Divergence of Ca\textsuperscript{2+} selectivity and equilibrium Ca\textsuperscript{2+} blockade in a Ca\textsuperscript{2+} release-activated Ca\textsuperscript{2+} channel

Megumi Yamashita and Murali Prakriya

Department of Molecular Pharmacology and Biological Chemistry, Northwestern University Feinberg School of Medicine, Chicago, IL 60611

Prevailing models postulate that high Ca\textsuperscript{2+} selectivity of Ca\textsuperscript{2+} release-activated Ca\textsuperscript{2+} (CRAC) channels arises from tight Ca\textsuperscript{2+} binding to a high affinity site within the pore, thereby blocking monovalent ion flux. Here, we examined the contribution of high affinity Ca\textsuperscript{2+} binding for Ca\textsuperscript{2+} selectivity in recombinant Orai3 channels, which function as highly Ca\textsuperscript{2+}-selective channels when gated by the endoplasmic reticulum Ca\textsuperscript{2+} sensor STIM1 or as poorly Ca\textsuperscript{2+}-selective channels when activated by the small molecule 2-aminoethoxydiphenyl borate (2-APB). Extracellular Ca\textsuperscript{2+} blocked Na\textsuperscript{+} currents in both gating modes with a similar inhibition constant (K_i $\sim$ 25 µM). Thus, equilibrium binding as set by the K_i of Ca\textsuperscript{2+} blockade cannot explain the differing Ca\textsuperscript{2+} selectivity of the two gating modes. Unlike STIM1-gated channels, Ca\textsuperscript{2+} blockade in 2-APB–gated channels depended on the extracellular Na\textsuperscript{+} concentration and exhibited an anomalously steep voltage dependence, consistent with enhanced Na\textsuperscript{+} pore occupancy. Moreover, the second-order rate constants of Ca\textsuperscript{2+} blockade were eightfold faster in 2-APB–gated channels than in STIM1-gated channels. A four-barrier, three–binding site Eyring model indicated that lowering the entry and exit energy barriers for Ca\textsuperscript{2+} and Na\textsuperscript{+} to simulate the faster rate constants of 2-APB–gated channels qualitatively reproduces their low Ca\textsuperscript{2+} selectivity, suggesting that ion entry and exit rates strongly affect Ca\textsuperscript{2+} selectivity. Noise analysis indicated that the unitary Na\textsuperscript{+} conductance of 2-APB–gated channels is fourfold larger than that of STIM1-gated channels, but both modes of gating show a high open probability (P_o $\sim$ 0.7). The increase in current noise during channel activation was consistent with stepwise recruitment of closed channels to a high P_o state in both cases, suggesting that the underlying gating mechanisms are operationally similar in the two gating modes. These results suggest that both high affinity Ca\textsuperscript{2+} binding and kinetic factors contribute to high Ca\textsuperscript{2+} selectivity in CRAC channels.

INTRODUCTION

Ca\textsuperscript{2+} is a multifunctional signaling messenger crucial for diverse biological processes. Among the various ways by which cellular Ca\textsuperscript{2+} signals are generated, store-operated Ca\textsuperscript{2+} release-activated Ca\textsuperscript{2+} (CRAC) channels are recognized as a widespread mechanism for regulating transcription, motility, and proliferation in many cells (Feske, 2009; Hogan et al., 2010; Lewis, 2011). CRAC channels produce sustained intracellular Ca\textsuperscript{2+} elevations and are implicated in a growing list of human diseases including immunodeficiency (Feske, 2009), allergy (Di Capite et al., 2011), cancer (Prevarskaya et al., 2011), thrombosis (Varga-Szabo et al., 2011), and inflammatory bowel disease (McCarl et al., 2010). The broad expression of CRAC channels and their involvement in many physiological processes has produced intense interest in CRAC channels as targets for drug development. Yet, our understanding of how CRAC channels operate at a mechanistic level is still rudimentary and, in particular, the molecular and structural mechanisms of ion permeation and channel gating are only now beginning to be elucidated.

A distinguishing feature of CRAC channels is high Ca\textsuperscript{2+} selectivity (P_{Ca}/P_{Na} = 1,000; Hoth and Penner, 1993). Current thinking about the origin of this selectivity is rooted in the idea of preferential Ca\textsuperscript{2+} binding to a high affinity binding site (K $\sim$ 20 µM) at the selectivity filter, which occludes Na\textsuperscript{+} flux through the pore (Prakriya, 2009). In support of this idea, a mutation at the predicted CRAC channel selectivity filter (E106D in Orai1) diminishes both Ca\textsuperscript{2+} selectivity as well as the affinity of Ca\textsuperscript{2+} blockade of Na\textsuperscript{+} flux (Prakriya et al., 2006; Vig et al., 2006; Yeromin et al., 2006; Yamashita et al., 2007), which would be expected if Ca\textsuperscript{2+} selectivity is primarily determined by the K_i of Na\textsuperscript{+} current blockade. Such a selection-through-affinity mechanism was originally described for voltage-gated Ca\textsuperscript{2+} channels, which, like CRAC channels, display exquisite Ca\textsuperscript{2+} selectivity (P_{Ca}/P_{Na} > 1,000; Sather and McCleskey, 2003). However, the apparent affinity of Ca\textsuperscript{2+} block is roughly 20-fold higher in voltage-gated Ca\textsuperscript{2+} channels than CRAC channels (K_i of $\sim$1 vs. 20 µM, respectively; Almers and McCleskey, 1984; Bakowski and Parekh, 2002; Su et al., 2004; Prakriya and...
Ca\(^{2+}\) selectivity in CRAC channels

Published February 24, 2014

Po's probability (stepwise recruitment of closed channels to a high open state, suggesting that the allosteric mechanisms that open the pore in response to ligand binding are operationally similar between the two gating modes. Collectively, these results provide new insights into the mechanisms of ion selectivity and gating in Orai channels.

MATERIALS AND METHODS

Cells

HEK293 cells were grown in medium consisting of 44% Dulbecco’s modified Eagle’s medium (Corning) and 44% Ham’s F12 (Corning), supplemented with 10% fetal calf serum (HyClone), 1% 200 mM glutamine, 1% 5000 U/ml penicillin, and 5,000 µg/ml streptomycin. The cells were maintained in log-phase growth at 37°C in 5% CO₂.

Solutions

The standard extracellular Ringer’s solution contained 130 mM NaCl, 4.5 mM KCl, 20 mM CaCl₂, 10 mM D-glucose, and 5 mM Na-HEPES, pH 7.4. The divalent-free (DFV) Ringer’s solution contained 150 mM NaCl, 10 mM HEDTA, 1 mM EDTA, and 10 mM HEPES, pH 7.4. pH was adjusted to 7.4 with NaOH or CsOH. 10 mM TEA-Cl was added to all extracellular solutions to prevent conduction of Na⁺-ICRAC by Ca²⁺. CaCl₂ was added to the standard DFV solution at the appropriate amount calculated from the MaxChelator software (WEBMAXC 2.10, available at http://www.stanford.edu/~cpatton/webmaxc2.htm). The 300- and 600-µM [Ca²⁺]i solutions were made by adding the indicated amount of CaCl₂ to a nominally Ca²⁺-free solution containing 150 mM NaCl and 10 mM HEPES, pH 7.4. For the pore-sizing studies described in Fig. 1 B, the following organic compounds were substituted for sodium methanesulfonate in the external solution: hydroxyamine HCl (NH₂OH-HCl), hydrazine HCl (NH₂NH₂-HCl), methylamine HCl (CH₃NH₂-HCl), dimethylamineHCl ((CH₃)₂NH-HCl), trimethylamineHCl ((CH₃)₃N-HCl), and tetramethylammonium chloride ((CH₃)₄NCl). These chemicals were purchased from Sigma-Aldrich. pH was adjusted to 7.4 with NMDG except in the case of hydrazine HCl (pH 6.4) and hydroxyamine HCl (pH 6.2), which were studied at acidic pH to increase the ionized concentration of the test ion.

Patch-clamp measurements

Patch-clamp recordings were performed using an Axopatch 200B amplifier (Molecular Devices) interfaced to an ITC-18 input/output board and an iMac G5 computer (Apple). Currents were filtered at 1 kHz with a 4-pole Bessel filter and sampled at 5 kHz. Stimulation and data acquisition and analysis were performed using routines developed on the Igor Pro platform by R.S. Lewis (Stanford University, Palo Alto, CA). The holding potential was...
+30 mV unless otherwise indicated. Two types of stimuli were used: (1) a 100-ms step to −100 mV followed by a 100-ms ramp from −100 to +100 mV usually applied every 1 s and (2) continuous recording holding potential at −100 mV. Current amplitudes were typically analyzed at −100 mV unless indicated otherwise.

Noise analysis

200-ms sweeps were acquired at the rate of 4 Hz at a constant holding potential of −100 mV, digitized at 20 kHz, low-pass filtered using a 10 kHz using the amplifier’s built-in Bessel filter, and recorded directly to hard disk. The mean current and variance were calculated from each sweep. For spectral analysis, data were low-pass filtered using a 20 Hz Gaussian filter and power spectra were computed from either 256- or 1,024-point segments using a Hamming window (Igor Pro; Wavemetrics) and averaged from 5–10 sweeps.

Data analysis

Unless noted otherwise, all data were corrected for leak currents collected in 20 mM Ca^{2+} + 50–100 µM La^{3+}. Averaged results are presented as the mean value ± SEM at −100 mV unless indicated otherwise. All curve fitting was done by least-squares methods using built-in functions in Igor Pro 5.0. The minimal pore diameter was estimated as previously described (Prakriya and Lewis, 2006; Yamashita et al., 2007). In brief, relative permeabilities to various organic cations estimated from the bionic GHK equation were plotted against ion size as in Fig. 1B and fit to the hydrodynamic relationship:

\[
P_{i}/P_{Na} = k \left(1 - \frac{d_{i}}{d_{pore}} \right)^{2},
\]

where \( P_{i}/P_{Na} \) is the relative permeability of the cation being tested, \( d_{i} \) is the diameter of the test cation, and \( d_{pore} \) is the minimal pore diameter. The relative permeabilities for the organic cations were determined from changes in reversal potential induced by replacing extracellular Na^{+} in the standard DVF solution with the test cation.

To analyze the voltage dependence of block of Na^{+}-ICRAC by Ca^{2+}, the model described by Guo and Lu (2000) was used. Unlike the older Woodhull (1973) model that assigns the voltage dependence of block solely to the valence of the blocking particle, the Guo and Lu (2000) model expresses voltage dependence in terms of an apparent valence, an empirical factor that encompasses the effects of blocker valence and the coupled movements of conducting ions displaced by blocker binding.

In this scheme, Ca^{2+} block is described in terms of second-order binding to a single site:

\[
\text{Ca}^{2+} + \text{Ch} \xrightarrow{k_{p}, Z_{1}} \text{Ch} \cdot \text{Ca} \xrightarrow{k_{p}, Z_{2}} \text{Ch} + \text{Ca}^{2+}
\]

(Scheme 1)

where Ch is the channel, Ca^{2+}, and Ca^{2+}; are extracellular and intracellular Ca^{2+}, \( k_{p} \) and \( k_{-} \) are the binding and unbinding rates from the extracellular side, \( k_{b} \) is the unbinding rate from the intracellular side, and each \( z_{i} \) represents the apparent valence for the corresponding transition. Block from the intracellular compartment is assumed to be negligible because of low (nanomolar) intracellular Ca^{2+} concentrations. With these assumptions, the fraction of unblocked current is given by (Guo and Lu, 2000):

\[
\frac{I}{I_{o}} = \left(1 + \frac{[Ca^{2+}]}{K_{i} e^{Rt}} \right)^{-1}
\]

where \( K_{i} = k_{-}/k_{p} \) is the equilibrium dissociation constant at 0 applied voltage, and \( Z_{1} \) and \( Z_{2} \) are the apparent valences. \( Z_{i} (= z_{i} + z_{-} \) provides a measure of the overall voltage dependence of Ca^{2+} block and arises from the movement of the charged blocker (Ca^{2+}) within the field as well as the possible displacement of permeant ions (Na^{+}) within the pore. \( k_{b}/k_{-} \) is the ratio of the rates of Ca^{2+} escaping into the cytoplasm versus returning to the extracellular solution from the pore, and thus provides a measure of Ca^{2+} permeation. The quantities \( k_{b}/k_{-} \) and \( z_{1} + z_{2} \) were treated as single adjustable parameters for fitting the data. To avoid complications arising from activation of Na^{+}-ICRAC during hyperpolarizing steps in 2-APB-gated channels (see Fig. S2), we measured block from the ratio of the steady-state currents in the presence and absence of extracellular Ca^{2+}, rather than from the ratio of the steady-state peak currents in Ca^{2+} as was done previously (Prakriya and Lewis, 2006; Yamashita et al., 2007).

RESULTS

Orai3 channels can be activated in a store-dependent manner by the ER Ca^{2+} sensor STIM1 or directly in a store-independent manner by high doses of 2-APB. In contrast to STIM1-activated Orai3 channels, however, 2-APB-activated Orai3 channels exhibit low Ca^{2+} permeability and readily conduct Cs^{+} ions (Schindl et al., 2008; Zhang et al., 2008; Yamashita et al., 2011). Here, we sought to understand differences in the interaction of conducting ions to the pores of STIM1- and 2-APB-activated Orai3 channels and compare their gating
behaviors. To carry out these studies, we expressed CFP-Orai3 either alone or together with unlabeled STIM1 in HEK293 cells and studied their functional properties by patch-clamp electrophysiology. STIM1-activated Orai3 currents were obtained by depleting ER Ca^{2+} stores in cells expressing STIM1 and CFP-Orai3 with 1 μM thapsigargin before patch-clamp recordings, whereas 2-APB–activated currents were elicited by administering 2-APB to cells overexpressing CFP-Orai3. A dose–response experiment indicated that 2-APB activates Orai3 channels with an EC_{50} of ~24 μM and a high Hill coefficient of ~8 (Fig. 1 A). Therefore, unless otherwise indicated, we used a concentration of 50 μM to elicit 2-APB–gated Orai3 currents.

Differences in the affinity of Ca^{2+} blockade of Na^+ flux cannot explain the differing Ca^{2+} selectivity of 2-APB– and STIM1-gated Orai3 channels

Unlike STIM1-activated Orai3 channels, 2-APB–gated Orai3 channels readily conduct many large cations (Schindl et al., 2008). In accordance with previous results postulating that an enlarged pore is responsible for this feature (Schindl et al., 2008), 2-APB–activated Orai3 channels display a wider apparent pore width compared with STIM1-activated Orai3 channels (Fig. 1 B). As a result of (or associated with) this structural change, 2-APB–gated Orai3 channels exhibit lower Ca^{2+} selectivity than STIM1-gated channels. This is reflected in the well-described leftward shift of the reversal potential (V_{rev}), which changed from 71 ± 6 mV (n = 7) for STIM1-gated channels to 25 ± 2 mV (n = 8) for 2-APB–gated channels in a Ringer’s solution containing 20 mM Ca^{2+}. 2-APB–gated Orai3 channels also showed large outward currents carried by intracellular Cs^+ (Fig. 1 C). Moreover, replacing extracellular Na^+ with choline, a large cation that is impermeable through most cationic channels, revealed significant Na^+ conduction at −100 mV (Fig. 1 D), reaffirming that 2-APB–gated channels are poorly Ca^{2+} selective under these experimental conditions.

Current models postulate that the high Ca^{2+} of CRAC channels originates from tight binding of Ca^{2+} to the CRAC channel selectivity filter (Prakriya and Lewis, 2003; Prakriya, 2009; McNally and Prakriya, 2012). On this basis, the widely different Ca^{2+} selectivities of STIM1- and 2-APB–activated channels should arise from differences in the energetics of Ca^{2+} binding to the Orai3 selectivity filter. We examined this question using several approaches: by estimating the thermodynamic stability of Ca^{2+} binding to the Orai3 channel pore from Ca^{2+} block measurements, from the voltage dependence of Ca^{2+} block, and from the rates of Ca^{2+} blockade of the monovalent current. Like voltage-gated Ca^{2+} channels, Orai channels readily conduct a variety of small monovalent cations including Na^+ upon removal of extracellular divalent cations. Therefore, we first applied a DVF solution to elicit Na^+ currents through Orai3 channels and examined the ability of micromolar concentrations of extracellular Ca^{2+} to block the Na^+ currents (Fig. 2).
At a membrane potential of $-100 \text{ mV}$, these tests indicated that Na$^+$ currents through STIM1-gated Orai3 channels are blocked dose dependently by extracellular Ca$^{2+}$ with an $K_i$ of $\sim 25 \mu \text{M}$ (Fig. 2, A and F). This $K_i$ is nearly identical to the sensitivity of native CRAC channels (Bakowski and Parekh, 2002; Su et al., 2004; Prakriya and Lewis, 2006) and Orai1 channels overexpressed in HEK293 cells (Yamashita et al., 2007). Thus, when gated by STIM1, the thermodynamic stability of Ca$^{2+}$ binding is similar in Orai1 and Orai3 channels, as expected given the high homology of the primary sequence in the pore regions of these paralogueous proteins. Furthermore, as previously described for Orai1 channels (Prakriya et al., 2006; Yamashita et al., 2007), mutating the predicted selectivity filter formed by the conserved amino acid Glu81 to Asp (E81D) lowered both Ca$^{2+}$ block (Fig. 2, B and F; $K_i = 111 \mu \text{M}$) and the Ca$^{2+}$ selectivity of STIM-activated Orai3 channels (Fig. S1 A), as expected if Ca$^{2+}$ selectivity is directly regulated by Ca$^{2+}$ binding at the selectivity filter.

Given that 2-APB–activated Orai3 channels are poorly Ca$^{2+}$ selective, we hypothesized that these channels should be less sensitive to Ca$^{2+}$ blockade than STIM1-gated Orai3 channels. Yet, much to our surprise, Na$^+$ currents through 2-APB–activated Orai3 channels were blocked with similar Ca$^{2+}$ sensitivity as STIM1-activated channels (Fig. 2, D and F; and Table 1). Moreover, as with STIM1-gated Orai3 channels, blockade of the monovalent current in the 2-APB gating mode was markedly lowered by the E81D mutation (Fig. 2, E and F; and Table 1; $K_i = 154 \mu \text{M}$), indicating that a pointed disruption of the Ca$^{2+}$ selectivity filter formed by E81 destabilizes Ca$^{2+}$ binding in both gating modes. Introducing the E165A mutation in the TM3 segment of Orai3 did not significantly alter the sensitivity of Ca$^{2+}$ blockade (Fig. 2 F) or Orai3 selectivity (Fig. S1, A and B), effectively ruling out a contribution for E165 and TM3 for Ca$^{2+}$ binding to the 2-APB–activated Orai3 pore. Collectively, these results indicate that the affinity of Ca$^{2+}$ binding to the Orai3 pore at the test potential used here ($-100 \text{ mV}$) is not different between the two gating modes, despite the lower Ca$^{2+}$ permeability of 2-APB–gated channels (Fig. 1 D).

Extracellular Na$^+$ concentration modulates sensitivity of Ca$^{2+}$ blockade of 2-APB–gated Orai3 channels

In many types of ion channels, the sensitivity and rate of open-channel block is affected by the concentration of permeant ions, either because of competition between

![Figure 2. Extracellular Ca$^{2+}$ blocks STIM1- and 2-APB–activated Orai3 Na$^+$ currents with similar sensitivity. (A, B, D, and E) Inhibition of STIM1- or 2-APB–activated Orai3 Na$^+$ currents by Ca$^{2+}$. In each case, the cell was voltage clamped to a constant potential of $-100 \text{ mV}$ and 200-ms sweeps were collected at 2 kHz. The mean current during each sweep is plotted against time. (C) Predicted topology of a single Orai3 subunit with the acidic residues (TM1 and TM3) highlighted. (F) Dose–response relationships of Ca$^{2+}$ blockade. Ca$^{2+}$ blockade of Na$^+$ currents through 2-APB–activated WT and E165A Orai3 channels occurred with a similar affinity as STIM1-activated Orai3 channels. E81D substitution increases the $K_i$ of block in both STIM1- and 2-APB–activated modes (also see Table 1). Block was quantified by measuring the Na$^+$ current immediately after application of a DVF solution supplemented with the indicated [Ca$^{2+}$]. Each dashed line is a least-squares fit of the Hill equation $block = max/[1 + (K_i/[Ca])^n]$, where max is the predicted maximal blockade at saturating Ca$^{2+}$ concentrations. Error bars represent SEM.](https://example.com/figure2)
blocker and permeant ions for pore binding sites or displacement of the blocker by permeant ions (Armstrong, 1971; Neyton and Miller, 1988b; Antonov and Johnson, 1999). In addition to revealing putative interactions between permeant ions and blockers in the pore, results from such experiments are often useful to delineate the position of pore sites for blockers and permeant ions (Neyton and Miller, 1988a). To approach this issue, we investigated the effects of varying the external Na+ concentration on Ca2+ blockade of inward Orai3 Na+ currents. These experiments revealed an interesting difference between STIM1- and 2-APB–gated channels. Lowering the extracellular Na+ concentration from 150 to 75 mM did not significantly alter the sensitivity of Ca2+ blockade of STIM1-gated Na+ fluxes (Fig. 3A). In 2-APB–gated channels, however, the K of inhibition of Na+ currents in 2-APB–gated channels was reduced threefold to ~9 µM (Fig. 3B). Thus, decreasing the Na+ occupancy of external pore sites by lowering [Na]o enhances Ca2+ binding to its high affinity site. Consistent with this interpretation, the blocking rate also increased twofold at the lower Na+ concentration (Fig. 3, D and E). These results indicate that competition between Na+ and Ca2+ ions in the pore influences the ability of Ca2+ to interact with its high affinity site in 2-APB–gated channels. One possibility is that Ca2+ has to dislodge Na+ ions from pore sites before it gains access to its high affinity site. This competition could occur either at the high affinity Ca2+ site (i.e., the selectivity filter) itself or at a more superficial location in the pore.

The outer vestibule of all Orai channels contains acidic residues that have been implicated in La3+ binding and position of pore sites for blockers and permeant ions (Armstrong, 1971; Neyton and Miller, 1988b; Antonov and Johnson, 1999). In addition to revealing putative interactions between permeant ions and blockers in the pore, results from such experiments are often useful to delineate the position of pore sites for blockers and permeant ions (Neyton and Miller, 1988a). To approach this issue, we investigated the effects of varying the external Na+ concentration on Ca2+ blockade of inward Orai3 Na+ currents. These experiments revealed an interesting difference between STIM1- and 2-APB–gated channels. Lowering the extracellular Na+ concentration from 150 to 75 mM did not significantly alter the sensitivity of Ca2+ blockade of STIM1-gated Na+ fluxes (Fig. 3A). In 2-APB–gated channels, however, the K of inhibition of Na+ currents in 2-APB–gated channels was reduced threefold to ~9 µM (Fig. 3B). Thus, decreasing the Na+ occupancy of external pore sites by lowering [Na]o enhances Ca2+ binding to its high affinity site. Consistent with this interpretation, the blocking rate also increased twofold at the lower Na+ concentration (Fig. 3, D and E). These results indicate that competition between Na+ and Ca2+ ions in the pore influences the ability of Ca2+ to interact with its high affinity site in 2-APB–gated channels. One possibility is that Ca2+ has to dislodge Na+ ions from pore sites before it gains access to its high affinity site. This competition could occur either at the high affinity Ca2+ site (i.e., the selectivity filter) itself or at a more superficial location in the pore.

The outer vestibule of all Orai channels contains acidic residues that have been implicated in La3+ binding and that are postulated to facilitate cation accumulation at the mouth of the channel (Fig. 3F; Yeromin et al., 2006; McNally et al., 2009). To examine the role of these residues that have been implicated in La3+ binding and conduction of intracellular Cs+ ions could arise if Ca2+ ions, we mutated these acidic sites (E85A/D87A/D89A) and tested how this maneuver affects Ca2+ blockade of monovalent conduction at positive voltages. In principle, the outward conduction of intracellular Ca2+ ions at positive voltages. In principle, the outward conduction of intracellular Ca2+ ions at positive voltages. In principle, the outward conduction of intracellular Ca2+ ions at positive voltages. In principle, the outward conduction of intracellular Ca2+ ions could arise if Ca2+ blockade of monovalent conduction at positive voltages is weaker in 2-APB– than in STIM1-gated channels. To test this possibility, we measured the voltage dependence of Ca2+ blockade in the two gating modes from a series of test potentials in 20 µM Ca2+. These experiments indicated that Na+ currents in both

## Table 1

<table>
<thead>
<tr>
<th>Channel type</th>
<th>Kᵢ (µM)</th>
<th>n</th>
<th>k on (M⁻¹·s⁻¹)</th>
<th>k off (s⁻¹)</th>
<th>Unitary current (pA)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Orai3 (STIM1-gated)</td>
<td>25</td>
<td>1.2</td>
<td>4 × 10⁶</td>
<td>35</td>
<td>71</td>
</tr>
<tr>
<td>Orai3 (2-APB–gated)</td>
<td>26</td>
<td>1.2</td>
<td>3 × 10⁷</td>
<td>246</td>
<td>283</td>
</tr>
<tr>
<td>E81D Orai3 (STIM1-gated)</td>
<td>111</td>
<td>1.0</td>
<td>4 × 10⁶</td>
<td>130</td>
<td>–</td>
</tr>
<tr>
<td>E81D Orai3 (2-APB–gated)</td>
<td>154</td>
<td>1.0</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>E165A Orai3 (2-APB–gated)</td>
<td>32</td>
<td>1.2</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
</tbody>
</table>

*–, not determined.
gating modes are blocked by extracellular Ca\(^{2+}\) in a voltage-dependent fashion, with negligible block at voltages positive to 0 mV (Fig. 4 D). However, the voltage dependence of Ca\(^{2+}\) block of 2-APB–activated channels was markedly steeper, and V\(_{50}\) more depolarized compared to STIM1-gated channels (Fig. 4 D). The anomalously high voltage dependence of Ca\(^{2+}\) block in 2-APB–gated Orai3 channels (Z\(_1\) = 3.4) cannot be explained exclusively in terms of blocking site location because the required site depth is far greater than that of a single Ca\(^{2+}\) (z = 2) moving all the way through the electric field (Woodhull, 1973). Instead, as described previously for K\(^+\) channels (Martínez-Francois and Lu, 2010), the most straightforward explanation is that Ca\(^{2+}\) block in this case is coupled to the displacement of permeant Na\(^+\) ions. This conclusion is further supported by a striking shift in the voltage dependence of block in the vestibule is expected to be absent. Here, the extent of blockade was essentially invariant in a large range of voltages tested (−120 to −20 mV), but declined gradually between 0 and +100 mV (Z\(_1\) = 0.8; Fig. 4 D). These results indicate that the steep voltage dependence of Ca\(^{2+}\) blockade in WT 2-APB–gated Orai3 channels is likely driven by the concurrent movement of Na\(^+\) ions that are pushed into the pore from the outer vestibule. When considered together with the finding that external Na\(^+\) ions affect Ca\(^{2+}\) binding to the 2-APB–gated pore (Fig. 3 B), these observations lead us to conclude that Ca\(^{2+}\) access to the high affinity site is hindered in 2-APB–gated channels as a result of the occupancy of external pore sites by Na\(^+\) ions. Ca\(^{2+}\) can block the pore but has to displace several Na\(^+\) ions into the pore to gain access to its binding site.

In contrast to 2-APB–gated channels, the lack of effect of extracellular Na\(^+\) concentration on Ca\(^{2+}\) block in

---

Figure 3. Reducing extracellular Na\(^+\) concentrations affects Ca\(^{2+}\) blockade of 2-APB– but not STIM1-gated Orai3 channels. (A and B) Blockade of Na\(^+\) currents through STIM1- or 2-APB–gated channels in 75 mM of external [Na\(^+\)]. The dashed line is a least-squares fit of the standard Hill equation

\[
\text{block} = \frac{\text{max}}{1 + (K_i / [Ca])^n},
\]

where max is the maximal blockade at saturating Ca\(^{2+}\) concentrations. The black dotted line in each case is the fit of the data at 150 mM [Na\(^+\)] from Fig. 2 F for comparison. Fit parameters for the 75-mM Na\(^+\) condition are: STIM1-activated current, K\(_i\) = 29 µM, n = 1.1; 2-APB–activated current, K\(_i\) = 9 µM, n = 1.2. (C) Blockade of Na\(^+\) currents through 2-APB–gated E85A/D87A/E89A triple mutant channels at normal (150 mM) and reduced (75 mM) extracellular Na\(^+\). The dashed line in each case is a Hill equation fit with one or two components:

\[
\text{block} = \frac{\text{max}_1}{1 + (K_{i1} / [Ca])^n_1} + \frac{\text{max}_2 - \text{max}_1}{1 + (K_{i2} / [Ca])^n_2},
\]

with the following parameters: max\(_1\) = 0.57, max\(_2\) = 0.87, K\(_{i1}\) = 33 µM, K\(_{i2}\) = 1,694 µM, n\(_1\) = 2.8, and n\(_2\) = 2.3. The 75-mM Na data were fit with a single Hill equation with a K of 64 µM and n = 1.4. (D) The rate of Na\(^+\) current blockade is accelerated by lowering extracellular [Na\(^+\)] in 2-APB–gated channels. Traces show the inward Na\(^+\) currents through 2-APB–gated channels during steps to −100 mV. 5 µM of extracellular Ca\(^{2+}\) was used to block the Na\(^+\) currents. Inset shows an exponential fit to the initial phase of blockade. (E) Summary of the time constants of Na\(^+\) current blockade by 5 µM [Ca\(^{2+}\)]. (P < 0.001). Error bars represent SEM. (F) Schematic representation of the pore-flanking TM1 segments of Orai3 and the acidic residues in the outer pore. The predicted selectivity filter (E81) and the La\(^{3+}\) binding sites in the outer vestibule are indicated.
STIM1-gated channels (Fig. 3A) and the shallower voltage dependence Ca\(^{2+}\) block (Fig. 4) suggest that far fewer (if any) Na\(^{+}\) ions have to be displaced for Ca\(^{2+}\) to access its site when the channel is gated by STIM1. This doesn’t mean that Ca\(^{2+}\) access to its high affinity site is energetically more favorable in STIM1-gated channels; in fact, as described in the next section, the rate constants indicate otherwise. Nonetheless, these results reaffirm the multi-ion nature of the Orai3 pore and are qualitatively consistent with the suggestion that pore occupancy by Na\(^{+}\) ions is greatly enhanced in 2-APB-gated Orai3 channels than in STIM1-gated Orai3 channels.

Although interesting, the distinct voltage dependencies of STIM1- and 2-APB-gated channels do not readily explain the inability of the 2-APB-gated pore to discriminate between Ca\(^{2+}\) and Na\(^{+}\) ions at negative voltages. This conclusion is supported by at least two aspects of the data. First, at a negative voltage (−100 mV) where STIM1-gated channels are Ca\(^{2+}\) selective, 2-APB-gated channels are blocked to a nearly identical extent as STIM1-gated channels, yet the experiments in Fig. 1 clearly show that a large fraction of the current at −100 mV is carried by Na\(^{+}\). Second and conversely, STIM1-gated channels do not readily conduct monovalent ions (e.g., Cs\(^{+}\)) even at positive voltages (>0 mV), where Ca\(^{2+}\) block in these channels is negligible. These characteristics of the voltage dependence indicate that the differing Ca\(^{2+}\) selectivities of STIM1- and 2-APB-gated channels cannot be explained directly in terms of the voltage dependence of Ca\(^{2+}\) binding to the pore.

The dwell time of Ca\(^{2+}\)-occupancy is lower in 2-APB-gated channels

In addition to differences in voltage dependence of blockade, STIM1- and 2-APB-activated Orai3 channels showed markedly different kinetics of Ca\(^{2+}\) blockade. At −100 mV, STIM1-activated Orai3 currents were blocked by external Ca\(^{2+}\) (20 µM) whose initial time course was fit with a single exponential function (Fig. 5, A and B). The time constant of blockade, 7 ± 1 ms (n = 5), closely

**Figure 4.** Voltage dependence of blockade of STIM1- and 2-APB-gated Orai3 channels by extracellular Ca\(^{2+}\). (A–C) Effects of extracellular Ca\(^{2+}\), on Na\(^{+}\) currents during voltage steps in WT (A and B) or E85A/D87A/E89A mutant (C) Orai3 channels gated by STIM1 or 2-APB. Hyperpolarizing voltage steps from −120 to +100 mV of 50-ms duration were applied from the holding potential (we used holding of +50 mV for STIM1-gated currents and −30 mV for 2-APB-gated currents). Block was induced by either 20 µM Ca\(^{2+}\) (STIM1- and 2-APB-gated WT Orai3) or 80 µM (triple mutant). (D) Voltage dependence of Ca\(^{2+}\)-block. Block was quantified from the ratio of the steady-state currents at the end of the 50-ms voltage steps in control and in the presence of extracellular Ca\(^{2+}\). The dashed line in each case is a least-squares fit to the Guo and Lu (2000) model (see Materials and methods) with the following key parameters: STIM1-activated Orai3 currents: \(Z_1 = 0.54\), \(K_1 = 187 \mu M\); 2-APB-activated Orai3: \(Z_1 = 3.39\), \(K_1 = 4523 \mu M\); 2-APB-gated E85A/D87A/E89A triple mutant: \(Z_1 = 0.78\), \(K_1 = 9.5 \mu M\). Error bars represent SEM.
Further, the blocked current fraction is given by the relationship:

\[ b_{\text{block}} = \frac{k_{\text{on}}[Ca^{2+}]}{(k_{\text{on}}[Ca^{2+}] + k_{\text{off}})}. \]  

Fig. 5 C plots the reciprocal of the averaged time constant at \(-100\) mV against different \(Ca^{2+}\) concentrations. These plots could be well fit with straight lines whose slope represents the apparent second-order on rate constant \((k_{\text{on}})\) and the intercept the off rate constant \((k_{\text{off}})\) for \(Ca^{2+}\) binding (Eq. 2). For STIM1-gated channels, the values of these parameters from the fit were \(k_{\text{on}} = 4 \times 10^6\) M\(^{-1}\)s\(^{-1}\) and \(k_{\text{off}} = 33\) s\(^{-1}\). These rate constants are similar to previously measured values for overexpressed Orai1 channels and native CRAC channels in Jurkat T cells (Prakriya and Lewis, 2006; Yamashita et al., 2007), indicating that the rates of \(Ca^{2+}\) entry and exit from the selectivity filter do not differ appreciably between STIM1-gated Orai1 and Orai3 channels. In contrast, \(k_{\text{on}}\) and \(k_{\text{off}}\) estimated from the data for 2-APB–gated currents were \(3 \times 10^7\) M\(^{-1}\)s\(^{-1}\) and 246 s\(^{-1}\), respectively, or approximately eightfold faster than the rate constants for STIM1-activated channels. These substantially faster rate constants imply that the energy barriers for \(Ca^{2+}\) entry and exit matches the block rate previously seen in STIM1-activated Orai1 currents (Yamashita et al., 2007) and native CRAC channels in Jurkat T cells (Prakriya and Lewis, 2006), suggesting that the blocking mechanism is operationally similar in both channel types. For 2-APB–gated channels, measurements of the time constant of \(Ca^{2+}\) blockade was complicated by a small degree of slow activation of the monovalent current during steps to \(-100\) mV (Fig. S2). However, lowering the holding potential from \(+50\) to \(-30\) mV diminished the hyperpolarization-induced activation of the 2-APB–gated monovalent current (Fig. S2), allowing us to directly examine the kinetics of \(Ca^{2+}\) blockade. These experiments showed that block kinetics were significantly faster in 2-APB–gated channels compared with STIM1-activated channels (Fig. 5, A and B; \(\tau = 1.2 \pm 0.1\) ms; \(n = 7\) cells).

We used the time constants of \(Ca^{2+}\) blockade from these experiments to ascertain the apparent second-order on and off rates of \(Ca^{2+}\) binding to the block site. If \(Ca^{2+}\) accesses a single binding site from the extracellular side at a rate \(k_{\text{on}}\) and exits the site at a rate \(k_{\text{off}}\), then the time constant \((\tau)\) of blockade can be described by the relation:

\[ \tau = \frac{1}{(k_{\text{on}}[Ca^{2+}] + k_{\text{off}})}. \]  

Figure 5. The kinetics of channel blockade is significantly faster in 2-APB–gated channels compared to STIM1-gated channels. (A) STIM1- and 2-APB–activated Na\(^+\) currents in the presence of 20 µM \(Ca^{2+}\). Currents were elicited using a step pulse to \(-100\) mV as described in Fig. 4. (B) Time constants of \(Ca^{2+}\) blockade of STIM1- and 2-APB–activated Orai3 currents as a function of voltage. Cells were stepped from the holding potential (+50 mV for STIM1-gated channels and \(-30\) mV for 2-APB–gated channels) to the indicated test pulse. (C) Plot of the reciprocal of the time constant obtained at \(-100\) mV against the blocking \(Ca^{2+}\) concentration. The slope and y-axis intercepts of the straight-line fits to the data are \(4 \times 10^6\) M\(^{-1}\)s\(^{-1}\) and 33 s\(^{-1}\) (STIM1-gated channels) and \(3 \times 10^7\) M\(^{-1}\)s\(^{-1}\) and 246 s\(^{-1}\) (2-APB–gated channels). (D) Relief from \(Ca^{2+}\) block at +100 mV. \(Ca^{2+}\) block was induced at 20 µM \(Ca^{2+}\), through a 200-ms step to \(-100\) mV, and the membrane voltage was stepped to +100 mV to induce relief of blockade. The normalized traces (normalized to the steady-state current at +100 mV) show the recovery from blockade after the step to +100 mV. (E) Summary of the dwell time of \(Ca^{2+}\) occupancy at \(-100\) and +100 mV. Dwell times were calculated as \(1/k_{\text{off}}\). The values at \(-100\) mV were derived from \(k_{\text{off}}\) estimates from the fit of the mean data shown in C; hence there are no error bars for these values. Data at +100 mV were determined from decay constants in experiments shown in D. Error bars represent SEM.
into/from the pore are lower in 2-APB–gated channels. Further, the faster off rate implies that the dwell time of Ca\(^{2+}\) occupancy at the block site \((1/k_{\text{off}})\) is significantly lower in 2-APB–activated channels compared with STIM1-activated channels (Fig. 5 E).

In contrast to the acceleration of \(k_{\text{on}}\) seen in 2-APB–gated Orai3 channels, perturbation of the selectivity filter by the E81D mutation decreased \(k_{\text{on}}\) tenfold \((k_{\text{on}} = 4 \times 10^{9} \text{M}^{-1} \text{s}^{-1}\) and \(k_{\text{off}} = 130 \text{ s}^{-1}\)). This is similar to the previously described behavior of E106D Orai1 mutant channels, which also exhibit substantial slowing of the on-rate constant (Yamashita et al., 2007). Together with the differences, which also exhibit substantial slowing of the on-rate constant, indicate that the mechanisms underlying diminished Ca\(^{2+}\) selectivity are likely quite different between Orai3 channels gated by 2-APB and E81D Orai3 channels gated by STIM1. Specifically, the lower Ca\(^{2+}\) selectivity of E81D Orai3 channels can be rationalized in terms of diminished Ca\(^{2+}\) binding to the selectivity filter, in turn related to diminished rate of Ca\(^{2+}\) entry into the pore. However, this does not readily explain the lower Ca\(^{2+}\) selectivity of 2-APB–gated WT Orai3 channels, which exhibit the same \(K_i\) of Ca\(^{2+}\) blockade at \(-100\) mV as STIM1-gated Orai3 channels (Fig. 2 E) but accelerated binding rate constants (Table 1). We will return to this issue in the Discussion.

To directly examine unblock kinetics, we depolarized the membrane to \(+100\) mV, where blockade is expected to be nonexistent after induction of block at \(-100\) mV. As shown in Fig. 5 D for STIM1-gated channels, a step to \(+100\) mV initiates current recovery with a time constant of \(\sim 10\) ms, reflecting unbinding of Ca\(^{2+}\) from the block site. This recovery time specifies a \(k_{\text{off}}\) \((= 1/\tau)\) of \(100 \text{ s}^{-1}\). Measurements of current recovery in 2-APB–activated channels were complicated by a small degree of deactivation of the control current (i.e., in the absence of blocking Ca\(^{2+}\) ions). When channels were blocked by 20 \(\mu\)M Ca\(^{2+}\), however, the deactivation phase was not seen, presumably because it is masked by recovery of current from Ca\(^{2+}\) blockade. The time course of current recovery in this phase occurred with a time constant of \(\sim 1\) ms. The true current recovery likely occurs with a faster time constant because, as noted above, current recovery in this case would be slowed by channel deactivation occurring simultaneously. Nevertheless, even this overestimated recovery time course specifies a \(k_{\text{off}}\) of \(1,000 \text{ s}^{-1}\), significantly faster than the \(k_{\text{off}}\) estimate for STIM1-gated channels. Taken together, these results complement findings observed at \(-100\) mV and indicate that Ca\(^{2+}\) binding to the 2-APB–gated pore is much more labile than that seen in STIM1-gated channels, and the bound Ca\(^{2+}\) comes off at a significantly higher rate.

2-APB– and STIM1-gated Orai3 channels exhibit different sensitivity to La\(^{3+}\) blockade

The results presented in Figs. 3 and 4 indicate that Na\(^{+}\) occupancy at external pore sites is enhanced in 2-APB–gated Orai3 channels relative to STIM1-gated channels. Moreover, tests with the E85A/D87A/E89A triple mutant suggest that this effect is at least partially explained by Na\(^{+}\) accumulation in the outer vestibule where these residues are positioned. These findings signify that the molecular and structural features of the outer vestibule formed by the TM1–TM2 loops differ in the two gating modes. We examined this issue further by assessing the sensitivity of 2-APB–activated Orai3 channels to blockade by the trivalent lanthanide ion La\(^{3+}\). CRAC channels are potently blocked by low concentrations of lanthanides (Mason et al., 1991; Veromin et al., 2006). Electrophysiological studies have indicated that high affinity La\(^{3+}\) blockade of Orai channels occurs primarily through binding of the trivalent ions to acidic residues in the TM1–TM2 loop segments (Fig. 3 F; Veromin et al., 2006; McNally et al., 2009). Hence, we rationalized that La\(^{3+}\) blockade may allow us to gauge probable alterations in the structure of the TM1–TM2 loops in 2-APB–gated Orai3 channels.

These tests revealed that 2-APB–activated Orai3 channels are significantly less sensitive to La\(^{3+}\) blockade than STIM1-activated channels (Fig. 6; apparent \(K_i\) = 470 nM in STIM1-gated channels and \(K_i = 6 \mu\)M in 2-APB–gated Orai3 channels, both at \(-100\) mV). Somewhat puzzlingly, the apparent Hill coefficient was also reduced from 1.1 in STIM1-activated currents to 0.7 in 2-APB–activated currents (Fig. 6 C). A Hill coefficient other than 1 is suggestive of multiple binding sites and could arise either because of negative cooperativity between multiple binding sites or progressive occupancy of multiple noninteracting sites with different binding affinities (Prinz, 2010). In the case of Orai channels, although electrophysiological studies indicate that high affinity lanthanide block is explained by blocker binding at the external vestibule (Veromin et al., 2006; McNally et al., 2009), the recent crystal structure of Drosophila melanogaster Orai depicts a Gd\(^{3+}\) electron density in close proximity to the Glu selectivity filter in crystals soaked with 1 mM GdCl\(_3\) (Hou et al., 2012). Thus, these studies raise the prospect that there are two lanthanide binding sites in the ion conduction pathway: a high affinity site formed by the TM1–TM2 loops, and a second, low affinity site formed by the Glu selectivity filter. Structural alterations in the TM1–TM2 loop residues may reduce but not eliminate high affinity binding entirely, increasing blocker occupancy of the second, low affinity binding site and causing an apparent reduction in the overall Hill coefficient. Consistent with this possibility, mutating the TM1–TM2 loop acidic residues (E85A/D87A/E89A) significantly reduced the sensitivity of La\(^{3+}\) blockade, but preserved low affinity blockade. This residual blockade could be well fit with a Hill relation of \(n = 1\) for both STIM1- as well as 2-APB–gated Orai3 channels, which is consistent with the presence of a lower affinity La\(^{3+}\) site deeper in the pore. Intriguingly,
the La$^{3+}$ sensitivity of the triple mutant was considerably lower when gated by 2-APB ($K_i = 189$ µM) than when gated by STIM1 ($K_i = 10$ µM). Assuming that the residual La$^{3+}$ binding occurs at the selectivity filter (E81), this result reveals that the affinity of ion binding (La$^{3+}$ in this case) at the selectivity filter does in fact differ between 2-APB- and STIM1-gated channels, even though this is not obviously detected in Ca$^{2+}$ at −100 mV (Fig. 2). Based on these observations, we conclude that the TM1–TM2 loops, which form the outer vestibule, adopt a different conformation in 2-APB–gated Orai3 channels, which diminishes La$^{3+}$ binding in the vestibule, but enhances Na$^+$ occupancy.

2-APB–activated Orai3 channels exhibit a higher Na$^+$ unitary conductance

The faster second-order Ca$^{2+}$ binding rate constants and increased Na$^+$ ion pore occupancy in 2-APB–gated channels led us to next consider whether the energy barriers for ion flow are diminished in this gating mode. To test this possibility, we estimated the unitary conductance of STIM1- and 2-APB–gated Orai3 channels using nonstationary fluctuation analysis (Sigworth, 1980). Orai3 channels were activated either by 2-APB (50 µM) or by STIM1 after store depletion, and monovalent current noise was analyzed in 200-ms sweeps acquired at −100 mV (Sigworth, 1980). The relationship between current variance ($\sigma^2$) and the $P_o$ of the channel can be described by the relation:

$$\sigma^2 = Ni P_o o,$$

(4)

where, $i$ is the unitary current amplitude and $N$ is the number of channels. Eq. 4 indicates that variance is related to $P_o$ by the well-described parabolic relationship, with variance reaching a maximum when $P_o$ is 0.5. Substituting the term for $N$ in terms of the total current ($I = iNP_o$), we get the relation:

$$\frac{\sigma^2}{I} = i(1 - P_o).$$

(5)

A representative $\sigma^2/I$ plot of Orai3 current activated by 2-APB is illustrated in Fig. 7 B. The data show that Orai3 channel activation increases $\sigma^2$ as expected. Yet, the $\sigma^2$ versus $I$ plots did not show a curvature expected from the canonical parabolic $\sigma^2/I$ relationship. Instead the data could be well fit with straight lines with a mean slope of $56 \pm 6$ fA ($n = 12$ cells; Fig. 7 B). As described previously (Prakriya and Lewis 2006), a linear $\sigma^2/I$ relationship can arise either because channel $P_o$ is always <<1 when the macroscopic current changes because of an increase in $P_o$ (Eq. 5) or, alternately, because of a change in channel number $N$ at any constant value of $P_o$ (Eq. 4). To distinguish between these possibilities and properly interpret the linear $\sigma^2/I$ relation, an estimate of $P_o$ is needed. For this, we used blockade of Orai3 currents by micromolar concentrations of extracellular Ca$^{2+}$ to define the position along the parabolic $\sigma^2/I$ relationship where Orai3 channels operate.

If 2-APB activates Orai3 channels to a $P_o > 0.5$, then partial blockade of current with a channel blocker should result in an increase in current variance, as the variance-mean current plot moves leftward on the parabolic $\sigma^2/I$ plot. Only after further blockade, when $P_o$ is
attenuated to values <0.5, should variance be expected to decline. Consistent with this prediction, modest blockade of the monovalent current by a relatively low dose of extracellular Ca\(^{2+}\) resulted in increase in current noise (Fig. 8 A). In contrast, strong blockade at higher Ca\(^{2+}\) concentrations decreased current variance. This result indicates that the 2-APB-activated current has a \(P_o > 0.5\). In fact, estimates of \(P_o\), \(i\), and \(N\) from fits of the data similar to that illustrated in Fig. 8 B revealed values of \(P_o = 0.7 \pm 0.05\), \(i = 283 \pm 61\) fA, and \(n = 21,444 \pm 4,521\) (\(n = 4\) cells). Power spectrum analysis of control and Ca\(^{2+}\)-blocked traces indicated that no high frequency components of current noise were missed under the 10-kHz low-pass filtering conditions used for these recordings (Fig. S3 B). These results indicate that 2-APB-activated Orai3 channels have a high \(P_o\).

Measurements of STIM1-gated Orai3 channel variance revealed that these channels also have a high \(P_o\) (Fig. 8, C and D). Estimates of \(P_o\), \(i\), and \(N\) from fits of the data similar to that illustrated in Fig. 8 D revealed values of \(P_o = 0.73 \pm 0.03\), \(i = 71 \pm 1\) fA, and \(n = 22,537 \pm 5,670\) (\(n = 5\) cells). Power spectrum analysis revealed that STIM1-gated currents exhibit lower frequency noise components than 2-APB–gated currents (Fig. S3), suggesting that despite a similar \(P_o\), 2-APB–gated channels exhibit flickery openings. Importantly, the estimated unitary current of STIM1-gated channels was significantly smaller (71 fA) than unitary current of 2-APB–gated channels (~280 fA).

These results indicate that in the absence of extracellular blocking divalents, 2-APB–gated channels exhibit a significant increase (fourfold) in the rate of Na\(^+\) conduction compared with STIM1-gated channels, which is indicative of a substantial decrease in the energy barriers for Na\(^+\) ion conduction. Moreover, the high \(P_o\) and linear \(\delta^2/I\) plots seen for both 2-APB as well as store-operated Orai3 channels indicate that both modes of activation (at least in the time scales of the 200-ms voltage sweeps used here) occur through a mechanism involving stepwise recruitment of channels to a high \(P_o\) mode, rather than a monotonic increase in \(P_o\). This suggests that the gating mechanisms in both modes of channel activation may be operationally similar.

**DISCUSSION**

In the prevailing model of CRAC channel selectivity, high Ca\(^{2+}\) selectivity is achieved through preferential binding of Ca\(^{2+}\) at the channel selectivity filter formed by a ring of Glu residues, causing occlusion of Na\(^+\) flux through the pore (McNally and Prakriya, 2012). Central to this idea is the notion that selectivity is governed by high affinity of Ca\(^{2+}\) binding to the selectivity filter. In agreement with this possibility, a previous study found that mutation of the selectivity filter (E106D Orai1) results in parallel decreases in both Ca\(^{2+}\) selectivity and the affinity of Ca\(^{2+}\) blockade of Na\(^+\) fluxes (Yamashita et al., 2007), as would be expected if the strength of Ca\(^{2+}\) binding directly influences the ability of bound Ca\(^{2+}\) ions to impede Na\(^+\) flux. Such a thermodynamically driven view of Ca\(^{2+}\) selectivity predicts that channels with lower relative Ca\(^{2+}\) selectivity should display diminished Ca\(^{2+}\) blockade of Na\(^+\) flux. Yet, here we find that at a voltage (~100 mV) where 2-APB–gated channels exhibit significant monovalent conduction, inhibition of Na\(^+\) Orai3 current by micromolar concentrations of extracellular Ca\(^{2+}\) is similar between the Ca\(^{2+}\)-selective STIM1-gated and the nonselective 2-APB–gated Orai3 channels. This result indicates that equilibrium blockade of Na\(^+\) flux, as measured by \(K_i\) of Na\(^+\)-CRAC inhibition, cannot account for the differing Ca\(^{2+}\) selectivity of the two gating modes.

Several explanations can, in principle, be considered for the different Ca\(^{2+}\) selectivity of STIM1- and 2-APB–gated channels. One explanation is that selectivity is...
channels exhibit considerably shorter dwell times than STIM1-gated channels. If this is also true at millimolar concentrations of extracellular Ca\(^{2+}\) where Ca\(^{2+}\) permeation occurs (which admittedly is an unsubstantiated assumption), the decreased dwell time of Ca\(^{2+}\) occupancy could lead to diminished Ca\(^{2+}\) selectivity by allowing a higher level of net Na\(^{+}\) permeation between Ca\(^{2+}\) binding events at the block site. Because each bound Ca\(^{2+}\) ion would be severalfold slower to leave in STIM1-gated channels, it could prevent many more Na\(^{+}\) ions from passing through than in 2-APB–gated channels. However, it is also possible that the decreased Ca\(^{2+}\) block dwell time is a consequence of enhanced Na\(^{+}\) permeation driven by repulsion from Na\(^{+}\) ions destabilizing Ca\(^{2+}\) binding (Fig. 3 B). In this case, a faster entry rate for Na\(^{+}\) would naturally bias selectivity toward Na\(^{+}\).

A third possibility is that the CRAC channel pore contains additional Ca\(^{2+}\) binding sites distinct from the single block site examined here. In this scenario, new Ca\(^{2+}\) binding sites that are not readily detected at low (micromolar) concentrations of extracellular Ca\(^{2+}\) used to gauge block come into play at millimolar concentrations to confer high Ca\(^{2+}\) selectivity of STIM1-gated channels. This argument is undermined by the fact that molecular and structural studies reveal only a single locus for Ca\(^{2+}\) influenced not only by the steady-state Ca\(^{2+}\) binding affinity to the selectivity filter but also by the kinetic properties of ion entry. Many studies have considered the idea that selectivity is governed by the rates of ion entry into the channel, with less selective channels exhibiting slower rates of ion entry into the selectivity filter (Grabe et al., 2006; Nimigean and Allen, 2011). Applying this idea to Orai3 channels, one scenario is that the weakly Ca\(^{2+}\)-selective 2-APB–gated Orai3 channels exhibit a slower rate of Ca\(^{2+}\) entry into the high affinity binding site than the highly Ca\(^{2+}\)-selective STIM1-gated channels. This appears to the case for E81D Orai3 mutant channels, which exhibit a 10-fold slower \(k_{on}(10^5 \text{M}^{-1}\text{s}^{-1})\), reaffirming the importance of high affinity binding at the selectivity filter for Ca\(^{2+}\) selectivity. However, this explanation cannot account for the lower Ca\(^{2+}\) selectivity of 2-APB–gated WT Orai3 channels because our estimates of the second-order on rates suggest that \(k_{on}\) is actually faster in 2-APB–gated channels (~10\(^7\) M\(^{-1}\text{s}^{-1}\)) than in STIM1-gated channels (~10\(^6\) M\(^{-1}\text{s}^{-1}\)).

A closely related kinetic possibility is that channels with lower Ca\(^{2+}\) selectivity exhibit transient (rather than stable) binding compared with channels with high Ca\(^{2+}\) selectivity. Indeed, estimates of Ca\(^{2+}\) dwell times extrapolated from the \(k_{off}\) measurements indicate that 2-APB–gated channels exhibit considerably shorter dwell times than STIM1-gated channels. If this is also true at millimolar concentrations of extracellular Ca\(^{2+}\) where Ca\(^{2+}\) permeation occurs (which admittedly is an unsubstantiated assumption), the decreased dwell time of Ca\(^{2+}\) occupancy could lead to diminished Ca\(^{2+}\) selectivity by allowing a higher level of net Na\(^{+}\) permeation between Ca\(^{2+}\) binding events at the block site. Because each bound Ca\(^{2+}\) ion would be severalfold slower to leave in STIM1-gated channels, it could prevent many more Na\(^{+}\) ions from passing through than in 2-APB–gated channels. However, it is also possible that the decreased Ca\(^{2+}\) block dwell time is a consequence of enhanced Na\(^{+}\) permeation driven by repulsion from Na\(^{+}\) ions destabilizing Ca\(^{2+}\) binding (Fig. 3 B). In this case, a faster entry rate for Na\(^{+}\) would naturally bias selectivity toward Na\(^{+}\).

A third possibility is that the CRAC channel pore contains additional Ca\(^{2+}\) binding sites distinct from the single block site examined here. In this scenario, new Ca\(^{2+}\) binding sites that are not readily detected at low (micromolar) concentrations of extracellular Ca\(^{2+}\) used to gauge block come into play at millimolar concentrations to confer high Ca\(^{2+}\) selectivity of STIM1-gated channels. This argument is undermined by the fact that molecular and structural studies reveal only a single locus for Ca\(^{2+}\) influenced not only by the steady-state Ca\(^{2+}\) binding affinity to the selectivity filter but also by the kinetic properties of ion entry. Many studies have considered the idea that selectivity is governed by the rates of ion entry into the channel, with less selective channels exhibiting slower rates of ion entry into the selectivity filter (Grabe et al., 2006; Nimigean and Allen, 2011). Applying this idea to Orai3 channels, one scenario is that the weakly Ca\(^{2+}\)-selective 2-APB–gated Orai3 channels exhibit a slower rate of Ca\(^{2+}\) entry into the high affinity binding site than the highly Ca\(^{2+}\)-selective STIM1-gated channels. This appears to the case for E81D Orai3 mutant channels, which exhibit a 10-fold slower \(k_{on}(10^5 \text{M}^{-1}\text{s}^{-1})\), reaffirming the importance of high affinity binding at the selectivity filter for Ca\(^{2+}\) selectivity. However, this explanation cannot account for the lower Ca\(^{2+}\) selectivity of 2-APB–gated WT Orai3 channels because our estimates of the second-order on rates suggest that \(k_{on}\) is actually faster in 2-APB–gated channels (~10\(^7\) M\(^{-1}\text{s}^{-1}\)) than in STIM1-gated channels (~10\(^6\) M\(^{-1}\text{s}^{-1}\)).

A closely related kinetic possibility is that channels with lower Ca\(^{2+}\) selectivity exhibit transient (rather than stable) binding compared with channels with high Ca\(^{2+}\) selectivity. Indeed, estimates of Ca\(^{2+}\) dwell times extrapolated from the \(k_{off}\) measurements indicate that 2-APB–gated channels exhibit considerably shorter dwell times than STIM1-gated channels. If this is also true at millimolar concentrations of extracellular Ca\(^{2+}\) where Ca\(^{2+}\) permeation occurs (which admittedly is an unsubstantiated assumption), the decreased dwell time of Ca\(^{2+}\) occupancy could lead to diminished Ca\(^{2+}\) selectivity by allowing a higher level of net Na\(^{+}\) permeation between Ca\(^{2+}\) binding events at the block site. Because each bound Ca\(^{2+}\) ion would be severalfold slower to leave in STIM1-gated channels, it could prevent many more Na\(^{+}\) ions from passing through than in 2-APB–gated channels. However, it is also possible that the decreased Ca\(^{2+}\) block dwell time is a consequence of enhanced Na\(^{+}\) permeation driven by repulsion from Na\(^{+}\) ions destabilizing Ca\(^{2+}\) binding (Fig. 3 B). In this case, a faster entry rate for Na\(^{+}\) would naturally bias selectivity toward Na\(^{+}\).

A third possibility is that the CRAC channel pore contains additional Ca\(^{2+}\) binding sites distinct from the single block site examined here. In this scenario, new Ca\(^{2+}\) binding sites that are not readily detected at low (micromolar) concentrations of extracellular Ca\(^{2+}\) used to gauge block come into play at millimolar concentrations to confer high Ca\(^{2+}\) selectivity of STIM1-gated channels. This argument is undermined by the fact that molecular and structural studies reveal only a single locus for Ca\(^{2+}\).
binding in the pore composed of conserved Glu residues in TM1 (E106 in Orai1 and E81 in Orai3). The reminder of the pore (TM1) is lined with hydrophobic and basic residues that are not supportive for Ca\(^{2+}\) binding. However, a key limitation of the current study is the experimental disconnect between permeability measurements, which can only be performed at high (millimolar) Ca\(^{2+}\) concentrations and Ca\(^{2+}\) block measurements, which are performed at low (micromolar) Ca\(^{2+}\) concentrations. This disconnect is exemplified by the vast difference in Ca\(^{2+}\) affinities seen at low and high extracellular Ca\(^{2+}\) concentrations: the \(K_d\) of Ca\(^{2+}\) blockade of Na\(^{+}\) flux is \(\sim 20 \mu M\), but saturation of I_{CRAC} occurs at millimolar Ca\(^{2+}\) concentrations \((K_m = 1–3 mM; Hoth and Penner, 1993; Premack et al., 1994; Fierro and Parekh, 2000). In fact, this is a common theme in many classes of ion channels: as multi-ion occupancy of the pore increases at high ionic strengths, the apparent affinity of Ca\(^{2+}\) binding to the pore declines (Hille 2001), likely through repulsive interactions between closely spaced ions in the pore. One plausible scenario through which this could occur is if the side chains of the six Glu residues in TM1 cluster into two groups along the axis of the pore to form two binding sites. Because these sites would be in close proximity, electrostatic repulsion between the closely spaced Ca\(^{2+}\) ions could reduce the apparent affinity of Ca\(^{2+}\) binding at the selectivity filter (Almers and McCleskey, 1984; Sather and McCleskey, 2003), potentially explaining the much lower dependence of I_{CRAC} on extracellular Ca\(^{2+}\).

A final possibility is that the change in selectivity arises directly as a consequence of a change in the charge/volume ratio because of structural enlargement of the pore. Some models of Ca\(^{2+}\) selectivity have concluded that Ca\(^{2+}\) selectivity in L-type voltage-gated Ca\(^{2+}\) channels is driven by a charge/space competition mechanism wherein selectivity arises from a balance of electrostatics and the excluded volume of ions in the crowded selectivity filter (Nonner et al., 2000; Sather and McCleskey, 2003; Malasics et al., 2009). Ca\(^{2+}\) is selected over Na\(^{+}\) because its higher charge more effectively neutralizes the negative charge within the narrow confines of the selectivity filter than the monovalent charge of Na\(^{+}\) ions. These models predict that squeezing the volume of the selectivity filter by narrowing the pore should generally result in preference for Ca\(^{2+}\) over Na\(^{+}\) ions. Conversely, increasing the pore size (as seen in the 2-APB–gated channels) should result in greater monovalent occupancy because of relaxation of the excluded volume and space/charge constraints. Such a model is certainly qualitatively consistent with our experimental observations and more work is needed to illuminate the extent to which volume exclusion and the higher charge/size ratio contributes to Ca\(^{2+}\) selectivity in CRAC channels.

Energetics of Ca\(^{2+}\) binding

To get an initial understanding of how alterations in the energetics of ion conduction influence Ca\(^{2+}\) selectivity, we considered a simple barrier model of the pore based binding in the pore composed of conserved Glu residues in TM1 (E106 in Orai1 and E81 in Orai3). The reminder of the pore (TM1) is lined with hydrophobic and basic residues that are not supportive for Ca\(^{2+}\) binding. However, a key limitation of the current study is the experimental disconnect between permeability measurements, which can only be performed at high (millimolar) Ca\(^{2+}\) concentrations and Ca\(^{2+}\) block measurements, which are performed at low (micromolar) Ca\(^{2+}\) concentrations. This disconnect is exemplified by the vast difference in Ca\(^{2+}\) affinities seen at low and high extracellular Ca\(^{2+}\) concentrations: the \(K_d\) of Ca\(^{2+}\) blockade of Na\(^{+}\) flux is \(\sim 20 \mu M\), but saturation of I_{CRAC} occurs at millimolar Ca\(^{2+}\) concentrations \((K_m = 1–3 mM; Hoth and Penner, 1993; Premack et al., 1994; Fierro and Parekh, 2000). In fact, this is a common theme in many classes of ion channels: as multi-ion occupancy of the pore increases at high ionic strengths, the apparent affinity of Ca\(^{2+}\) binding to the pore declines (Hille 2001), likely through repulsive interactions between closely spaced ions in the pore. One plausible scenario through which this could occur is if the side chains of the six Glu residues in TM1 cluster into two groups along the axis of the pore to form two binding sites. Because these sites would be in close proximity, electrostatic repulsion between the closely spaced Ca\(^{2+}\) ions could reduce the apparent affinity of Ca\(^{2+}\) binding at the selectivity filter (Almers and McCleskey, 1984; Sather and McCleskey, 2003), potentially explaining the much lower dependence of I_{CRAC} on extracellular Ca\(^{2+}\).

A final possibility is that the change in selectivity arises directly as a consequence of a change in the charge/volume ratio because of structural enlargement of the pore. Some models of Ca\(^{2+}\) selectivity have concluded that Ca\(^{2+}\) selectivity in L-type voltage-gated Ca\(^{2+}\) channels is driven by a charge/space competition mechanism wherein selectivity arises from a balance of electrostatics and the excluded volume of ions in the crowded selectivity filter (Nonner et al., 2000; Sather and McCleskey, 2003; Malasics et al., 2009). Ca\(^{2+}\) is selected over Na\(^{+}\) because its higher charge more effectively neutralizes the negative charge within the narrow confines of the selectivity filter than the monovalent charge of Na\(^{+}\) ions. These models predict that squeezing the volume of the selectivity filter by narrowing the pore should generally result in preference for Ca\(^{2+}\) over Na\(^{+}\) ions. Conversely, increasing the pore size (as seen in the 2-APB–gated channels) should result in greater monovalent occupancy because of relaxation of the excluded volume and space/charge constraints. Such a model is certainly qualitatively consistent with our experimental observations and more work is needed to illuminate the extent to which volume exclusion and the higher charge/size ratio contributes to Ca\(^{2+}\) selectivity in CRAC channels.

Energetics of Ca\(^{2+}\) binding

To get an initial understanding of how alterations in the energetics of ion conduction influence Ca\(^{2+}\) selectivity, we considered a simple barrier model of the pore based
on Eyring rate theory. Rate theory descriptions of the pore in which the ion conduction pathway is depicted as a series of discrete energy wells separated by barriers that limit ion conduction have been widely used to understand the major forces that regulate selectivity and conduction in ion channels (Begenisich and Cahalan, 1980; Hille, 2001). Such models admittedly have limitations in their ability to capture the molecular attributes of the pore such as locations and chemistry of binding sites and the physical basis of energy barriers (Hille, 2001). Nonetheless, they often yield useful insights on the energetic influences of binding sites and barriers and can provide qualitative forecasts of the effects of their changes on ion selectivity. We first considered the simplest model possible: a two-barrier, one binding site model to examine blockade of Na+ ions by micromolar concentrations of Ca2+. The Gibbs free energy of the Ca2+ site can be calculated from the relation:

$$G_{Ca} = RT \ln K_d.$$  

(6)

Substituting the observed values of $K_d$ at $-100 \text{ mV}$ (Table 1), the relationship specifies a well depth of 11–12 RT units (depending on the extracellular Na+ concentration) for the Ca2+ site. $G_{Ca}$ declines to 9 RT units in the E81D Orai3 mutant channels ($K_d$ of $\sim 110 \mu M$).

The rate at which an ion moves from one site to another equals the product of the rate constant for this transition and the probability of occupancy of the source site by that ion. For the energy profile diagrammed in Fig. 9 A, the rate constant $k_i$ is given by:

$$k_i = v e^{-G_i/RT},$$  

(7)

indicating that entry rate declines exponentially with increasing barrier height. $v$ represents the maximum possible reaction rate set by the thermal vibrational frequency and has the value of $5.8 \times 10^{12} \text{ s}^{-1}$ at room temperature ($v = kT/h$, where $k$ is the Boltzmann constant and $h$ is the Planck’s constant). For STIM1-gated channels, if the second-order $k_{on}$ is approximated as the rate of Ca2+ entry into the pore, then the energy of this transition is given by relation:

$$G = RT \ln \frac{k_i}{v},$$  

(8)

yielding an outer barrier height of 14–14 RT. Thus, a Ca2+ ion lodged at the well faces a total energy barrier of 26 RT units to escape back outside. To estimate the height of the cytoplasmic barrier, we used $k_{off}$ estimated from the single-site model ($33 \text{ s}^{-1}$). Under our experimental conditions ($V_m = -100 \text{ mV}$), if we assume that this is due escape of Ca2+ into the intracellular compartment, this rate specifies an energy of 26 RT units, reflecting the total energetic barrier faced by Ca2+ ions lodged at the binding site. Thus, the height of the inner barrier is 26 − 12 or 14 RT units, roughly identical to the external barrier. The values of $k_{on}$ and $k_{off}$ increase to $3 \times 10^7 \text{ M}^{-1}\text{s}^{-1}$ and 246 s$^{-1}$, respectively, in 2-APB–gated channels. From Eq. 8, these correspond to energies of 12 and 24 RT units for the entry and exit of Ca2+ ions from the binding site. Thus, barrier heights for Ca2+ decline by roughly 2 RT units in 2-APB–gated channels compared with STIM1-gated channels.

**Energetics of Ca2+ permeation**

The single-site model yields Ca2+ unbinding rates ($33 \text{ s}^{-1}$ in STIM1-gated channels and 245 s$^{-1}$ in 2-APB–activated channels) that are vastly lower than the Ca2+ flux rates observed at millimolar extracellular Ca2+ concentrations (11,000 s$^{-1}$ calculated from a unitary current of $\sim 3.7 \text{ pA at } -80 \text{ mV}$ [Zweifach and Lewis, 1993]). This paradox, well described in voltage-gated Ca2+ channels (Sather and McCleskey, 2003), indicates that single-site models cannot model permeation of Ca2+ at high Ca2+ concentrations. We therefore considered a four-barrier three-site model similar to the one previously described for voltage-gated Ca2+ channels with a single high affinity binding site flanked by two low affinity sites, allowing occupancy of the pore by multiple ions (Dang and McCleskey, 1998). Like voltage-gated Ca2+ channels (Sather and McCleskey, 2003), CRAC channels exhibit two Ca2+ affinities—a relatively high affinity of 25 µM (−11 RT units) for Ca2+ blockade of Na+ currents (Su et al., 2004; Prakriya and Lewis, 2006; Yamashita et al., 2007) and a low affinity (we used a value of 3.3 mM or −6 RT units [Hoth and Penner, 1993]) for saturation of the Ca2+ current. Thus, as extracellular Ca2+ is raised from micro- to millimolar concentrations, the apparent affinity of the pore declines by several orders of magnitude, ensuring that ions are not permanently trapped in the pore. In studies of voltage-gated Ca2+ channels, this change has been modeled as arising either because of electrostatic repulsion between two closely spaced Ca2+ ions located at the selectivity filter (Almers and McCleskey, 1984; Friel and Tsien, 1989) or because of the presence of multiple shallow, low affinity sites flanking a single high affinity site (Dang and McCleskey, 1998), conferring in effect a staircase of multiple rising steps at the exits of the free-energy profile. We used the latter model to evaluate whether changes in barrier heights influence the relative Ca2+ selectivity of CRAC channels.

Values of the barrier heights (~14 RT units) and the central well depth (~12 RT units) were kept close to estimates in the single-site model. The validity of these energies for the high Ca2+ condition was confirmed from analysis of the rates of permeation at high Ca2+ concentrations. At high (millimolar) Ca2+ concentrations under which Ca2+ permeation occurs, the off rate for Ca2+ conduction is $k_{off} = k_{on} \times K_{M}$. Substituting the values of $k_{on}$ ($4 \times 10^6 \text{ M}^{-1}\text{s}^{-1}$) and $K_{M}$ (3.3 mM) yields a $k_{off}$ of $\sim 12,000 \text{ ions s}^{-1}$. This rate is very close to the
observed flux rate of ions from the unitary Ca\textsuperscript{2+} current amplitude determined from noise measurements (∼3–4 fA at −80 to −100 mV, or ∼11,000 s\textsuperscript{−1}; Zweifach and Lewis, 1993; Prakriya and Lewis, 2002), indicating that the \( k_{in} \) estimate obtained from the single-site model provides a reasonable rate of Ca\textsuperscript{2+} entry into the pore even at high Ca\textsuperscript{2+} concentrations. Likewise, a flux rate of 12,000 ions/s corresponds to a total energy barrier of ∼20 RT units from Eq. 8. As noted in the previous paragraph, Ca\textsuperscript{2+} flux saturates with a \( K_{M} = 3.3 \) mM, which specifies a well depth of −6 RT units during Ca\textsuperscript{2+} permeation (Eq. 6). With a total energy barrier of 20 RT units from the deepest to the highest point of the energy landscape during Ca\textsuperscript{2+} permeation and an apparent well of −6 RT units, the inner barrier height should be \( 20 - 6 = 14 \) RT units, identical to value estimated from the single-site model. We placed the wells toward the extracellular region of the pore (at electrical distances of 0.15, 0.2, and 0.3 units) to account for the known positions of E81 and the TM1–TM2 acidic residues (E85/D87/E89) in the outer vestibule (Fig. 3 F).

For Na\textsuperscript{+} ions, the unitary Na\textsuperscript{+} currents of STIM1- and 2-APB–gated channels from noise analysis are 0.08 and ∼0.3 pA, respectively, corresponding to off-rate energies of 17 and 14 RT units, respectively. Our tests indicate that the Na\textsuperscript{+} current saturates with a \( K_{M} \) of ∼90 mM (or −2.4 RT units; unpublished data). With the reasonable assumption that this binding is predominantly governed by the selectivity filter, the barrier heights thus can be determined to be ∼14 and 12 RT units in STIM1- and 2-APB–gated channels, similar to the values for Ca\textsuperscript{2+}. The major difference in energy profiles experienced by Ca\textsuperscript{2+} and Na\textsuperscript{+} is thus the absence of a high affinity central binding site for Na\textsuperscript{+}.

With these values of barrier heights and well depths, we next computed the I–V profiles using the four-barrier, three-well model (Dang and McCleskey, 1998). For STIM1-gated channels, the simulated I–V profiles qualitatively matched the experimental I–Vs seen in Orai3 channel activated by STIM1 (Fig. 9, B and C). As predicted from the deep well depth and high barriers for ion flow, the I–Vs showed strong inward rectification with \( V_{m} \) at very positive voltages, typical of STIM1-gated Orai channels. The proportion of current carried by monovalent ions was negligible both at negative and positive voltages (Fig. 9 B). Decreasing the well depth by 3 RT units to mimic the diminished Ca\textsuperscript{2+} block affinity of the E81D Orai3 mutant reduced Ca\textsuperscript{2+} selectivity and resulted in greater monovalent permeation as seen experimentally (Fig. 9 B). This latter finding reaffirms the critical importance of the high affinity Ca\textsuperscript{2+} binding site for Ca\textsuperscript{2+} selectivity. Thus, the model qualitatively reproduces many key experimental features of STIM1-activated Orai3 currents.

To understand why the 2-APB–gated channels exhibit lower Ca\textsuperscript{2+} selectivity, we next altered the parameters of the model to mimic those seen in 2-APB–gated channels. Lowering the outer barriers by 3 RT units for both Na\textsuperscript{+} and Ca\textsuperscript{2+} ions while leaving the well depths unchanged elicited profound changes in ion selectivity (Fig. 9 C). The theoretical I–Vs revealed a large leftward shift in the \( V_{m} \) and a significant outwardly rectifying monovalent current, in accordance with the properties observed in 2-APB–gated channels. Importantly, the fraction of inward current carried by Ca\textsuperscript{2+} was significantly diminished compared with the model for STIM1-gated channels. A shallower well depth resulted in even less Ca\textsuperscript{2+} selectivity and greater monovalent ion permeation (unpublished data), as seen in the E81D mutant, again reaffirming the importance but not the exclusive role of the high affinity site for Ca\textsuperscript{2+} selectivity. Thus, these findings reveal that in addition to the well-established influence of the well depth, barrier heights are critical determinants of Ca\textsuperscript{2+} selectivity in CRAC channels. In particular, the results indicate that structural changes that produce broad alterations in barrier heights for permeant ions can elicit robust effects on Ca\textsuperscript{2+} selectivity, even if the well depth for Ca\textsuperscript{2+} binding is unchanged. We consider a possible mechanistic basis of this feature in the following paragraph.

Ca\textsuperscript{2+} selectivity in voltage-gated Ca\textsuperscript{2+} channels, which have a relatively large pore, yet are highly selective for Ca\textsuperscript{2+}, has been traditionally viewed as a prime example of selectivity by affinity. In these channels, the high pore affinity for Ca\textsuperscript{2+} (\( K_{M} \) of ∼0.7 µM) is believed to form the basis of the physiologically important selectivity of the channels for Ca\textsuperscript{2+} over the more prevalent Na\textsuperscript{+} and K\textsuperscript{+} ions (Sather and McCleskey, 2003). In CRAC channels, affinity of Ca\textsuperscript{2+} binding (\( K_{M} \) of Na\textsuperscript{+} current blockade) is at least 25-fold lower, yet these channels exhibit comparably high Ca\textsuperscript{2+} selectivity, suggesting that CRAC channels achieve selectivity through additional mechanisms.

Based on the experimental and modeling results presented in this study, we propose that in addition to specific Ca\textsuperscript{2+} binding, high Ca\textsuperscript{2+} selectivity of CRAC channels may be attributed, at least in part, to the rejection of all ions (both preferred and non-preferred) by high energy barriers. Enhancing the Na\textsuperscript{+} and Ca\textsuperscript{2+} flux rates by lowering the entry and exit barriers paradoxically reduces Ca\textsuperscript{2+} selectivity as seen in the 2-APB–gated channels. This may be related to the decreased residence time of Ca\textsuperscript{2+} occupancy at the block site, thereby increasing opportunistic flow of Na\textsuperscript{+} ions between block events. Alternately, it could be related to enhanced Na\textsuperscript{+} ion entry rates into the pore, which could destabilize Ca\textsuperscript{2+} binding through a knockoff effect. More quantitative and modeling studies are needed to examine the mechanistic underpinnings of this effect, but the results presented in this study provide a basis for testing these and other models.

What is the physical basis of the wells and barriers in the Eyring model? It is straightforward to expect that the high affinity Ca\textsuperscript{2+} binding site is formed by the selectivity
filter, and the superficial cation binding site can also be plausibly assigned to the vestibule acidic residues (E85/D87/D89; Fig. 3 F). As argued above, an inner cation binding site can also be envisioned to arise at high Ca$^{2+}$ concentrations if the carboxylate side chains of the Glu residues at E81 cluster into two groups to form separate binding sites. It is more difficult, however, to envision the physical basis of the energy barriers, which may in fact not have any tangible physical correlate. The outer “barrier” may simply reflect the ease with which ions are dehydrated. The inner barrier may simply arise from a location in the pore where ions pause momentarily before moving to the next energy minimum. Still, these unknowns do not diminish the lessons of the Eyring rate analysis, for they illustrate the plausibility that both kinetic and thermodynamic factors shape Ca$^{2+}$ selectivity of Orai channels.

Biophysical similarity of 2-APB and STIM1 gating

Analysis of current noise indicates that activation of Orai3 channels by 2-APB occurs through a mechanism qualitatively similar to the stepwise recruitment of silent channels to a high $P_o$ mode described for gating of native CRAC channels by store depletion (Prakriya and Lewis, 2006) and activation of Orai3 channels by STIM1 (this study). In both gating modes, channel opening occurs slowly over time scales of seconds and involves recruitment of closed channels to a long-lasting high $P_o$ state ($P_o$ of $\sim$0.7). One simple explanation is that the slow gating mode of Orai channels is a channel intrinsic behavior, independent of the activation stimulus. However, given the strong nonlinearity of 2-APB activation (Hill coefficient of $\sim$8; Fig. 1 A), an alternative possibility is that the abrupt opening of single channels to the high $P_o$ state reflects the concerted action of multiple ligand molecules (two STIM1 molecules per Orai monomer for STIM1-gated channels [Li et al., 2010; Hoover and Lewis, 2011] and at least eight 2-APB molecules per channel for 2-APB-gated channels) that triggers stepwise channel opening. Either way, this operational similarity suggests that despite differences in the permeation and selectivity of open channels, STIM1 and 2-APB both use a similar molecular mechanism for gating the channel, possibly through related transduction mechanisms wherein the steps between ligand binding and pore opening are identical. This scenario is also consistent with a recent suggestion that 2-APB and STIM1 both use a graded activation mechanism to cause pore opening (Amcheslavsky et al., 2013). More studies are needed to elucidate the complete story of the concerted action of STIM1 and 2-APB on channel opening, taking into account the channel stoichiometry and the presence of multiple ligand binding sites on each Orai subunit.

The authors are grateful to Ted Begenisich for providing the computer program for the four-barrier, three-site Eyring rate model and to Ted Begenisich, Jon Sack, Chris Lingle, and Ed McCleskey for helpful consultations during the course of this work.

This research was supported by National Institutes of Health (grant NS057499).

The authors declare no competing financial interests.

Sharona E. Gordon served as editor.

Submitted: 24 September 2013
Accepted: 30 January 2014

REFERENCES


Yamashita and Prakriya 341

Downloaded from on June 20, 2017

Published February 24, 2014


Figure S1. Current–voltage relationships of WT Orai3 and Orai3 mutants in 20 mM Ca\textsuperscript{2+} Ringer’s solution when activated by STIM1 (A) or 2-APB (B). The reversal potentials ($V_{\text{rev}}$) of E81D and the E85A/D87A/E89A Orai3 mutants when activated by STIM1 are significantly left shifted compared with WT Orai3 channels ($V_{\text{rev}}$ of STIM1-gated WT: 71 ± 6 mV; STIM1-gated E81D: 5 ± 3 mV; STIM1-gated E85A/D87A/E89A: 20 ± 1 mV; 2-APB–gated WT: 25 ± 2 mV; 2-APB–gated E81D: 10 ± 2 mV; 2-APB–gated E85A/D87A/E89A: 25 ± 1 mV).

Figure S2. Time course of STIM1- and 2-APB–gated Orai3 Na\textsuperscript{+} currents in response to hyperpolarizing steps to $V_h$ = −100 mV. (A and B) Unlike STIM1-gated Orai3 channels, the hyperpolarizing step to $V_h$ = −100 mV induces a slow phase of current activation in 2-APB–gated channels. (C) The slow activation of Na\textsuperscript{+}-ICRAC current in 2-APB–gated channels at $V_h$ = −100 mV is diminished by hyperpolarizing the holding potential to $V_h$ = −30 mV, presumably by preactivating channels at the more hyperpolarized holding potential.
Figure S3. Power-spectrum analysis of Orai3 channels activated by STIM1 or 2-APB. (A and B). Spectra were collected either in 20 mM Ca$^{2+}$ or in DVF Ringer’s solutions containing the indicated free [Ca$^{2+}$]. Each trace is the mean of 3–10 spectra. There is negligible Orai3 current power above background levels at frequencies >10 kHz, indicating that current noise occurs well within the recording bandwidth. In B, the 20-mM Ca$^{2+}$ traces were collected before application of 2-APB (50 µM).