Cell Culture**

The human T cell line, Jurkat E6-1 was cultured in RPMI 1640 with 10% fetal calf serum, 1 mM glutamine, and 25 mM HEPES in a 5% CO\(_2\) incubator at 37°C.

**Whole-Cell Recordings**

Patch clamp experiments were performed at room temperature in the standard whole-cell recording configuration (Hamill et al., 1981). Pipettes were pulled from soft glass capillaries (Accucap, 90 Micropipets; Becton Dickinson and Co., Parsippany, NJ), coated with Sylgard (Dow Corning Corp., Midland, MI), and fire polished (Dow Corning Corp., Midland, MI), and fire polished to a resistance of 2–5 MΩ when filled with internal solutions. Membrane currents were recorded using an EPC-9 patch-clamp amplifier (HEKA, Lambrecht, Germany). Data were sampled at a rate of 5–10 kHz and digitally filtered at 0.7 kHz for analysis and display. Fast and slow capacitative transients were canceled by the compensation circuitry of the EPC-9. The membrane capacitance of cells selected for recording was 6.3 ± 1.8 nF (n = 300). Command potentials were corrected for liquid junction potentials. The series resistance (4–10 MΩ) was not compensated. The membrane potential was trimmed at 0 mV, and 200-ms voltage ramps from −120 to +50 mV were delivered every second. Leak currents before activation of CRAC channels were averaged and subtracted from subsequent current records. With Cl\(^{−}\) replacement, input resistances determined before store depletion were >10 GΩ. L_{BRK} was induced by passive Ca\(^{2+}\) store depletion by using 12 mM BAPTA. External solutions were changed by puffer pipettes as described (Lepple-Wienhues and Cahalan, 1996).

**Solutions**

Methanesulfonate was the main anion in the external solution, to reduce the permeability through Cl\(^{−}\) channels (Lewis et al., 1993). For measurement of relative permeabilities, Na\(^+\) in the bathing solution was replaced by alkylated ammonium derivatives (see below). EGTA and HEDTA saturated with Ca\(^{2+}\) were prepared using a pH–metric method (Neher, 1988). The low [Ca\(^{2+}\)]\(_o\) external solution had the following composition (mM): 150 X\(^+\), 150 methane sulfonate, 2 mM EGTA, where X\(^+\) is Na\(^+\), K\(^+\), Li\(^+\), Cs\(^+\), NH\(_4\)\(^+\), methylammonium, dimethylammonium, trimethylammonium, tetramethylammonium (TMA\(^+\)), ethylammonium, isopropylammonium, hydrizine, or N-methyl-D-glucamine (NMG\(^+\)). External solutions containing various [Ca\(^{2+}\)]\(_o\) were buffered with 2 mM HEDTA (1 μM, 10 μM free Ca\(^{2+}\)) or with 2 mM EGTA (1 μM, 0.1 μM free Ca\(^{2+}\)). Nominally divalent-free solution contained 150 mM Na\(^+\) methane sulfonic acid. All solutions contained 10 mM HEPES. The osmolarity was adjusted to
300 mosmol with glucose, and the pH was titrated to pH 7.2. NH₄⁺, methylamine, dimethylamine, trimethylamine, and TMA⁺ were purchased from Aldrich Chemical Co. (Milwaukee, WI); ethylamine, isopropylamine, hydrazine, and methane sulfonic were purchased from Sigma Chemical Co. (St. Louis, MO).

The pipette solution usually contained (mM): 128 Cs aspartate, 10 Cs-HEPES, 12 BAPTA, 0.9 CaCl₂, 3.16 MgCl₂, pH 7.2. In some pipette solutions, Cs⁺ ions were substituted by Na⁺ or NMDG⁺. Solutions titrated to 6.2 and 6.8 sometimes contained 10 mM Tris instead of HEPES. In Mg²⁺-free solutions, MgCl₂ was omitted from the internal solution.

RESULTS

CRAC Channels are Permeable to Monovalent Cations when External Ca²⁺ Is Lowered

In physiological solutions with external Ca²⁺ concentration ([Ca²⁺]₀) in the millimolar range, CRAC channels are highly selective for Ca²⁺ over monovalent cations. However, previous studies have shown that when external divalents are reduced to the micromolar range, Na⁺ ions carry a large, transient inward current through CRAC channels (Hoth and Penner, 1993; Lepple-Wienhues and Cahalan, 1996). In the present study, we activated CRAC channels by dialyzing the cell with BAPTA-buffered low [Ca²⁺]ᵢ solutions ([Ca²⁺]ᵢ₀ = 5 nM) to deplete intracellular Ca²⁺ stores passively. Currents were recorded during 200-ms voltage ramps from −120 to +50 mV delivered every second. In the presence of high [Ca²⁺]ᵢ₀, a small inwardly rectifying Ca²⁺ current (I.CRAC) was induced during dialysis (Fig. 1, A–C). In the absence of external Mg²⁺ and immediately upon reducing [Ca²⁺]ᵢ₀ to 1 µM, a large inwardly rectifying Na⁺ current developed, and then slowly declined over tens of seconds (Fig. 1 D). This current was carried by Na⁺ since it vanished when NMDG⁺ was substituted for Na⁺ in the bath. Similar large monovalent currents were observed in cells dialyzed with Cs⁺, Na⁺, or NMDG⁺ (Fig. 1, A–C), but only if CRAC channels were already activated by Ca²⁺ store depletion during dialysis. The parallel development of monovalent and Ca²⁺ current during the initial phase of CRAC channel activation provides evidence that the monovalent current is carried through CRAC channels, rather than through a nonspecific “leak” (Lepple-Wienhues and Cahalan, 1996). With NMDG⁺ as the main internal cation, the current density measured at −80 mV was 0.8 ± 0.2 pA/pF (n = 4) when the channel...
carried $\text{Ca}^{2+}$ in 20 mM $\text{Ca}^{2+}$, and the peak Na current upon lowering $\text{Ca}^{2+}$ to 1 $\mu$M was $3.7 \pm 0.7 \text{ pA/pF}$ ($n = 4$). The ratio of monovalent to divalent current indicates that the CRAC channel can conduct monovalent ions much more readily than $\text{Ca}^{2+}$ ions. Later in this paper, we show that the measured monovalent to divalent current ratio is even higher if the decline of monovalent current upon $[\text{Ca}^{2+}]_o$ removal is prevented.

Although varying the internal or external monovalent ionic species did not affect the development of the monovalent current, reversal potentials and current magnitudes through CRAC channels depended upon the current-carrying species. At positive potentials, a small outward current was observed in low $[\text{Ca}^{2+}]_o$ using $\text{Cs}^+$- or $\text{Na}^+$-containing internal solutions (Fig. 1, A and B). This outward current was carried by $\text{Cs}^+$ or $\text{Na}^+$ through CRAC channels, since it activated with the same time course as the inward current, was blocked by $\text{La}^{3+}$, and was not present in experiments using NMDG$^+$ in the internal solution (Fig. 1 C). Upon lowering $[\text{Ca}^{2+}]_o$, inward current magnitudes varied substantially depending upon the external species of monovalent cation. For example, $\text{Na}^+$ carried a much larger inward current through CRAC channels than $\text{Cs}^+$ did; the ratio of $\text{Na}^+$ to $\text{Cs}^+$ inward currents was $26 \pm 3$ (SD; $n = 5$), even though the measured reversal potentials were similar and outward $\text{Na}^+$ and $\text{Cs}^+$ currents were of comparable magnitude. NMDG$^+$ inward current could not be detected.

These results verify and extend a previous report (Lepple-Wienhues and Cahalan, 1996) that, under conditions of low $[\text{Ca}^{2+}]_o$, CRAC channels become permeable to monovalent cations, a property shared with volt-
age-gated Ca\(^{2+}\) channels. The permeability of CRAC channels to Na\(^{+}\) and other alkali cations diminishes with time after exposure to low [Ca\(^{2+}\)]\(_o\). The current carried by Na\(^{+}\) immediately after lowering [Ca\(^{2+}\)]\(_o\) is 5- to 10-fold larger than the preceding Ca\(^{2+}\) current. Cs\(^{+}\), although nearly as permeant as Na\(^{+}\) from reversal potential measurements, carries much less inward current than Na\(^{+}\). In the following experiments, we compare the reversal potentials, permeabilities relative to Na\(^{+}\), rectification, and kinetics of the CRAC channel carrying monovalent organic cations.

**Permeability of CRAC Channels to Organic Cations**

We used a series of organic monovalent cations to obtain further information about the selectivity of CRAC channels and to estimate the minimal cross-sectional diameter of the conducting pore. We substituted the organic cations for Na\(^{+}\) in the external solution, measured reversal potentials \(E_X\) and \(E_{Na}\), and calculated the permeability relative to Na\(^{+}\) using the following equation:

\[
E_X - E_{Na} = \frac{RT}{F} \ln \frac{P_X[X]_o}{P_{Na}[Na]_o},
\]

where \(X\) specifies the ion substituted for Na\(^{+}\). To test for possible contaminating “leak” currents, control experiments with four of the test cations (NH\(_4^+\), hydrazine, methylammonium, and dimethylammonium) were performed to ensure that the monovalent current is not observed under “nondepleted” conditions before CRAC channels activate. Again, as with Na\(^{+}\), monovalent current carried by organic cations was measured only if CRAC channels were already open. To establish biionic conditions, we chose Na\(^{+}\) instead of Cs\(^{+}\) as the internal cation because it passes through the CRAC channel more readily and is less permeant through K\(^{+}\) channels than Cs\(^{+}\). Similar results were obtained using internal Cs\(^{+}\) (data not shown). Assuming that internal concentrations remain constant when external solutions are exchanged, the permeability ratio \(P_X/P_{Na}\) for the test cation relative to Na\(^{+}\) can be calculated from the change in reversal potential, \(E_X - E_{Na}\). Since monovalent ions are only permeant through CRAC channels under conditions of low divalence, we performed all experiments with organic monovalent ions in 1 \(\mu\)M [Ca\(^{2+}\)]\(_o\).

The organic cations varied substantially in their permeability through CRAC channels. Fig. 2 demonstrates that increasing the number of methyl groups on NH\(_4^+\) shifted the reversal potentials to the left, indicating re-
duced permeability. The most permeant ion tested was NH$_4^+$ ($P_{NH_4}/P_{Na} = 1.37$; Fig. 2 A), and the least measurably permeant ion was TMA$^+$ ($P_{TMA}/P_{Na} = 0.09$; Fig. 2 F). Ethylammonium (Fig. 2 G) and isopropylammonium (Fig. 2 H) were more permeant than the symmetrical and bulkier TMA$^+$ (Fig. 2 F). With external NMDG$^+$, inward currents were not measurable (Fig. 2 H), suggesting that NMDG$^+$ is not permeant through CRAC channels.

In parallel with the shift of the reversal potential to the left, the magnitude of the inward current decreased. Note that the scales in Fig. 2 vary depending on the cation being tested. Inward ramp currents measured at $-80$ mV carried by NH$_4^+$ or TMA$^+$ differed by a factor of 100. Outward currents carried by Na$^+$ were less affected. A direct comparison of TMA$^+$ and NMDG$^+$ is shown in Fig. 2 J.

Table I and Fig. 3 summarize reversal potentials and permeability ratios for the organic cations tested. The permeability ratios can be used to estimate the diameter of the selectivity filter. The largest ion used, TMA$^+$, has a diameter of 0.55 nm, indicating that the cross-sectional diameter of the conducting path through a CRAC channel at its narrowest region is at least this size. We calculated permeability ratios and estimated the size of the selectivity filter, assuming that steric hindrance determined permeability differences but not the electrostatic interaction between ion channel and cation. The ion channel is considered as a water-filled pore, obeying a simple hydrodynamics equation:

$$P_X/P_{Na} = \kappa \left[1 - \left( \frac{d_{ion}}{d_{pore}} \right)^2 \right],$$

where $P_X/P_{Na}$ is the permeability ratio, $\kappa$ is a proportionality constant, $d_{ion}$ is the diameter of the ion, and $d_{pore}$ is the diameter of the pore (Dwyer et al., 1980; Burnashev et al., 1996). The line drawn in Fig. 3 relates only to the size of the pore but does not include energetic changes associated with the permeation of the ion through the pore. The estimated diameter of the pore is at least 0.58 nm. Although CRAC channels are amazingly selective for Ca$^{2+}$ over monovalent ions in physiologic ion solutions, they become relatively nonselective when [Ca$^{2+}$] is reduced to micromolar levels, indicative of a rather large pore.

**Table I**

<table>
<thead>
<tr>
<th>Ion</th>
<th>$\Delta V$</th>
<th>$P_{X}/P_{Na}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Na$^+$</td>
<td>0 ± 3 (n = 10)</td>
<td>—</td>
</tr>
<tr>
<td>K$^+$</td>
<td>0 ± 3 (n = 3)</td>
<td>1.0</td>
</tr>
<tr>
<td>Li$^+$</td>
<td>0 ± 4 (n = 4)</td>
<td>1.0</td>
</tr>
<tr>
<td>Cs$^+$</td>
<td>0 ± 4 (n = 5)</td>
<td>1.0</td>
</tr>
<tr>
<td>Ammonium</td>
<td>+8 ± 2 (n = 6)</td>
<td>1.37</td>
</tr>
<tr>
<td>Methylammonium</td>
<td>-11 ± 4 (n = 9)</td>
<td>0.65</td>
</tr>
<tr>
<td>Dimethylammonium</td>
<td>-33 ± 3 (n = 9)</td>
<td>0.28</td>
</tr>
<tr>
<td>Trimethylammonium</td>
<td>-52 ± 1 (n = 3)</td>
<td>0.13</td>
</tr>
<tr>
<td>Tetramethylammonium</td>
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<td>0.09</td>
</tr>
<tr>
<td>Ethylammonium</td>
<td>-15 ± 2 (n = 8)</td>
<td>0.56</td>
</tr>
<tr>
<td>Isopropylammonium</td>
<td>-22 ± 1 (n = 3)</td>
<td>0.42</td>
</tr>
<tr>
<td>Hydrazine</td>
<td>-6 ± 2 (n = 4)</td>
<td>0.79</td>
</tr>
<tr>
<td>N-methyl-$\alpha$-glucamine</td>
<td>-120 (n = 6)</td>
<td>&lt;0.0085</td>
</tr>
</tbody>
</table>

*Figure 3.* Estimation of the pore size of CRAC channels. Permeability ratios $P_{X}/P_{Na}$ of ammonium and its methylated derivatives were calculated from measured reversal potentials using Eq. 1 and plotted as a function of the geometric mean diameter of the organic cation. The line represents a fits to Eq. 2. The extrapolated diameter of the pore is 0.58 nm.

**pH Dependence of Kinetics and Current Magnitude**

After the development of $I_{CRAC}$ in 20 mM [Ca$^{2+}$]$_o$, exposure to 1 µM [Ca$^{2+}$]$_o$ produced an inward Na$^+$ current that declined towards zero within several tens of seconds (Fig. 1 D). For simplicity, we will refer to this decline of monovalent current as “inactivation.” In contrast, with NH$_4^+$ and related organic cations in the external solution, inward and outward currents through CRAC channels were sustained (Fig. 2 B). Ammonium and related cations exist in equilibrium with neutral molecules that may cross the membrane in their uncharged form and subsequently be reprotonated, resulting in alkalinization of the internal solution. Therefore, we investigated the effects of varying internal pH on the kinetics of the Na$^+$ current upon valent withdrawal. We controlled intracellular pH (pHi) by dialyzing the cell with internal solution buffered between pH 6.2 and 8.2. The current time courses and representative I-V curves are illustrated in Fig. 4. Increasing pH did indeed reduce the inactivation of Na$^+$ current through CRAC channels, as predicted from the hypothesis that NH$_3$ crossing the membrane alkalinized the cy-
toplasmic side of the membrane (Fig. 4 A). Conversely, decreasing pHi accelerated the rate of decline (Fig. 4 E), and also reduced the current amplitude.

To assess the effects of intracellular pH on current amplitude and kinetics, we measured current amplitudes and inactivation rates over a range of pipette pH values, using either HEPES- or Tris-buffered pipette solutions, with identical results. The time course of inactivation upon lowering [Ca$^{2+}$]o cannot be fitted well by a single exponential function. For a semi-quantitative index of the inactivation rate, the value of the Na$^{+}$ current through CRAC channels 50 s after the maximum activation (I$_{50}$), relative to the maximum Na$^{+}$ current (I$_{max}$) through CRAC channels, was determined and plotted as a function of pHi in Fig. 5 A. This ratio increased from 0.15 ± 0.07 (n = 8) at pH 6.2 to 0.49 ± 0.17 (n = 6) at pH 8.2. We conclude that raising intracellular pH can partially inhibit inactivation with an apparent pKa value above 8. In addition to the effect on inactivation of monovalent current through CRAC channels, pHi modulated the magnitude of the current (Fig. 5 B). In agreement with the assumption that Na$^{+}$ and Ca$^{2+}$ pass through the same CRAC channels, Na$^{+}$ and Ca$^{2+}$ current were equally reduced by lowering pHi. The Ca$^{2+}$ current density was reduced from 1.6 ± 0.2 pA/pF at pH 8.2 (n = 6) to 0.3 ± 0.1 pA/pF at pH 6.2 (n = 10). The pH$_i$ effect on current magnitude was fitted with an apparent pK$_a$ of 6.8 (Fig. 5 B). I-V shapes were similar with high and low pH$_i$. Both pK$_a$ values could be influenced by the fact that true current magnitudes may be underestimated due to rapid compo-

![Figure 4](image-url) ![Figure 5](image-url)
ments of inactivation that may occur during the solution exchange at low or normal intracellular pH. We conclude that there are at least two distinct sites where intracellular protons modulate the CRAC channel, one favoring inactivation and the other decreasing the magnitude of current through the channel.

**Mg**\(^{2+}\)-dependent Block of CRAC Channels

Both divalent and monovalent currents through CRAC channels exhibit inward rectification. In several other channel types, Mg\(^{2+}\) has been shown to block open channels in a voltage-dependent manner and contribute to rectification (Nowak et al., 1984; Vandenberg, 1987; Pusch, 1990; reviewed by Nichols and Lopatin, 1997; Bara et al., 1993). To test whether intracellular Mg\(^{2+}\) modulates rectification in CRAC channels, we introduced Mg\(^{2+}\)-free internal solution into cells bathed in nominally Ca\(^{2+}\)- and Mg\(^{2+}\)-free external solution. Large outward currents developed exactly in parallel with inward CRAC currents, as shown in Fig. 6 A. Fig. 6 B illustrates the I-V shape with very large outward currents that normally are not observed. Although Cs\(^{+}\) normally conducts poorly through CRAC channels, even when [Ca\(^{2+}\)]\(_i\) is lowered (e.g., Fig. 1, A and E), several lines of evidence point to the large outward current being carried by Cs\(^{+}\) through CRAC channels when cytoplasmic Mg\(^{2+}\) is lowered. First, the outward and inward currents activate with the same time course as stores are passively depleted, as illustrated by the scaled records in Fig 6 A. Second, in seven experiments with variable current amplitudes, relative current amplitudes at positive and negative potentials were highly consistent (Fig. 6, legend), reflecting a consistent I-V shape. If leak currents were responsible for the outward currents, the I-V shapes should vary considerably from cell to cell. Instead, a characteristic sigmoid shape was observed in cells without Mg\(^{2+}\) inside, instead of the normal inwardly rectifying I-V observed when Mg\(^{2+}\) was present. Third, when NMDG\(^{+}\) was used instead of Cs\(^{+}\) inside the pipette, the outward currents were much smaller (Fig. 7, E and F). Fourth, both outward and inward currents were blocked by addition of La\(^{3+}\) (300 \(\mu\)M, data not shown). In addition, when Ca\(^{2+}\) was included in the external solution, both the inward and outward currents were reduced (see below). These results demonstrate that when Mg\(^{2+}\) is excluded from the internal solution, both inward and outward monovalent currents are carried readily through CRAC channels when external Ca\(^{2+}\) is lowered. We conclude that internal Mg\(^{2+}\) normally blocks outward current through CRAC channels.

In addition to altering I-V characteristics of CRAC channels, removal of internal Mg\(^{2+}\) eliminates the slow decline of monovalent current through CRAC channels, a result qualitatively similar to the effect of increasing pH\(_i\). Both inward and outward monovalent currents were sustained upon divalent removal. Similar results were obtained in several experiments with 100 \(\mu\)M [Mg\(^{2+}\)].

Fig. 7 gives an overview of Ca\(^{2+}\), Na\(^{+}\), and Cs\(^{+}\) current through CRAC channels using cells dialyzed with or without internal Mg\(^{2+}\). Recordings from a cell dialyzed with Mg\(^{2+}\)-free external solution (Fig. 7, A and B) revealed inward currents with magnitudes that varied in the sequence Na\(^{+}\) > Ca\(^{2+}\) > Cs\(^{+}\) at \(-80\) mV. At \(+30\) mV, small outward currents were carried by Cs\(^{+}\). Despite the huge difference in the magnitude of the inward Na\(^{+}\) and Cs\(^{+}\) currents, both had nearly the same reversal potential (Fig. 7 B). Thus, with Mg\(^{2+}\) inside, Cs\(^{+}\) carries current poorly, despite being very permeant judging from reversal potentials. However, when Mg\(^{2+}\) was excluded from the internal solution, inward and outward current magnitudes were larger, the currents were sustained, and the difference between Na\(^{+}\) and Cs\(^{+}\) current magnitudes was erased (Fig. 7, C and D with Cs\(^{+}\) aspartate inside, and E procedure was done in seven cells, with a consistent scaling factor of 5.9 \(\pm\) 0.3 (mean \(\pm\) S.D.). (B) Representative current traces taken from the cell shown in A before and activation of CRAC channels with Cs\(^{+}\) aspartate, 0 Mg\(^{2+}\) inside.

![Figure 6](https://example.com/fig6.png)

**Figure 6.** Removal of internal Mg\(^{2+}\) permits large, sustained inward and outward monovalent currents through CRAC channels. Whole-cell dialysis with Cs\(^{+}\) aspartate, zero Mg\(^{2+}\) pipette solution. The bath contained nominally Ca\(^{2+}\)-free external solution. (A) Development of outward currents at \(+30\) mV and inward currents at \(-80\) mV. The inward current at \(-80\) mV was inverted, scaled, and superimposed (light trace) to the current at \(+30\) mV for comparison. This scaling procedure was done in seven cells, with a consistent scaling factor of 5.9 \(\pm\) 0.3 (mean \(\pm\) S.D.). (B) Representative current traces taken from the cell shown in A before and activation of CRAC channels with Cs\(^{+}\) aspartate, 0 Mg\(^{2+}\) inside.
The inward current magnitudes varied in the sequence Na$^+$ > Ca$^{2+}$, Cs$^+$. We conclude that internal Mg$^{2+}$ affects channel rectification and the relative ease with which Na$^+$ and Cs$^+$ carry current through the CRAC channel. These results can be qualitatively explained by competition between Mg$^{2+}$ bound to the channel and Na$^+$ moving inward, with Na$^+$ having a greater ability than Cs$^+$ to clear the channel of bound Mg$^{2+}$, resulting in large Na$^+$ currents but small Cs$^+$ currents when Mg$^{2+}$ is present. Upon removal of internal and external divalents, Na$^+$ and Cs$^+$ carry current equally well.

Fig. 8 provides evidence that internal Mg$^{2+}$ also affects the I-V shape of Ca$^{2+}$ current through CRAC channels. With Mg$^{2+}$ inside, Ca$^{2+}$ current through CRAC channels rectifies inwardly (Fig. 8 A). Perfusion of the cell with Mg$^{2+}$-free internal solution results in a more linear I-V curve through CRAC channels (Figs. 1 and 8 B).

Mg$^{2+}$ removal from the inside permits large outward
as well as inward monovalent currents, thus enabling the block of monovalent currents by Ca\(^{2+}\) to be evaluated at positive as well as negative membrane potentials (Fig. 9). At the beginning of the recording, the cell was superfused with 20 mM Ca\(^{2+}\). Under this condition, only an inward Ca\(^{2+}\) current was detectable. Decreasing the external Ca\(^{2+}\) concentration to 100 \(\mu\)M revealed an inward current at −80 mV and an outward current at +30 mV. Decreasing [Ca\(^{2+}\)]\(_i\), further increased both inward and outward currents. Current magnitudes were normalized to the maximum current at or below 1 \(\mu\)M Ca\(^{2+}\) and fitted to a simple equation based upon the assumption that binding of a single Ca\(^{2+}\) blocks inward Na\(^{+}\) current. At −80 mV the \(K_d\) value was 5 \(\mu\)M, while at +30 mV the \(K_d\) was 100 \(\mu\)M. The results suggest that the apparent \(K_d\) may be influenced by the distance that a Ca\(^{2+}\) ion must travel to bind to its site within the membrane (Woodhull, 1973).

When [Mg\(^{2+}\)]\(_o\), or [Ca\(^{2+}\)]\(_o\), were varied, corresponding I-V shapes suggested voltage-dependent block of monovalent current through CRAC channels by divalent ions. Mg\(^{2+}\) preferentially blocks outward current from the inside (Fig. 7, A and D), and Ca\(^{2+}\) preferentially blocks inward current from the outside (Fig. 9 B). Assuming that Ca\(^{2+}\) or Mg\(^{2+}\) may block in a voltage-dependent manner from the outside and inside, respectively, the voltage dependence of block can be assessed by dividing monovalent ramp currents in pairs of cells with or without the divalent blocking ion. To assess the steepness of block, I-V ratios were fitted to a Boltzmann distribution,

\[
\frac{I_x}{I} = A/\left\{1 + \exp \left[ \frac{(E_h - E)}{k} \right] \right\},
\]

where \(I_x\) is the current with internal Mg\(^{2+}\) or external Ca\(^{2+}\) relative to the unblocked current \(I\) in the absence of divalents, \(A\) is the maximum current ratio, \(E_h\) is the voltage at which half of the channels are blocked, and \(k\) is the steepness of the block. Fig. 10 A shows an example of an I-V ratio, illustrating Mg\(^{2+}\) block from the inside. Mg\(^{2+}\) induced inward rectification by blocking outward currents at positive potentials. The steepness factor \(k\) was −13.3 ± 1.5 mV (\(n = 3\)), suggesting the existence of a Mg\(^{2+}\) binding site within the membrane electric field. In a simple blocking model, the factor \(k\) is equivalent to RT/\(z\delta F\), where R, T, and F have their usual meanings, \(z\) is the charge of the blocking ion, and \(\delta\) is the fractional distance across the electric field that the ion would travel to reach its binding site (Woodhull, 1973). The mean \(z\delta\) product for Mg\(^{2+}\) block of 1.88 would correspond to a single Mg\(^{2+}\) ion moving across 94% of the membrane electric field from the inside, or half this distance if two Mg\(^{2+}\) ions move.

To analyze the voltage dependence of external Ca\(^{2+}\) block, we calculated ratios of I-V curves when [Ca\(^{2+}\)]\(_o\), was varied from 1 to 100 \(\mu\)M (Fig. 10 B). The steepness factor \(k\) was 15.4 ± 0.9 mV (\(n = 5\)), corresponding to a mean \(z\delta\) product of 1.62 and an “equivalent electrical
distance” of 81% across the membrane electric field from the outside. Thus, both Mg^{2+} from the inside and Ca^{2+} from the outside block monovalent current through CRAC channels in a steeply voltage-dependent manner.

**Discussion**

We first present a cartoon to illustrate several properties of CRAC channels described in this paper. Fig. 11 depicts three distinct sites, including a selectivity filter, a site facing the cytoplasm that regulates conduction, and a basic residue that regulates inactivation. In addition, Ca^{2+}, Mg^{2+}, Na^{+}, Cs^{+}, and H^{+} ions are shown as they interact with these sites.

Our experiments demonstrate that CRAC channels carry large and sustained inward and outward monovalent currents when internal and external divalent ion concentrations are reduced to the micromolar level. In the absence of external divalents, the CRAC channel displays a large, weakly selective pore that discriminates among organic cations by molecular sieving. Internal Mg^{2+} appears to exert three effects upon the CRAC channel. First, Mg^{2+} as an internal voltage-dependent blocking ion, sculpts the I-V relationship by preferentially reducing the outward current, thereby inducing inward rectification. Second, Mg^{2+} modulates the relative currents carried by different monovalent cations, perhaps by competing with specific monovalent ions, making the current for Cs^{+} small compared with that for Na^{+}. Third, Mg^{2+} is required for inactivation of monovalent current upon lowering [Ca^{2+}]_{o}. Raising pH_{i} exerts effects similar to the removal of internal Mg^{2+}, whereas reducing pH_{i} inhibits divalent or monovalent current through the CRAC channel. External Ca^{2+} regulates inactivation.
lates channel selectivity by selective binding. As \([\text{Ca}^{2+}]_o\) is raised in the micromolar range, monovalent currents are reduced as \(\text{Ca}^{2+}\) blocks in a voltage-dependent manner within the pore. As \([\text{Ca}^{2+}]_o\) is raised further into the millimolar range, \(\text{Ca}^{2+}\) moves selectively across the channel, but \(\text{Ca}^{2+}\) currents are small compared with the monovalent currents.

**Sizing the CRAC Channel**

What is the minimal size of the conducting pore? To obtain an estimate, we reduced extracellular \([\text{Ca}^{2+}]\) to permit monovalent conduction and measured reversal potentials using a series of nine organic compounds as probes of varying size (Figs. 1 and 2; Table I). Our results show that ammonium derivatives exhibit a well-ordered permeability sequence depending upon ionic size, ranging from \(\text{NH}_4^+\) with \(P_{\text{NH}_4^+}/P_{\text{Na}} = 1.37\) to \(\text{TMA}^+\) with \(P_{\text{TMA}}/P_{\text{Na}} = 0.09\). The largest permeant cation used, \(\text{TMA}^+\), has a diameter of 0.55 nm, indicating that the pore of the CRAC channel is at least this size. Assuming only volume exclusion, and neglecting the friction between the ion channel and the organic cation and the viscosity of the medium surrounding the ion (Dwyer et al., 1980), the extrapolated diameter of the pore is 0.58 nm (Fig. 3). We attempted to measure current with \(\text{NMDG}^+\), an asymmetric molecule with a minimal cross-section corresponding to \(0.5 \times 0.64\) nm. With external \(\text{NMDG}^+\), inward currents were not detected above -120 mV, placing the value of \(P_{\text{NMDG}}/P_{\text{Na}} < 0.0085\). Although highly selective for \(\text{Ca}^{2+}\) and with an extremely small unitary conductance, the CRAC channel pore is physically large enough to accommodate molecules up to roughly 0.6 nm in diameter.

**Inactivation of Monovalent Current**

What type of process accounts for the decline (inactivation) of monovalent current after removal of extracellular \(\text{Ca}^{2+}\)? Our results indicate that both protons and \(\text{Mg}^{2+}\) ions in the cytoplasm are required (Figs. 4–7). In low \(\text{Ca}^{2+}\) Ringer, the \(\text{Na}^+\) current through CRAC channels is transient, gradually declining over tens of seconds with at least two exponential components. For simplicity, but without mechanistic implication, we refer to the decline of monovalent current as “inactivation.” After inactivation and upon readdition of extracellular \(\text{Ca}^{2+}\), the \(\text{Ca}^{2+}\) current increases with complex kinetics, including a very rapid initial phase followed by a slower increase that has been termed “\(\text{Ca}^{2+}\)-dependent potentiation” (Zweifach and Lewis, 1996). In the course of examining the permeability of the channel to organic cations, we noticed that inactivation was greatly reduced when \(\text{NH}_4^+\) was substituted for \(\text{Na}^+\). We hypothesized that movement of neutral \(\text{NH}_4\) across the membrane might raise the cytoplasmic pH, despite the presence of intracellular buffer, and that alkalinization of the cytoplasm near the CRAC channel could prevent inactivation. Consistent with this hypothesis, we found that raising internal pH by dialysis of pH-buffered solutions reduced the extent of inactivation; the pKa for this kinetic effect is above 8 (Figs. 4 and 5).

In addition, we found that the inactivation process requires internal \(\text{Mg}^{2+}\) (Figs. 6 and 7). With internal \(\text{Mg}^{2+}\) reduced to 100 \(\mu\)M or lower, monovalent currents were sustained, regardless of the current carrier or the direction of current flow. Inactivation and the reverse process of \(\text{Ca}^{2+}\)-dependent potentiation may be due to the binding and unbinding, respectively, of \(\text{Mg}^{2+}\) ions to a site or sites associated with the CRAC channel. Let us assume that the binding site for \(\text{Mg}^{2+}\) is located at a selectivity filter region near the external membrane surface, consistent with strong voltage-dependent block shown in Fig. 10. Upon readdition, \(\text{Ca}^{2+}\) would have to compete with \(\text{Mg}^{2+}\) to pass through the channel. This competition may be reflected in the time course of calcium-dependent potentiation (see Figs. 1, 4, and 7A). External \(\text{Ni}^{2+}\) can substitute for \(\text{Ca}^{2+}\) in mediating potentiation (see Fig. 9 in Zweifach and Lewis, 1996). \(\text{Ni}^{2+}\) may bind with low affinity to the same binding site as \(\text{Mg}^{2+}\), dislodging \(\text{Mg}^{2+}\) but allowing \(\text{Ca}^{2+}\) to carry current immediately. At depolarized potentials, \(\text{Mg}^{2+}\) binds more strongly, consistent with the reduced extent of calcium-dependent potentiation at depolarized potentials (see Fig. 4 in Zweifach and Lewis, 1996). To summarize, \(\text{Ca}^{2+}\)-dependent potentiation may arise from competition between internal \(\text{Mg}^{2+}\) and external \(\text{Ca}^{2+}\) for a site near the outer membrane surface.

**Mechanism of Inward Rectification and Block by Divalent**

What controls inward rectification of \(\text{Ca}^{2+}\) and monovalent current through CRAC channels? Is it an intrinsic property of the conducting pore, or do blocking ions contribute? There is ample precedent for \(\text{Mg}^{2+}\) ions playing a role in rectification of other channels. For example, cytoplasmic \(\text{Mg}^{2+}\) causes fast voltage-dependent block in inward rectifying \(K^+\) and \(\text{Na}^+\) channels (Vandenberg, 1987; Pusch, 1990). Inward rectification through CRAC channels is observed whether the inward current is carried by \(\text{Ca}^{2+}\) or by \(\text{Na}^+\). Our results indicate that internal \(\text{Mg}^{2+}\) blocks outward current through CRAC channels. Removal of \(\text{Mg}^{2+}\) ions from the pipette solution resulted in much larger outward monovalent currents through CRAC channels, consistent with a voltage-dependent \(\text{Mg}^{2+}\) block mechanism for rectification. In the simplest model for voltage-dependent block (Woodhull, 1973), an ion is attracted towards or repelled from a site within the membrane according to the electric field. By fitting a Boltzmann relation to ratios of the I-V relations with and without internal \(\text{Mg}^{2+}\), we identify a putative \(\text{Mg}^{2+}\)-binding site
within the membrane. The steepness of block (zδ = 1.88) would be equivalent to one Mg$^{2+}$ moving almost completely across the membrane from the inside (Fig. 10 A).

Ca$^{2+}$ blocks the monovalent current through CRAC channels at an externally accessible site, with affinity in the micromolar range (Figs. 9 and 10). With the inactivation process and block of outward currents eliminated by removal of internal Mg$^{2+}$, we examined the voltage dependence of Ca$^{2+}$ block over a wide range of potentials and found that the apparent affinity is reduced at positive potentials. At −80 mV, Ca$^{2+}$ blocks the inward current with a $K_d$ of 5 μM, but at +30 mV the $K_d$ was 100 μM. Our $K_d$ value of 5 μM at −80 mV (internal Mg$^{2+}$ removed) is in excellent agreement with the $K_d$ value for Ca$^{2+}$ block determined previously (4 μM at −80 mV, with internal Mg$^{2+}$ present; Lepple-Wienhues and Cahalan, 1996), suggesting that internal Mg$^{2+}$ does not alter the affinity of external Ca$^{2+}$ for the blocking site within the channel. To examine the voltage dependence of Ca$^{2+}$ block in greater detail, we analyzed I-V shapes at varying [Ca$^{2+}$]$_o$ levels in the absence of internal Mg$^{2+}$. Block by external Ca$^{2+}$ is steeply voltage dependent (zδ = 1.62), corresponding to an externally accessible site located at an equivalent electrical distance of 81% across the membrane from the outside.

Both Mg$^{2+}$ block from the inside and Ca$^{2+}$ block from the outside are steeply voltage dependent. The steepness of block suggests movement of a divalent ion most of the way across the membrane to a blocking site. How can we reconcile the existence of steeply voltage-dependent block by internal Mg$^{2+}$ and external Ca$^{2+}$? Are there two distinct sites on opposite sides of the membrane electric field where divalent ions can bind? Alternatively, the voltage dependence would also be compatible with a single site or sites (the selectivity filter?) located midway through the channel that can attract two divalent ions, with Mg$^{2+}$ or Ca$^{2+}$ able to bind to this site, gaining access from opposite sides of the membrane. Finally, we cannot yet be certain whether the internally accessible Mg$^{2+}$ binding site(s) controlling rectification, relative Cs$^+$ conductance, and inactivation are one and the same. It is uncertain whether a Mg$^{2+}$ ion can function as the inactivation gate or instead alters the access to an inactivated conformation of the protein.

**Permeation Properties of CRAC and Voltage-gated Ca$^{2+}$ Channels**

Our data permit a detailed comparison of ion permeation in two very different types of Ca$^{2+}$-selective ion channels, as summarized in Table II. Although entirely different gating mechanisms are responsible for channel activation (depolarization vs. depletion of intracellular Ca$^{2+}$ stores), several similarities and some interesting differences exist. Upon reducing [Ca$^{2+}$]$_o$, to micromolar levels, voltage-gated Ca$^{2+}$ channels in various

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**Table II**

**Ion Permeation in Voltage-gated Ca$^{2+}$ and CRAC Channels**

<table>
<thead>
<tr>
<th>Property</th>
<th>Voltage-gated</th>
<th>CRAC</th>
</tr>
</thead>
<tbody>
<tr>
<td>$K_d$ (Ca$^{2+}$ block)</td>
<td>0.7 μM*</td>
<td>4; 5 μM§§</td>
</tr>
<tr>
<td>Voltage dependence of $K_d$</td>
<td>Increases at negative $E$‡</td>
<td>Decreases at negative $E$</td>
</tr>
<tr>
<td>Unitary Ca$^{2+}$ conductance</td>
<td>8 pS§</td>
<td>24 pS**</td>
</tr>
<tr>
<td>Unitary Na$^+$ conductance</td>
<td>85 pS§</td>
<td>2.6 pS§</td>
</tr>
<tr>
<td>Monovalent conductance sequence</td>
<td>Na$^+$ &gt; Li$^+$</td>
<td>Na$^+$ &gt; Li$^+$ = K$^+$ = Rb$^+$ &gt; Ca$^{2+}$</td>
</tr>
<tr>
<td>Monovalent permeability sequence</td>
<td>Li$^+$ &gt; Na$^+$ &gt; K$^+$ &gt; Ca$^{2+}$</td>
<td>Na$^+$ &gt; Li$^+$ = K$^+$ = Ca$^{2+}$</td>
</tr>
<tr>
<td>Divalent conductance sequence</td>
<td>Ba$^{2+}$ &gt; Ca$^{2+}$ = Sr$^{2+}$</td>
<td>Ca$^{2+}$ &gt; Ba$^{2+}$ = Sr$^{2+}$ &gt; &gt; Mg$^{2+}$</td>
</tr>
<tr>
<td>Divalent permeability sequence</td>
<td>Ca$^{2+}$ &gt; Sr$^{2+}$ &gt; Ba$^{2+}$ &gt; &gt; Mg$^{2+}$</td>
<td>?</td>
</tr>
<tr>
<td>Conductance sequence affected by Mg$^{2+}$,</td>
<td>?</td>
<td>Yes, Na$^+$ &gt; Ca$^{2+}$</td>
</tr>
<tr>
<td>Rectification affected by Mg$^{2+}$,</td>
<td>Yes, inward rectification**</td>
<td>Yes, inward rectification</td>
</tr>
<tr>
<td>Pore size</td>
<td>0.6 nm*</td>
<td>0.6 nm</td>
</tr>
<tr>
<td>pK$_a$ (external pH block)</td>
<td>8.5‡</td>
<td>8.2‡‡</td>
</tr>
<tr>
<td>pK$_a$ (internal pH block)</td>
<td>6.9*</td>
<td>6.8</td>
</tr>
</tbody>
</table>

permeation in voltage-gated Ca\(^{2+}\) channels is revealed. The relationship between current and the shape of the relationship between current and \([Ca^{2+}]_o\), as Table II illustrates diagrammatically. With \([Ca^{2+}]_o\) in the micromolar range, the \(K_d\) values for block of monovalent current are very similar, suggesting a site with a similar energy well for both channels. However, as \([Ca^{2+}]_o\) is elevated and Ca\(^{2+}\) ions begin to carry significant current, current increases much more steeply for voltage-gated than for CRAC channels.

Under similar ionic conditions, the single-channel conductance of L-type voltage-gated Ca\(^{2+}\) channels is 300\(\times\) larger than that of CRAC channels. The relative ease with which Ca\(^{2+}\) moves through the voltage-gated Ca\(^{2+}\) channel may also account for the difference in voltage dependence for Ca\(^{2+}\) block. In the voltage-gated Ca\(^{2+}\) channel, hyperpolarization increases the \(K_d\) for Ca\(^{2+}\) block, perhaps because Ca\(^{2+}\) has a greater tendency to go through the channel rather than simply blocking at negative potentials (Lansman et al., 1986). In CRAC channels, the \(K_d\) for Ca\(^{2+}\) block decreases as the membrane potential is made more negative, as one would expect for simple voltage-dependent block, perhaps also a consequence of a higher barrier for movement through the membrane.

The ability of voltage-gated Ca\(^{2+}\) channels to carry Ca\(^{2+}\) current is thought to involve interactions between two or more Ca\(^{2+}\) ions inside the channel. Early energy barrier models for Ca\(^{2+}\) channel permeation depicted two distinct sites with repulsion between bound Ca\(^{2+}\) ions (Hess and Tsien 1984; Almers and McCleskey, 1984). Recent molecular evidence favors a single site consisting of a ring of glutamates contributed by each of four Ca\(^{2+}\) channel domains (Ellinor et al., 1995). The Ca\(^{2+}\) binding affinity of the glutamate ring may be altered by an approaching Ca\(^{2+}\) ion, as proposed before molecular identification of the site (Armstrong and Neyton, 1991). Regardless of mechanistic details, interactions between Ca\(^{2+}\) ions would facilitate Ca\(^{2+}\) influx. We propose that a similar mechanism operates in CRAC channels, but that the repulsive interaction between Ca\(^{2+}\) ions is reduced, or that the overall energy barrier is higher than in the voltage-gated Ca\(^{2+}\) channel. This would account for the shallower I-[Ca\(^{2+}\)]_o relation, the lower single channel conductance, and the difference in voltage dependence for Ca\(^{2+}\) block in CRAC channels.

**Conductance and Permeability Sequences**

As summarized in Table II, CRAC channels under low divalent conditions exhibit differences in permeability sequences (defined from reversal potentials) and conductance sequences (from current magnitudes) among the alkali metal ions Na\(^{+}\), K\(^{+}\), Li\(^{+}\), and Cs\(^{+}\). In addition, the conductance sequence and rectification of the CRAC channel can be modulated by internal Mg\(^{2+}\) (Fig. 7). Cs\(^{+}\) represents the clearest anomaly. Although
highly permeant based upon reversal potential measurements, $Cs^+$ carries inward current very poorly with internal $Mg^{2+}$ present. In terms of Eyring rate theory and simple barrier models for permeation, this difference can be accounted for by $Cs^+$ ions having a deeper energy well to traverse, but similar energy barriers, relative to $Na^+$. In other words, $Cs^+$ may bind more tightly to a site within the channel, resulting in smaller current magnitudes. In contrast, with $Mg^{2+}$ removed, $Cs^+$ and $Na^+$ were equally effective in carrying inward current. We envision a competitive interaction between $Mg^{2+}$ bound at or near the selectivity filter and $Na^+$ as it approaches the site from the outside. In this view, $Na^+$, but not $Cs^+$, would be able to compete with $Mg^{2+}$ and move through the channel. An alternative view would be that $Mg^{2+}$ binding allosterically affects the relative $Na^+$ and $Cs^+$ affinities, making $Cs^+$ sticky by lowering an energy well. These proposals are amenable to further experimental tests. $Mg^{2+}$ does not carry appreciable currents through the CRAC channel; for now we assume that it cannot go through, although its presence inside the channel can regulate the relative ability of $Cs^+$ to carry inward current compared with $Na^+$.

**Rectification and Block by Internal $Mg^{2+}$**

Internal $Mg^{2+}$ blocks $Ca^{2+}$ and monovalent $Na^+$ and $Li^+$ current through voltage-gated $Ca^{2+}$ channels (Agus and Morad, 1991; Kuo and Hess, 1993). Block of the monovalent current is strongly voltage dependent, resulting in inward rectification (Kuo and Hess, 1993). To account for the strong voltage dependence of the on-rate (e-fold increase per $\sim$15 mV), a high affinity binding site for $Mg^{2+}$ close to the external mouth of the pore was proposed (Kuo and Hess, 1993). Internal $Mg^{2+}$ is responsible for inward rectification of CRAC channels, with an e-fold increase in block per 13 mV also suggesting a binding site close to the outside. Therefore, CRAC and voltage-gated $Ca^{2+}$ channels may exhibit a similar energy profile for $Mg^{2+}$ block inside the pore.

**Pore Size**

When probed with organic cations of varying size, both voltage-gated and CRAC channels are revealed to be large, nonselective cation pores of $\sim$0.6 nm in dimension. Even TMA$^+$ can be accommodated and carries a measurable current in both channels. Unitary $Ca^{2+}$ currents of voltage-gated $Ca^{2+}$ channels are estimated to be 300-fold larger than in CRAC channels, despite the similarity in pore dimension. $Ca^{2+}$ and $Na^+$ have ionic radii of 0.099 and 0.095 nm, respectively. Unlike the molecular sieving of organic compounds with varying size, selectivity between $Ca^{2+}$ and $Na^+$ must arise as a result of interactions between the ion, the channel, and water molecules. $Ca^{2+}$ (normally) or $Na^+$ (if $[Ca^{2+}]_i$ is low) would be able to permeate with at least one associated water molecule, and multiple ions would be able to fit into the large selectivity filter region.

**Modulation by pH**

CRAC channels and voltage gated $Ca^{2+}$ channels have similar sensitivities to external and internal pH, as summarized in Table II. Our data indicate that $pH_i$ modulates current magnitudes through CRAC channels, as well as the rate of inactivation. Current magnitudes for both $Ca^{2+}$ and monovalent currents are reduced as $pH_i$ is lowered, with a $pK_a$ of 6.8 suggesting a histidine; no voltage dependence was observed. Reducing $pH_i$ has been reported to reduce $Ca^{2+}$ current through voltage-gated $Ca^{2+}$ channels with a similar $pK_a$ (Kaibara and Kameyama, 1988; Klöckner and Isenberg, 1994). The rate of CRAC channel inactivation of monovalent current was accelerated by lowering $pH_i$, with a $pK_a$ of $>8$, suggesting a separate site of action. External pH has previously been shown to alter current magnitudes in both CRAC and voltage-gated $Ca^{2+}$ channels. Malayev and Nelson (1995) reported that raising extracellular pH increases the magnitude of CRAC channels in macrophages with a $pK_a$ of 8.2. In voltage-gated $Ca^{2+}$ channels at the single-channel level with $Na^+$ as a charge carrier, increasing external pH increases the relative frequency of high conductance substates with a similar $pK_a$ of $\sim$8.5 (Chen et al., 1996). A possible target is the ring of glutamate residues within the channel, because mutation to glutamine made the channel behave as if it were protonated. Although glutamate has a $pK_a$ of $\sim$4.4, it was suggested for L-type $Ca^{2+}$ channels that multiple hydrogen-bonded carboxylates may have a much higher $pK_a$ than unpaired carboxylates (Chen et al. 1996). Assuming similar glutamate residues in the pore of CRAC channels, protonation of these amino acids could affect the currents through CRAC channels.

**Possible Relation of CRAC Channels to TRP**

Recently, homologues of the *Drosophila trp* gene have been identified in human (Wes et al., 1995; Birnbaumer et al., 1996; Zhu et al., 1996; Zitt et al., 1996). TRPC1A shares with CRAC channels the property of being activated by depletion of $Ca^{2+}$ stores, but in contrast to CRAC channels, TRP channels are nonselective cation channels (Hardie and Minke 1992; Phillips et al., 1992; Hu et al., 1994; Vaca et al., 1994; Zitt et al., 1996). In the present study, we showed that CRAC channels are basically nonselective cation channels and obtain their $Ca^{2+}$ selectivity by their affinity to $Ca^{2+}$. Therefore, CRAC channels may represent $Ca^{2+}$-selective variants of TRP channels. We anticipate that the biophysical characterization of permeation, rectifica-
tion, and block will be of value in identifying candidate genes encoding the CRAC channel.

Significance of I-V Shape and I-[Ca^{2+}]_o for T-Cell Activation

The shape of the CRAC channel I-V relation is of considerable importance for [Ca^{2+}]_i signaling, gene expression, and proliferation in T lymphocytes. In contrast to Ca^{2+} influx through voltage-gated Ca^{2+} channels, Ca^{2+} current through CRAC channels is reduced by membrane depolarization. Membrane depolarization resulting from elevated levels of external K+ or by application of specific K+ channel blockers inhibits T cell activation and Ca^{2+} signaling indirectly by reducing Ca^{2+} influx through CRAC channels (reviewed in Lewis and Cahalan, 1995). The inhibition is stronger than one might expect simply from the change in driving force E_m - E_{Ca} because the I-V relation is not linear. To obtain an estimate of the Ca^{2+} influx at the resting potential compared with a fully depolarized cell, we analyzed relative inward current amplitudes at -60 and 0 mV, using cells with NMDG+ inside to prevent contamination by outward currents. Ca^{2+} currents at -60 mV were 4.0 ± 0.7-fold larger than at 0 mV (n = 5). As a result of inward rectification mediated by Mg^{2+} block, depolarization near 0 mV would reduce Ca^{2+} influx through CRAC channels by 75%, to a level where existing Ca^{2+} store pump mechanisms reduce the residual [Ca^{2+}]_i to a level below that required for gene expression.

Since CRAC channels provide the trigger for activation of signaling pathways inside T cells leading to gene expression and activation or apoptosis, a low conductance channel may be beneficial to avoid inadvertent signaling. Because of its small volume and low resting [Ca^{2+}]_o of 100 nM, a human T cell contains fewer than 10^4 free Ca^{2+} ions in cytoplasm. Even considering the ability of cytoplasm to buffer Ca^{2+}, a very small Ca^{2+} current (order of 1 pA) will result in a large change in [Ca^{2+}]_i. Thus, to control the rise in [Ca^{2+}]_i, it may be advantageous to limit Ca^{2+} influx through a CRAC channel to thousands rather than millions of ions per second. The low Ca^{2+} throughput may be seen in the very low single-channel conductance of a CRAC channel, as well as in the I-[Ca^{2+}]_o relation.

The authors acknowledge the continued excellent assistance of Dr. Lu Forrest in cell culture.

This work was supported by National Institutes of Health grants NS-14609 and GM-41514.

Original version received 28 August 1997 and accepted version received 21 January 1998.

REFERENCES


Hoth, M., and R. Penner. 1992. Depletion of intracellular calcium...
Kuo, C., and P. Hess. 1993. Block of the L-type Ca2+


Lewis, R.S., M.D. Cahalan. 1996. Regulation of depletion-activated Ca2+ channels by intracellular protons in single ventricular myocytes of the guinea-pig. J. Physiol. (Camb.) 496:1463–1475.