Stable Activation of Single Ca\(^{2+}\) Release-activated Ca\(^{2+}\) Channels in Divalent Cation-free Solutions*

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The regulation of store-operated, calcium-selective channels in the plasma membrane of rat basophilic leukemia cells (RBL-2H3 m1), an immortalized mucosal mast cell line, was studied at the single-channel level with the patch clamp technique by removing divalent cations from both sides of the membrane. The activity of the single channels in excised patches could be modulated by Ca\(^{2+}\), Mg\(^{2+}\), and pH. The maximal activation of these channels by divalent cation-free conditions occurred independently of depletion of intracellular Ca\(^{2+}\) stores, whether in excised patches or in whole cell mode. Yet, a number of points of evidence establish these single-channel openings as amplified store-operated channel events. Specifically, (i) the single channels are exquisitely sensitive to inhibition by intracellular Ca\(^{2+}\), and (ii) both the store-operated current and the single-channel openings are completely blocked by the capacitative calcium entry blocker, 2-aminoethoxydiphenyl borane. In addition, in Jurkat T cells single-channel openings with lower open probability have been observed in the whole cell mode with intracellular Mg\(^{2+}\) present (Kerschbaum, H. H., and Cahalan, M. D. (1999) Science 283, 836–839), and in RBL-2H3 m1 cells a current with similar properties is activated by store depletion.

In a variety of cells, binding of ligands such as neurotransmitters, hormones, or growth factors to receptors on the cell surface generates intracellular calcium signals. Receptor-mediated release of Ca\(^{2+}\) from IP\(_3\)-sensitive intracellular stores triggers an influx of Ca\(^{2+}\) into the cell via store-operated channels (SOCs) in the plasma membrane, a process termed “capacitative calcium entry” (1, 2). The resulting increase in intracellular Ca\(^{2+}\) concentration regulates important processes, ranging from cell growth and differentiation to apoptosis and cell death. Molecular candidates for SOCs are the mammalian homologues of the Drosophila TRP (transient receptor potential) protein (3). Expression of the related genes, however, has resulted in varying patterns of response, including constitutive activity, augmentation of capacitative calcium entry, apparent direct activation by IP\(_3\), and activation by diacylglycerol (4). Therefore, the mechanism by which emptying of intracellular Ca\(^{2+}\) stores activates SOCs remains elusive. Recent findings have favored a “direct coupling” model, whereby IP\(_3\) receptors in the store membranes sense the Ca\(^{2+}\)-filling status of the stores and transfer this information via direct coupling to sub-units of SOCs in the plasma membrane (5–8).

For the present, the best knowledge of SOCs comes from electrophysiological studies of a specific current associated with SOCs, known as the Ca\(^{2+}\) release-activated calcium current (I\(_{\text{crac}}\)) (9). Despite extensive characterization of the macroscopic CRAC currents, the single-channel signature is poorly understood. This is because the channels are presumed to have a minute single-channel conductance, too low to be resolved at the single-channel level (10). During whole cell measurements in Jurkat T-lymphocytes, recently, Kerschbaum and Cahalan (11) were able to detect for the first time single-channel events which they attributed to CRAC channels. Their approach was to measure currents in the complete absence of intra- and extracellular divalent cations. They reasoned that under these conditions CRAC channels, like voltage-gated Ca\(^{2+}\) channels should pass monovalent cations indiscriminately and with enhanced single-channel conductance (12–14). Indeed, in the absence of divalent cations large nonselective cation currents were observed, and during the initial stage of activation, single-channel conductances of 36 to 40 pS were measured in whole cell mode (11). However, the relationship of these large nonselective cation currents to the process of store depletion, and thus their identity as CRAC channels, has not been definitively established.

Here we report similar single-channel activities measured for the first time in excised plasma membrane patches, using a mast cell line (RBL-2H3 m1) and divalent cation-free solutions. Our whole cell data are similar in some respects to those from Jurkat T-lymphocytes. In addition, because of direct access to the cytoplasmic surface of the channels, we have elucidated kinetic and regulatory properties of the channels. In particular, we have sought to determine whether or not these cation channels truly represent CRAC channels. In divalent cation-free solutions excised channels of 25 to 39 pS conductance showed stable activation, independent of IP\(_3\) or IP\(_3\) receptors, and were blocked by 2-APB, a relatively specific inhibitor of SOC activation (15, 16). The effects of 2-APB, as well as the modulation of channel activity by Ca\(^{2+}\) provide evidence that the Na\(^{+}\)-conducting channels are in fact single CRAC channels. Hence, this report offers new insight into the possible regulation mechanism of native SOCs and provides a novel approach to investigate these channels under low divalent conditions.

MATERIALS AND METHODS

Cell Culture—Rat basophilic leukemia cells (RBL-2H3 m1), an immortalized mucosal mast cell line expressing m1 muscarinic receptors, were obtained from Dr. M. Beaven, National Institutes of Health (17). The cells were cultured in Earle’s minimal essential medium with Earle’s salts, 10% fetal bovine serum, 4 mM l-glutamine, 50 units/ml penicillin, and 50 mg/ml streptomycin (37 °C, 5% CO\(_2\)). For experi-

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ments, cells were plated onto glass coverslips and used 12–36 h there after.

Electrophysiology—Patch clamp experiments were performed at 20–22 °C in the tight-seal whole cell, cell-attached and inside-out configurations (19). Patch pipettes were pulled from borosilicate glass (Corning glass, 7052) and fire polished. Membrane currents, filtered at 1–2 kHz, were recorded using an Axopatch-200B amplifier (Axon Instruments, Burlington, CA). Voltage clamp protocols were implemented and data acquisition performed with pCLAMP 7.0 Software (Axon Instruments). Solution changes were accomplished by bath perfusion. The time required for a complete change was around 2 s. All voltages were corrected for a liquid junction potential.

For whole cell experiments with Ca\(^{2+}\) as charge carrier, unless stated otherwise, the patch pipette (2–5 megohm) solutions had the following composition (in mM): 140 Cs\(^{+}\)-aspartate, 2 MgCl\(_2\), 1 MgATP, 10 Cs\(^{+}\)-BAPTA (with free calcium set to 100 nM, calculated using MaxChelator software, version 6.60), 10 HEPS (pH 7.2 with CsOH). The bath solutions contained (in mM) 140 NaCl, 4.7 KCl, 10 CsCl, 1.1 MgCl\(_2\), 10 glucose, 10 CaCl\(_2\), 10 HEPS (pH 7.2 with NaOH). Divalent free whole cell measurements, with Na\(^+\) as charge carrier, were done with pipettes containing (in mM) 128 Cs\(^{+}\)-aspartate, 12 Cs\(^{+}\)-BAPTA, 0.9 CsCl\(_2\) (free Ca\(^{2+}\)~5 nM), 10 HEPS (pH 7.2 with NaOH), and bath solutions containing (in mM) 150 Na\(^+\) methanesulfonate, 2 EDTA, 10 HEPS (pH 7.2 with NaOH). CRAC channels were opened by passive store-depletion with high BAPTA (12 mM) in the pipette solution. With external Ca\(^{2+}\) present (Fig. 1a), Ca\(^{2+}\) ions permeate through the open CRAC channels and the Ca\(^{2+}\)-influx during progressive channel activation could be monitored as a developing small inward current, of 30 to 60 pA (2–3 pA/pF) \((n = 5)\). Single-channel currents underlying the macroscopic Ca\(^{2+}\) currents were too small to be resolved, even at ~120 mV membrane potential.

With divalent free solutions and Na\(^{+}\) as the permeant ion, however, single-channel events have been observed during whole cell measurements in Jurkat T-lymphocytes (11). We first sought to determine whether similar currents could be observed with rat basophilic leukemia (RBL-2H3 m1) cells. The current development in Fig. 1b illustrates activation of a large inward current under divalent cation-free conditions \((n = 6)\). The latency for current activation after break in, and the development time to reach the peak macroscopic current, were characteristically 1.5 to 2 times longer for the current in divalent-free solutions, than for \(I_{\text{crac}}\) in Ca\(^{2+}\)-containing solutions. In the absence of divalent cations, dialysis of the cell with the pipette solution typically activated macroscopic currents up to 2000 pA (~130 pA/pF), or ~40-fold greater than \(I_{\text{crac}}\). The current-voltage relationship of the fully developed current showed a modest inward rectification. External addition of 10 \(\mu\)M Mg\(^{2+}\) inhibited the monovalent currents in a voltage-dependent manner (data not shown).

During the initial activation of the large cation current (see Fig. 1b), we were able to detect single-channel openings in RBL-2H3 m1 cells (Fig. 1c). We recorded single-channel inward currents of 3.3 to 4 pA, during voltage steps to ~100 mV, giving conductances of 33 to 40 pS \((n = 6)\), assuming a reversible potential of 0 mV, see below). Based on the whole cell current amplitudes and the unitary current of the single-channels, we calculated the number of channels per cell to be 260 to 500

**RESULTS**

**CRAC Currents and Monovalent Currents in RBL-2H3 m1 Cells**—Ca\(^{2+}\) release-activated Ca\(^{2+}\) currents can be activated by a variety of procedures that share the common property of emptying intracellular IP\(_3\)-sensitive stores. Stores can be depleted actively by exposure to receptor agonists that elevate IP\(_3\) levels, external application of Ca\(^{2+}\)-ionophores like ionomycin, or the presence of IP\(_3\) in the patch pipette solution (19). Passive methods for store depletion rely on a constitutive and ill-defined leak of Ca\(^{2+}\) from stores. If store refilling is prevented by inhibition of SERCA pumps or by high concentrations of cytoplasmic Ca\(^{2+}\) chelators, the stores gradually lose their Ca\(^{2+}\).

In the whole cell measurements shown in Fig. 1, we used the latter method and activated CRAC channels by breaking into the cell, while including high concentrations of the Ca\(^{2+}\)-chelator, BAPTA (12 mM), in the pipette solution. With external Ca\(^{2+}\) present (Fig. 1a), Ca\(^{2+}\) ions permeate through the open CRAC channels and the Ca\(^{2+}\)-influx during progressive channel activation could be monitored as a developing small inward current, of 30 to 60 pA (2–3 pA/pF) \((n = 5)\). Single-channel currents underlying the macroscopic Ca\(^{2+}\) currents were too small to be resolved, even at ~120 mV membrane potential.

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channels ($n = 6$). This suggests an average surface density of ~0.22 channels per $\mu m^2$.

**Stable Activation of Single Cation Channels in Excised Plasma Membrane Patches**—To gain access to the cytoplasmic surface of the channels, and to limit the number of investigated channels, we attempted to record the activity of individual channels in excised plasma membrane patches. Sodium ions (150 mM) were used as the primary charge carriers. Starting from cell-attached patches with no channel activity, we excised the patches into divalent cation-free solutions, while clamping the cell’s membrane potential to ~73 mV. In 65 out of 95 inside-out patches this procedure induced a channel activity, with an average conductance of 31 pS (25 pS to 39 pS) (Fig. 2, a and b). We conclude that the channel activity seen after excision corresponds to the channels underlying the macroscopic current activated by divalent cation-free solutions; the single-channel conductance found in excised patches was similar to the conductance recorded in the whole cell mode (Fig. 1c), the channels appear to have similar lack of selectivity, and the channels have similar, high open probability under total divalent cation-free conditions.

When patches were excised into divalent-free media, the channels apparently needed no additional factors to be activated, such as IP$_3$ or exogenously added IP$_3$ receptors (7, 20). However, if fragments of endoplasmic reticulum stores were still attached to the patches, the absence of ATP, Ca$^{2+}$, and Mg$^{2+}$ from the intracellular solutions could have induced store depletion, and thus caused or contributed to channel activation.

Continuous channel activity began an average of 70 to 80 s after excision (Fig. 2c) and was stable during recording time for at least 20 min, even after extensive washing. In 32% of the experiments the progressive opening of 6 and more channels in a patch could be observed, which may suggest clustering of CRAC channels in specific membrane domains.

**Single-channel Properties of Excised Channels, Activated by Divalent Cation-free Solutions**—We examined the single-channel properties of channels in inside-out patches, in divalent cation-free solutions. Fig. 3a shows recordings of 2 active channels in an excised inside-out patch, at different membrane potentials. The channels had a high open probability (0.94 ± 0.03 at ~113 mV, $n = 5$). The duration of the closures from the open state decreased with hyperpolarization, from $\tau = 1.19 \pm 0.13$ (± S.E.) ms at ~73 mV to $\tau = 0.50 \pm 0.06$ (± S.E.) ms at ~113 mV ($n = 7$ cells). From traces such as those shown in Fig. 3a, current amplitudes were measured manually or determined from all points amplitude histograms (Fig. 3b), over a voltage range from ~113 to +67 mV. The resulting current-voltage relationships (Fig. 3c) gave an average single-channel conductance of 31 pS (25 to 39 pS). Moreover, the IV-relationship was linear with a reversal potential close to 0 mV. As the major cation in the intracellular solution was K$^+$, while that in the pipette was Na$^+$, this is indicative of a channel that passes monovalent cations indiscriminately (12). The IV-relationship is similar to that for the whole cell current, except the latter is slightly inwardly rectifying, due to a small effect of voltage on open probability (not shown). Another specific channel feature, the mean open time, was calculated to be $14.9 \pm 1.2$ (± S.E.) ms ($n = 7$) (Fig. 3d).

**Effect of Ca$^{2+}$-store Depletion on the Activation of Monovalent Currents and CRAC Currents**—The results in Fig. 1 show that the activation of the large cation current by divalent cation-free solutions was considerably slower than the activation of $I_{\text{crac}}$, despite the expectation that passive store emptying by BAPTA should occur at a similar rate in the two conditions. This would indicate that the activation of cation channels by divalent cation-free solutions involves a mechanism other than, or in addition to store depletion. Alternatively, it is possible that the absence of divalent cations slows the mechanism for coupling store depletion to channel activation. To address these possibilities, we carried out experiments in which Ca$^{2+}$-stores were emptied and CRAC channels thus activated prior to going whole cell or excision into divalent cation-free media. Before sealing on the cells, we applied either 1 $\mu M$ of the SERCA inhibitor, thapsigargin plus 500 nM of the ionophore ionomycin, or 500 nM ionomycin alone. The incubation time with these drugs was minimally 5 min, by which time stores should be completely depleted and the currents should have fully developed (21). Subsequently, we patched the cells, and established either the whole cell configuration with either Ca$^{2+}$ or Na$^+$ as charge carriers, or excised the patch (inside-out mode) at ~73 mV into divalent-free solutions. In the whole cell experiments, the pipette solutions included high concentrations of BAPTA to passively deplete stores in the control cells.
Prior depletion of Ca\textsuperscript{2+}-stores resulted in preactivation of \( I_{\text{crac}} \) with about 2 pA/pF (7/7) when measured with Ca\textsuperscript{2+} as the charge carrier (Fig. 4, a and b, inset). However, directly after switching to Na\textsuperscript{+} in divalent free solutions, the current only increased 4-fold (Fig. 4b, inset), corresponding to about 10% of activation compared with the peak macroscopic divalent free currents (Fig. 4b). This 4-fold activation may correspond to the current seen in Jurkat cells by Kerschbaum and Cahalan (11) in the absence of external divalent cations, but with Mg\textsuperscript{2+} present in the cytoplasm. However, in the RBL-2H3 cells no larger, transient inward current was observed. Similar kinetics for RBL-1 cells have been reported by Fiero et al. (22). Following this 4-fold jump, which was only seen in the preactivated cells, a slower activation to the full, maximally conducting state was observed, and the kinetics of this process were similar in control and preactivated cells. Likewise, the appearance of single-channel openings following excision into divalent cation-free solutions occurred with a similar delay when stores were previously emptied (Fig. 4c).

Taken together, these results demonstrate that the delay time for whole cell current activation by divalent cation-free solutions, as well as the delay time for channel activation following excision into divalent cation-free solutions does not appear to depend on depletion of Ca\textsuperscript{2+}-stores. However, they do not rule out a requirement for initial store depletion, followed by a subsequent amplification step, which is rate-limiting. Nonetheless, the mechanism by which observable single-channel activation occurs appears to be at least somewhat distinct from the mechanism of activation of \( I_{\text{crac}} \) by store depletion. Note, however, that activation of an intermediate activity state of the channels, similar to that seen with low divalent cations outside and with Mg\textsuperscript{2+} inside (11, 22), did appear to be hastened by prior depletion of Ca\textsuperscript{2+}-stores. The significance of this observation will be considered subsequently under “Discussion.”

Modulation of Channel Activity in Excised Patches by Internal Ca\textsuperscript{2+}, Mg\textsuperscript{2+}, and pH—To further address the issue of whether the channels activated by divalent cation-free solutions represent CRAC channels, we next examined the modulation of these channels by internal divalent cations and by pH to compare their behavior which was shown previously for CRAC channels.

Krause et al. (23) reported from whole cell studies in RBL-1 cells, that \( I_{\text{crac}} \) activation changed from spontaneous to store-operated mode, if the cytosolic [Ca\textsuperscript{2+}] is elevated to more than 50 nM. The spontaneous \( I_{\text{crac}} \) was inactivated at a resting cytosolic [Ca\textsuperscript{2+}] of 105 nM. On the basis of their finding, after reaching full channel activity in the inside-out mode, we perfused the bath with intracellular solution containing 100 nM free Ca\textsuperscript{2+} (Fig. 5a). This concentration completely inhibited channel activity in 50.5 ± 9.5 s in 4 patches, while in 3 other patches channel activity was unaffected. In the 4 patches in which channel activity was inhibited, restoration of low Ca\textsuperscript{2+} conditions reversed the inhibition in only 1 of the 4 experiments. The inhibitory effect of Ca\textsuperscript{2+} was rather specific and not due to inhibition of monovalent currents by divalent ions per se, because similarly low concentrations of free Mg\textsuperscript{2+} failed to inhibit. However, if 100 \( \mu \)M Mg\textsuperscript{2+} or more was added to the inner surface of the active channels, inhibition developed in 13.4 ± 3.1 s (12 out of 12 patches) (Fig. 5b). The activity was restored by divalent free buffers (not shown). In both cases, as the inhibition developed, single channel conductance was unaffected indicating that the divalent cations suppress open probability.

In the whole cell mode in Jurkat T-lymphocytes, the inactivation of Na\textsuperscript{+} current by intracellular Mg\textsuperscript{2+} could be reduced by increasing the intracellular pH (14). The experiment in Fig. 5c demonstrates a similar effect of increasing pH for the channels in excised patches. After inhibition by 100 \( \mu \)M Mg\textsuperscript{2+} (pH 7.2), channel activity could be rescued by increasing the pH in the perfusion solution to pH 8.2 (4 out of 7 cells). The time needed for complete reversal in the cells that responded to the pH change was 42 ± 10 s. We also carried out experiments similar to those described by Kerschbaum and Cahalan (14) in which the channels were inhibited by addition of a much higher concentration of Mg\textsuperscript{2+} (2.4 mM). Channel activity was blocked in 7 of 7 experiments, but in three attempts there was no recovery on shifting to pH 8.0 (not shown).

From the experiments depicted in Fig. 5, some interesting observations can be made. First, the results provide evidence that the single-channels are the channels underlying the large whole cell current in divalent cation-free solutions. Second, the inhibition of the channels with Ca\textsuperscript{2+} and Mg\textsuperscript{2+} concentrations in the physiological range explains the inability to detect the same channels in the cell-attached mode. In the cell-attached mode, neither addition of 1 \( \mu \)M thapsigargin (\( n = 14 \)) nor addition of 500 nM ionomycin (\( n = 13 \)) evoked detectable single-channel activity. Note that under these conditions, whole cell...
excision into divalent cation-free buffers (73 mV $V_2$) the pH to pH 8.2. The time needed was 42 $10^6$ (12 out of 12).

Breaking in at 0 s was performed with pipette solutions containing 12 mM BAPTA ($[Ca^{2+}]$ present, is shown in the inset (0–25 s). Directly after switching to Na$^+$ in divalent free solutions the current density, compared with controls, increased 4-fold (inset). The ensuing current kinetics were similar in preactivated (average of 6 ± S.E.) and control cells (average of 6 ± S.E.). c, also, the appearance of single-channel openings following excision into divalent cation-free buffers (−73 mV $V_m$) occurred with a similar delay when stores were previously depleted with 500 nM ionomycin (72 ± 22 s, $n = 3$), compared with controls (70–80 s, see Fig. 2).

**FIG. 4. Effects of Ca$^{2+}$ store depletion on the activation of monovalent currents and CRAC currents.** a and b, the time courses are taken from whole cell measurements of control cells and cells preincubated for a minimum of 5 min with 1 μM thapsigargin, 500 nM ionomycin, added to the bath solution. Breaking in at 0 s was performed with pipette solutions containing 12 mM BAPTA ([Ca$^{2+}$] = 5 mM). a, with Ca$^{2+}$ as charge carrier $I_{crac}$ could be preactivated, with a current density of −2pA/pF (tracing in the figure is representative of three), compared with control cells (representative of six). To establish baseline current in control and preactivated cells, at 370 s Ca$^{2+}$ was replaced with equimolar Mg$^{2+}$ which does not permeate CRAC channels. b, preactivation of the same extent, with extracellular Ca$^{2+}$ present, is shown in the inset (0–25 s). Directly after switching to Na$^+$ in divalent free solutions the current density, compared with controls, increased 4-fold (inset). The ensuing current kinetics were similar in preactivated (average of 6 ± S.E.) and control cells (average of 6 ± S.E.). c, also, the appearance of single-channel openings following excision into divalent cation-free buffers (−73 mV $V_m$) occurred with a similar delay when stores were previously depleted with 500 nM ionomycin (72 ± 22 s, $n = 3$), compared with controls (70–80 s, see Fig. 2).

**Effects of the IP$_3$-receptor Antagonist 2-APB (2-Aminoethoxydiphenyl Borane) on $I_{crac}$ Activity**—Recent reports have supported a conformational coupling model for SOC activation, whereby IP$_3$ receptors in the endoplasmic reticulum sense the Ca$^{2+}$ filling status of the stores and transfer this information via direct coupling to SOCs in the plasma membrane (5–7). Ma et al. (16) concluded from their studies, using the membrane permeant IP$_3$ receptor inhibitor 2-APB that IP$_3$ receptors are essential not only for opening of SOCs, but also for maintaining their activation. On this basis, we examined the effects of 2-APB on macroscopic currents, and on channel activity in excised patches.

The results shown before in Fig. 2 demonstrate that no added IP$_3$ or added IP$_3$ receptors are necessary to maintain activity of CRAC channels in excised patches under divalent cation-free conditions. This is in contrast to previous reports demonstrating that Trp-3 channel activity (24) and store-operated channel activity in A431 cells (7) were rapidly lost in the absence of added IP$_3$ and IP$_3$ receptors. This may indicate that once activated under divalent-free conditions, CRAC channel activity is independent of Ca$^{2+}$-store depletion and coupling to IP$_3$ receptors (although we cannot unequivocally rule out the stable retention of fragments of endoplasmic reticulum and IP$_3$ receptors). Thus, we were initially not surprised to find that addition of 100 μM 2-APB to the medium surrounding activated channels in excised patches in most instances failed to inhibit channel activity (for example, Fig. 6d). However, with this protocol 2-APB is added to the cytoplasmic side of the plasma membrane, while in previous studies of Ma et al. (16), the drug was always added to the outside. Experiments illustrated in Fig. 6, a and b, show that in the whole cell configuration, with either Ca$^{2+}$ or Na$^+$ as charge carrier, 2-APB blocked the current completely when applied externally, but had significantly less effect when applied in the pipette. Finally, although 2-APB blocked channel activity when applied to the cytoplasmic side of excised patches in only 2 of 11 experiments (Fig. 6d), if

**FIG. 5. Modulation of channel activity by Ca$^{2+}$, Mg$^{2+}$, and pH.** a–c, representative open probability plots picture the time course of channel activity in inside-out patches, following application of the indicated ions to the inner surface of the cell membrane. The plots were created from recordings after patch excision into divalent free solutions, while the membrane was held at a potential of −73 mV. The additions were made several minutes after excision (time 0 does not correspond to the time of excision). a, addition of 100 mM Ca$^{2+}$ to the patch was enough to inhibit the channels in 50.9 ± 9.5 s (± S.E.), in 4 out of 7 trials. b, the inhibition time with 100 μM Mg$^{2+}$ (pH 7.2) was faster, with 13.4 ± 3.1 s (12 out of 12). c, the full channel activity could be rescued by increasing the pH to pH 8.2. The time needed was 42 ± 10 s (4 out of 7).

currents ($I_{crac}$, pA magnitude) can be seen indicating that single channels must be opening; but these channels are not detectable because in the presence of Ca$^{2+}$ or Mg$^{2+}$ the single channel conductance and/or frequency of opening is below the level of detection. Third, the ability of low, physiological concentrations of Ca$^{2+}$ to inhibit the channels is consistent with their identity as CRAC channels.
2-APB may be relatively selective for CRAC channels among plasma membrane Ca$^{2+}$ channels. The current study shows that CRAC channels are directly blocked by this drug, but L-type calcium channels (15), Trp-3 channels (16), and arachidonic acid-activated channels are not.\textsuperscript{2} Thus, the sensitivity of the channels to 2-APB seen under divalent cation-free conditions can be taken as an additional piece of evidence that they do in fact represent a high conductance state of CRAC channels. Also, based on the stability of channel activity in divalent cation-free solutions, we conclude that when activated in this way, interactions between channels and IP$_3$-liganded IP$_3$ receptors are not required for activation, or at least for maintenance of the activated state.

**DISCUSSION**

Our studies on RBL-2H3 m1 cells have shown that in the complete absence of intra- and extracellular divalent cations, large macroscopic inward currents develop, when measured in whole cell mode. This behavior is similar to that described for Jurkat T-lymphocytes by Kerschbaum and Cahalan (11). During the initial phase of activation, single-channels with conductances of 33 to 40 pS could be detected, resulting in an estimate of 260 to 500 channels per cell, calculated from the ratio of macroscopic to unitary currents. By excision into divalent cation-free media we found similar channels in inside-out patches, with conductances of 25 to 35 pS (averaging 31 pS). Since the conductances for single-channels in excised patches and in whole cell measurements are similar to each other, and the channels have similar open probabilities and lack of selectivity, we conclude that they are the same channels.

Whole cell currents as well as currents in excised patches were blocked by the drug 2-APB, a relatively specific inhibitor of SOC activation (16). Channel activity in excised patches was also inhibited by low Ca$^{2+}$ concentrations, as low as 100 nM. Similarly low concentrations of Mg$^{2+}$ did not inhibit, showing that the inhibiting effect of Ca$^{2+}$ is specific, and not due to a general inhibition of monovalent currents by divalent cations. Furthermore, the inhibition of channel activity in excised patches caused by 100 mM Mg$^{2+}$ could be rescued by increasing the pH in the intracellular solution. A similar pH effect was shown for whole cell monovalent currents in Jurkat cells (14); however, in contrast to the findings in Jurkat cells, in RBL-2H3 cells inhibition by higher (2.4 mM) concentrations of Mg$^{2+}$ could not be reversed.

We have also carried out experiments to investigate the role of intracellular Ca$^{2+}$ store depletion on the kinetics of activation of I$_{\text{crac}}$ and the large currents and single-channels activated under divalent cation-free conditions. Our results indicate that two currents were preactivated by store depletion, I$_{\text{crac}}$, and an intermediate-sized current which is similar to one seen with no divalent cations outside but with Mg$^{2+}$ inside. However, the large current in whole cell mode, and the single-channel activity in excised patches did not develop more rapidly when stores were first depleted. So, given this independence of store depletion for the current and single-channel kinetics, what is the evidence that the channels underlying the large monovalent currents are in fact CRAC-channels? We believe that they are indeed CRAC channels due to the following observations. (i) The ability of low, physiological concentrations of Ca$^{2+}$ to inhibit the channels in excised patches is consistent with their identity as CRAC channels. We know of no other channel which is so exquisitely sensitive to inhibition by Ca$^{2+}$. (ii) We found that the channels observed under divalent cation-free conditions are sensitive to 2-APB; this drug

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\textsuperscript{2}D. Luo, L. M. Broad, G. St. J. Bird, and J. W. Putney, Jr., unpublished observation.
appears to be relatively selective for CRAC channels among membrane Ca\(^{2+}\) channels in inhibiting SOC activation; for example, the drug does not block voltage-dependent calcium channels or arachidonic acid-activated channels (15).\(^2\) Therefore, although the investigated channels seem likely to be CRAC channels, activation by store depletion may be unnecessary in the complete absence of divalent cations. Alternatively, activation by store depletion may precede subsequent further activation by divalent cation-free solutions such that the latter is rate-limiting.\(^3\)

Although the kinetics of currents activated by divalent cation-free solutions appear independent of store depletion, there is another line of evidence that may link these currents to the process of calcium store depletion. While the large, macroscopic current was not pre-activated by store depletion, a current much smaller than the maximal macroscopic, yet larger than \(I_{\text{crac}}\) was observed in the pre-activated cells when extracellular divalent cations were removed. This current may correspond to the intermediate sized current seen by Kerschbaum and Cahalan (11) in the absence of external divalent cations, but with intracellular Mg\(^{2+}\) present. In the latter case, it was shown that the intermediate current resulted from single channel openings identical to those underlying the larger macroscopic current. In our experiments, an intermediate current was activated by calcium store depletion (Fig. 4B, *inset*), and if the single channels underlying this current are the same as those for the macroscopic current, then this is further evidence that the large openings are CRAC channels. However, we have not been successful in observing single channel openings in RBL-2H3 cells with Mg\(^{2+}\) present.

According to the conformational coupling model for SOC activation, IP\(_3\) receptors are essential for activation and maintenance of channel activity (5, 6, 16). Recent findings have also implicated a requirement for IP\(_3\) in this activation mechanism (7). However, there is data in conflict with this idea, at least in some cell types. For example, heparin is known not to inhibit SOC activation (25) or \(I_{\text{crac}}\) (9), and SOC activation occurs in cells apparently devoid of IP\(_3\) receptors (26). In the current study, once excised single-channels were activated by divalent cation-free solutions, they did not run down for at least 20 min. This stable activation, elicited solely by excision into divalent cation-free media, was independent of added IP\(_3\) or IP\(_3\) receptors. However, the previous studies implicating coupling of SOCs to IP\(_3\) receptors were not performed in the complete absence of divalent cations. Thus, two distinct modes of activation may exist, one requiring coupling to IP\(_3\) receptors, and one dependent on removal of divalent cations and independent of IP\(_3\) receptors. This conclusion is similar to one drawn by Krause et al. (23) who concluded that low cytoplasmic Ca\(^{2+}\) per se was sufficient to activate an \(I_{\text{crac}}\)-like current in RBL-1 cells.

Another important finding from the current work, with significant impact on the interpretation of results in previous investigations, is the effect of 2-APB on the divalent cation-free activated CRAC channels. The relatively specific blockade of CRAC channels by 2-APB has until now been attributed to its antagonistic effect on IP\(_3\) receptors (15), thereby blocking their regulation of the channels depends on divalent cations. Soluble factors do not seem to be absolutely required for inactivation by Ca\(^{2+}\) and Mg\(^{2+}\) since these cations blocked channel activity when added several minutes after excision. However, we were generally unsuccessful in reversing the Ca\(^{2+}\) block by returning the patches to a low-Ca\(^{2+}\) medium. Furthermore, we found that following excision of patches in media containing 100 nm [Ca\(^{2+}\)], lowering of [Ca\(^{2+}\)] did not lead to the appearance of channel activity (data not shown). This may indicate that Ca\(^{2+}\) binding to inhibitory sites is irreversible in the absence of a factor or structure that is lost following patch excision.

Regardless of the underlying mechanism, the use of divalent cation-free solutions has provided conditions for the first measurement and characterization of single CRAC channel activity in excised plasma membrane patches. Understanding the behavior of CRAC channels in the absence of divalent cations may provide an important first step for the design of future experiments addressing the mechanisms of regulation of CRAC channels under more physiological conditions.

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\(^3\) While this paper was under review, a manuscript appeared describing studies of single channel behavior in Jurkat and T-cells by utilizing divalent cation-free solutions in the whole cell patch clamp mode (Fomina, A. F., Fanger, C. M., Kozak, J. A., and Cahalan, M. D. (2000) J. Cell Biol. 150, 1435–1444). In contrast to the current study, Fomina et al. found that the appearance of the large macroscopic current was hastened by pre-activation of SOCs, and they concluded, as have we, that the observable channels underlying the large macroscopic currents are the channels responsible for \(I_{\text{crac}}\).

\(^2\) Since the drug does not block voltage-dependent calcium channels or arachidonic acid-activated channels (15).

\(^1\) In our experiments, an intermediate current was activated by calcium store depletion (Fig. 4B, *inset*), and if the single channels underlying this current are the same as those for the macroscopic current, then this is further evidence that the large openings are CRAC channels. However, we have not been successful in observing single channel openings in RBL-2H3 cells with Mg\(^{2+}\) present.

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