Calcium Inhibition and Calcium Potentiation of Orai1, Orai2, and Orai3 Calcium Release-activated Calcium Channels*

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The recent discoveries of Stim1 and Orai proteins have shed light on the molecular makeup of both the endoplasmic reticulum Ca2⁺ sensor and the calcium release-activated calcium (CRAC) channel, respectively. In this study, we investigated the regulation of CRAC channel function by extracellular Ca2⁺ for channels composed primarily of Orai1, Orai2, and Orai3, by co-expressing these proteins together with Stim1, as well as the endogenous channels in HEK293 cells. As reported previously, Orai1 or Orai2 resulted in a substantial increase in CRAC current (Icrac), but Orai3 failed to produce any detectable Ca2⁺-selective currents. However, sodium currents measured in the Orai3-expressing HEK293 cells were significantly larger in current density than Stim1-expressing cells. Moreover, upon switching to divalent free external solutions, Orai3 currents were considerably more stable than Orai1 or Orai2, indicating that Orai3 channels undergo a lesser degree of depotentiation. Additionally, the difference between depotentiation from Ca2⁺ and Ba2⁺ or Mg2⁺ solutions was significantly less for Orai3 than for Orai1 or -2. Nonetheless, the Na⁺ currents through Orai1, Orai2, and Orai3, as well as the endogenous store-operated Na⁺ currents in HEK293 cells, were all inhibited by extracellular Ca2⁺ with a half-maximal concentration of ~20 μM. We conclude that Orai1, -2, and -3 channels are similarly inhibited by extracellular Ca2⁺, indicating similar affinities for Ca2⁺ within the selectivity filter. Orai3 channels appeared to differ from Orai1 and -2 in being somewhat resistant to the process of Ca2⁺ depotentiation.

Store-operated or capacitative calcium entry channels sub-end or support important Ca2⁺ signaling pathways in a wide variety of cell types (1). The best characterized store-operated mechanism involves a current termed calcium release-activated calcium (CRAC) current or Icrac. The recent discoveries of Stim1 as an endoplasmic reticulum Ca2⁺ sensor (2, 3) and Orai1 as a pore-forming subunit of the T-cell CRAC channel (4–6) provide powerful tools for understanding the mechanisms of activation and regulation of these channels, as well as their specific roles in various physiological pathways. In addition to Orai1, two homologs, Orai2 and -3, have been shown to be capable of forming store-operated channels (7). Knowledge of the properties of these three Orai forms is necessary to understand the specific functions of the subunits and to implicate specific molecular components of native store-operated channels. In this study, we focused on the well characterized modulatory effects of extracellular Ca2⁺ on CRAC channels (8, 9). Extracellular Ca2⁺ has two effects on CRAC channels. It reduces conductance, or blocks the channels, by binding to a selectivity filter in the channel pore (10), but it also augments channel function by binding to an undefined extracellular site, a process known as potentiation (11). The latter role of extracellular Ca2⁺ can be revealed by protocols in which extracellular divalent cations are removed. The removal of Ca2⁺ block results in rapid increases in inward current, and then, as Ca2⁺ dissociates from the potentiating sites, current declines, a process termed depotentiation (11–13). Thus, we investigated the sensitivity of Orai channels to block by extracellular Ca2⁺, and the sensitivity of Orai channels to the process of depotentiation. In the course of this work, we developed a technique that permits reproducible assessment of very small Icrac such as encountered in wild type HEK293 cells or in cells transfected with Stim1 and Orai3. Our results indicate remarkable similarity among the three Orai forms with respect to selectivity and sensitivity to extracellular Ca2⁺ block. However, we find that Orai3 differs strikingly from Orai1 and -2 in being relatively resistant to the process of depotentiation.

MATERIALS AND METHODS

Cell Culture—HEK293 cells were obtained from ATCC and cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% heat-inactivated fetal bovine serum and 2 mM glutamine. The HEK293 cells were maintained at 37 °C in a humidified incubator set at 5% CO2. All experiments were performed on HEK293 cells plated onto 30-mm round glass coverslips mounted in a Teflon chamber.

Plasmids—Full-length Orai cDNA plasmids were purchased from Origene in the pCMV6-XL4 (Orai1 and Orai3) and pCMV6-XL5 (Orai2) vectors. An EFYP-Stim1 plasmid was

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The abbreviations used are: CRAC, calcium release-activated calcium; EFYP, enhanced yellow fluorescent protein; RNAi, RNA interference; CFP, cyan fluorescence protein; HBSS, HEPES-buffered saline solution; DVF, divalent free solution; IP3, inositol 1,4,5-trisphosphate; siRNA, short interfering RNA; BAPTA, 1,2-bis(2-aminophenoxy)ethane-N,N,N′,N′-tetraacetic acid; NMDG, N-methyl-D-glucamine; TEA, tetraethylammonium; SOC, store-operated current; pF, picofarad.
obtained from Tobias Meyer, Stanford University. N-terminal enhanced CFP-tagged Orai plasmids were constructed using human Orai 1, -2, and -3 purchased from Invitrogen in the pENTR™/221 vector, as part of the Ultimate ORF Clone collection. The Orai open reading frames were tagged at the N terminus using Gateway system LR reaction (Invitrogen) and the destination vector pDEST501. Successful recombination was confirmed by sequencing.

siRNA Knockdown—Orai1 siRNA (Invitrogen) was performed as described previously (7). Briefly, HEK293 cells were plated in a 6-well plate on day 1. On day 2, cells were transfected with 100 nM siRNA using Metafectine (Biontex Laboratories). After 6–8 h of incubation, the bathing medium was changed with complete Dulbecco’s modified Eagle’s medium. On day 3, siRNA-treated cells were transfected with cDNA as described below.

cDNA Transfection—HEK293 cells were transferred to 6-well plates and allowed to grow to 80–90% confluence, followed by transfection using Lipofectamine 2000 (Invitrogen); 2 μl per well with EFYP cDNA (0.5 μg/well), EFYP-Stim1 cDNA (0.5 μg/well), or EFYP-Stim1 cDNA together with Orai cDNA (Orai1, 0.5 μg/well; Orai2, 2 μg/well; Orai3, 2 μg/well), as described previously (7). After a 6–8-h incubation period, the medium bathing the cells was replaced with complete Dulbecco’s modified Eagle’s medium and maintained in culture. The next day, cDNA-treated cells were transferred to 30-mm glass coverslips in preparation for electrophysiological studies as described below. All transfections of cells with Orai constructs involved co-transfection with EFYP-Stim1, the fluorescence of which was used to identify successfully transfected cells.

Electrophysiology—Whole-cell currents were investigated at room temperature (20–25 °C) in HEK293 cells using the patch clamp technique in the whole-cell configuration. The standard HEPES-buffered saline solution (HBSS) contained (mM): 140 NaCl, 3 KCl, 1.2 MgCl2, 2.0 CaCl2, 10 glucose, and 10 HEPES (pH to 7.4 with NaOH). The standard divalent free solution (DVF) was prepared by removing the CaCl2 and MgCl2 from HBSS and adding 0.1 mM EGTA. The divalent cation substitution experiments used the standard DVF solution throughout, with Ca2+, Ba2+, or Mg2+ added to 2 mM final concentration. Monovalent permeation experiments were performed using 145 NaCl, 3 KCl, 10 CsCl, 10 HEPES, 2 EDTA, 10 glucose; pH was adjusted to 7.4 with NaOH. Where stated, NaCl was substituted by equimolar LiCl, RbCl, CsCl, TEA-Cl, or NMDG; pH was adjusted to 7.4. The DVF solution used in experiments examining Ca2+ inhibition of Na+ currents through Orai1, Orai2, or Orai3 was described previously by Prakriya and Lewis (14) and contained (pH 7.4) 10 mM HEDTA and 1 mM EDTA. CaCl2 was added to this DVF solution at the amount calculated using the Maxchelator program (WEBMAXC). Fire-polished pipettes fabricated from borosilicate glass capillaries (WPI, Sarasota, FL) with 3–5-megohm resistance were filled with the following (in mM): 145 cesium methanesulfonate, 20 BAPTA, 10 HEPES, and 8 MgCl2 (pH to 7.2 with CsOH).

In most experiments, the pipette also contained 25 μM inositol 1,4,5-trisphosphate (IP3, hexasodium salt; Sigma). Voltage ramps (−100 to +100 mV) of 250 ms were recorded every 2 s immediately after gaining access to the cell from a holding potential of 0 mV, and the currents were normalized based on cell capacitance. Leak currents were subtracted by taking an initial ramp current before Icrac developed and subtracting this from all subsequent ramp currents. Access resistance was typically between 5 and 10 megohms. The currents were acquired with pCLAMP-10 (Axon Instruments) and analyzed with Clampfit (Axon Instruments) and Origin 6 (Microcal) software. All solutions were applied by means of a gravity-based multibarrel local perfusion system with an extremely low dead volume common delivery port (Perfusion pencil, Automate Scientific, Inc.). Flow rates were set at ~0.25 ml/min.

In cells transfected with Orai1 and Stim1, we noticed one anomaly that limited the experimental protocols to some degree. When we broke into these cells with IP3 in the pipette, but with extracellular solutions ranging from nominally Ca2+-free to ~100 μM [Ca2+], large currents developed with unusual doubly rectifying properties (supplemental Fig. 1). We carried out experiments in nontransfected HEK293 and RBL cells, and in neither case did such currents develop. They thus may reflect a degree of instability of the channels in this highly overexpressed state. As a result, in all experiments, we initially broke in with an extracellular Ca2+-containing medium, and we then switched to DVF media or to media containing other cations, as indicated in specific protocols. As long as the experiments were initiated in a Ca2+-containing medium (mM range) or DVF solution, the unusual currents were never observed, even if subsequently the solution was changed to one containing low Ca2+. Also, note that our usual extracellular medium contained 2 mM Ca2+ rather than the 10 mM Ca2+ generally used for Icrac measurements. We found that with overexpressed Orai1 and Stim1, with 10 mM Ca2+ the resulting store-operated currents were somewhat unstable and tended to run down rapidly. With 2 mM Ca2+, the currents were generally stable for prolonged periods (see for example, Fig. 1).

RESULTS

Fig. 1 illustrates experiments in which HEK293 cells were transiently transfected with cDNA encoding the endoplasmic reticulum Ca2+ sensor, EFYP-Stim1 alone, or EFYP-Stim1 co-transfected with the plasma membrane proteins Orai1, Orai2, or Orai3. Successfully transfected cells were identified by the fluorescence from EFYP-Stim1 or by transfected EYFP for endogenous HEK293 CRAC currents. For Orai-transfected cells, EFYP-Stim1 was always present. Whole-cell electrophysiological experiments were performed with IP3 and BAPTA in the pipette to deplete endoplasmic reticulum Ca2+ stores, and the extracellular solution in these studies contained 2 mM Ca2+. Fig. 1A depicts representative inward currents collected from −100 mV voltage steps every 2 s in HEK293 cells expressing the indicated Orai constructs. In our hands, neither EFYP controls, Stim1 alone, nor Orai3-expressing cells developed any detectable Icrac-like Ca2+ currents. Other laboratories have measured, or attempted to measure, endogenous Icrac in wild type HEK293 cells and have concluded the currents to be very small (~0.5 pA/pF) and inconsistently detectable (5, 7, 15, 16). However, large inward currents were recorded from cells in which Orai1 or Orai2 and Stim1 were co-expressed, as described previously (6, 7, 17, 18). Voltage ramps (250 ms) from −100 to
+100 mV were used to compare the current-voltage relationships under extracellular Ca\(^{2+}\) conditions for Orai1 and Orai2. Fig. 1B shows the strong inward rectification and very positive reversal potential (> +50 mV), indicative of the Ca\(^{2+}\)-selective currents. Fig. 1C shows a summary of the data, where the mean inward Ca\(^{2+}\) currents activated by store depletion were −0.15 ± 0.03 pA/pF (YFP), −0.37 ± 0.10 pA/pF (Stim1), −0.27 ± 0.11 pA/pF (Orai1 + Stim1), −3.65 ± 0.36 pA/pF (Orai2 + Stim1), and −11.55 ± 0.78 pA/pF (Orai1 + Orai2 + Stim1). Unlike Stim1 alone or Orai3 and Stim1, both Orai1 and Orai2 co-expressed with YFP-Stim1 produced SOC currents that were significantly larger than YFP.

The inability to detect endogenous $I_{crac}$ currents in HEK293 cells is a recurring problem in cell types with small store-operated currents. This prevents quantitative comparisons of these currents to those produced by expression of Orai1 and -2. It is also difficult to assess the effects of expressing Stim1 alone or to characterize the currents produced by Orai3. One biophysical hallmark of $I_{crac}$ is development of a transient Na\(^{+}\) current upon removal of extracellular Ca\(^{2+}\) (10). Fig. 2 shows the results of whole-cell electrophysiological experiments utilizing a DVF-switching technique to readily reveal these small currents. A DVF Na\(^{+}\)-containing solution was perfused onto the cells following store depletion with IP\(_3\) and BAPTA in the pipette. In Fig. 2, A–E, representative current traces are depicted showing that robust and reproducible Na\(^{+}\) currents are readily apparent for endogenous (A; EYFP control), EYFP-Stim1 (B), and Orai1- (C), Orai2- (D), and Orai3- (E) HEK293 cells. Note that the current scales for YFP and Stim1 are different from the scales for Orai-expressing cells. Thus, it is now readily apparent that Stim1 expression alone causes a significant increase in $I_{crac}$ in this cell type, and that co-expression of Orai3 and Stim1 results in Na\(^{+}\) currents greater than those in wild type cells or with Stim1 alone. The rapid perfusion of divalent free solution onto the cells caused an initial augmentation of the Na\(^{+}\) currents, because of removal of Ca\(^{2+}\) block, followed by a depotentiation of these currents, similar to the Na\(^{+}\)-CRAC currents seen with native channels in RBL and Jurkat cells (10, 11). This depotentiation is believed to result from the dissociation of Ca\(^{2+}\) from an external site that is necessary for full CRAC channel activation (11, 13). However, in HEK293 cells expressing Orai3 and EYFP-Stim1, the depotentiation appeared significantly less pronounced (Fig. 2E). Fig. 2F shows representative current-voltage relationships for each of the experiments performed in A–E. Similar to the I-V relationships under extracellular Ca\(^{2+}\) conditions seen in Fig. 1B, the I-V relationships under divalent free conditions remain strongly inward rectifying with a reversal potential of approximately +50 mV for YFP controls (endogenous), Stim1 alone, and Orai1, Orai2, and Orai3 cells co-expressed with Stim1. Fig. 2G summarizes peak divalent free Na\(^{+}\) currents; the mean inward Na\(^{+}\) currents (depicted by horizontal line in scatter plot) activated by store depletion were −3.09 ± 0.36 pA/pF (YFP), −10.91 ± 0.64 pA/pF (Stim1), −25.34 ± 1.26 pA/pF (Orai3 + Stim1), −34.33 ± 1.89 pA/pF (Orai2 + Stim1), and −66.48 ± 3.90 pA/pF (Orai1 + Stim1). The summary data confirm the statistically significant increase in current by Stim1 alone and greater increase when co-expressed with each of the Orai forms.

To assess more quantitatively the extent of depotentiation of the Na\(^{+}\)-CRAC currents seen with the Orai-expressing HEK293 cells, we calculated the inactivation indices for each condition. The inactivation index was determined by dividing the Na\(^{+}\)-CRAC currents at steady state (30 s after application of DVF solution) by the peak Na\(^{+}\)-CRAC currents, and subtracting this from one. This method is similar to that described by Zweifach and Lewis (19) for quantitating degree of fast inactivation. Fig. 2H shows a scatter plot of the inactivation indices for Stim1 (0.51 ± 0.03), Orai1 and Stim1 (0.61 ± 0.02), Orai2 and Stim1 (0.63 ± 0.02), and Orai3 + Stim1 (0.28 ± 0.01). Thus, although ~60% of the Na\(^{+}\) currents depotentiated in YFP-, Stim1-, Orai1-Stim1-, and Orai2-Stim1-expressing HEK293 cells, only ~30% depotentiation was seen in Orai3-Stim1-co-expressing cells.

As in our previous study, the rank order of $I_{crac}$ activation by the three Orai with this Stim1 co-transfection paradigm was Orai1 > Orai2 > Orai3. We considered the possibility that
these differences reflected differences in expression of the three proteins. To address this issue, we constructed N-terminal CFP fusions of each of the Orai. The results were consistent with the observations of current, i.e. Orai1 was expressed to a greater level than Orai2 or -3. The relative expression of the three proteins was determined by flow cytometry and was as follows (in arbitrary units): CFP-Orai1, 3041 ± 753; CFP-Orai2, 1494 ± 122; CFP-Orai3, 1,086 ± 51 (n = 3 transfections, 10,000 cells per transfection per condition). For the most part, these constructs behaved in a manner similar to their wild type counterparts when co-transfected with Stim1. However, unlike the results with unmodified Orai3, CFP-Orai3 + Stim1-transfected cells revealed detectable store-operated Ca$^{2+}$ currents and significantly larger Na$^{+}$ currents. In addition, these larger CFP-Orai3 currents tended to depotentiate in a manner similar to Orai1 and -2. The reason for these differences in behavior for the N-terminal fusion version of Orai3 was not further investigated. However, because of this, we did not use the tagged constructs for any experiments other than for determination of expression level and localization. All three localized similarly at the cell periphery, presumably in the plasma membrane (supplemental Fig. 2). Taken together, these results suggest the smaller Orai2 and Orai3 currents might result at least in part from differences in expression levels in HEK293 cells. However, a caveat is that the tagged versions of the proteins did not behave identically to the wild type proteins, either quantitatively or qualitatively. Thus, comparison of both behavior and expression of unmodified Orai must await development of isoform-specific antibodies.

In previous work examining Ca$^{2+}$ signaling responses, overexpression of Orai1 and -2 appeared to inhibit entry, whereas Orai3 had no effect. Thus, we next examined the effects of overexpression of each of the three Orai on store-operated Na$^{+}$ currents. As shown in Fig. 3, overexpression of Orai1 or -2 alone significantly inhibited store-operated Na$^{+}$ currents in divalent free solutions. However, overexpression of Orai3 caused a modest but statistically significant increase in the current. In contrast to
of store-operated Na⁺ currents following expression of Orai1 and -2 with Stim1, and Orai3 with and without Stim1 in cells with endogenous Orai1 reduced by RNAi, as described previously (7). The results of these experiments are shown in Fig. 4. Knockdown of Orai1 resulted in an almost complete loss of store-operated Na⁺ current (−0.63 ± 0.11 pA/pF; not shown), even with overexpression of Stim1 (−0.64 ± 0.22 pA/pF; Fig. 4B). Expression of Orai1 and -2 with Stim1 produced currents similar to those seen without Orai1 knockdown, although somewhat smaller (Fig. 4, C, D, and G). Expression of Orai3 alone in these cells restored Na⁺ currents to near control levels (−2.46 ± 0.61 pA/pF), consistent with our previous report showing that Orai3 can rescue Ca²⁺ entry following knockdown of Orai1 (7). Expression of Orai3 with Stim1 in these cells resulted in larger currents but still considerably smaller than those seen when Orai3 and Stim1 were expressed in cells containing endogenous Orai1 (−6.05 ± 0.49 pA/pF versus −25.34 ± 1.26 pA/pF), consistent with the idea that the current from cells expressing their endogenous complement of Orai1 results from a combination of Orai1 and Orai3. As predicted, the inactivation index for Orai3 in cells lacking Orai1 was 0.25 ± 0.04 in the absence of Stim1 and was not significantly different from that for the larger currents when Orai1 remained (0.28 ± 0.01).

It is generally believed that depotentiation results from dissociation of Ca²⁺ from a regulatory extracellular site, i.e. it is the reciprocal of the results in the presence of excess Stim1, when expressed alone, the inactivation index for Orai3-expressing cells was not significantly different from control (EYFP-expressing cells).

We considered that the apparent difference in depotentiation of Orai3 in the presence or absence of Stim1 might reflect the relative contribution of endogenous Orai1 under the two conditions, i.e. if Orai1 were better able to interact with Stim1 (or Stim1-dependent signals) than Orai3, then with Stim1 limiting, the functional channels might be largely Orai1 homomers and Orai1–Orai3 heteromers (20, 21). When Stim1 is not limiting, the effects of Orai3 homomers may contribute to a greater degree, as in Fig. 2E. Thus, we next examined the characteristics of Ca²⁺ potentiation (11, 13, 22). Other divalent cations can occupy this site but fulfill the potentiating effects of Ca²⁺ less well (11, 13). We thus compared the potentiating effects of Ba²⁺ and Mg²⁺ to those of Ca²⁺. Fig. 5, A–C, shows representative current traces of HEK293 cells expressing EYFP-Stim1 co-expressed with Orai1 (A), Orai2 (B), or Orai3 (C), in which extracellular DVF solution was focally applied to the cells after pre-exposing them to 2 mM Ca²⁺, Ba²⁺, or Mg²⁺ outside. All experiments were performed on cells in which stores were actively depleted by IP₃ and BAPTA in the pipette. In Orai1-Stim1 and Orai2-Stim1 co-expressing HEK293 cells, the focal perfusion of 2 mM Ba²⁺ and Mg²⁺ significantly reduced the

![FIGURE 3. Orai1 and Orai2 but not Orai3 expression in HEK293 cells reduces endogenous store-operated inward currents revealed by switching to divalent free solutions. Time courses of the transient Na⁺ currents that occur under DVF-extracellular conditions in HEK293 cells expressing YFP alone (A), or co-expressed with Orai1 (B), Orai2 (C), and Orai3 (D) after stores were actively depleted using IP₃ and BAPTA in the pipette. E, scatter plot of the maximal current developed under DVF conditions for YFP- (−0.49 ± 0.06 pA/pF; n = 6), Orai1- (−1.21 ± 0.20 pA/pF; n = 6), Orai2- (−1.75 ± 0.17 pA/pF; n = 4), and Orai3- (−6.12 ± 0.32 pA/pF; n = 11)-expressing HEK293 cells. Orai1 (p < 0.001) and Orai2 (p < 0.01) but not Orai3 expression significantly reduced Na⁺-currents when compared with YFP alone. Orai3 expression significantly (p < 0.01) increased Na⁺-currents. F, scatter plot of the inactivation indices for Na⁺-CRAC currents in HEK293 cells expressing YFP alone (0.48 ± 0.04; n = 6) or co-expressed with Orai3 (0.44 ± 0.04; n = 11).]
Ca\(^{2+}\) Regulation of Orai

**FIGURE 4.** Orai1, Orai2, and Orai3 rescue store-operated Ca\(^{2+}\) currents after RNAi knockdown of Orai1. A–F depict whole-cell currents recorded from HEK293 cells in which Ca\(^{2+}\) stores were actively depleted with IP\(_3\) and BAPTA. The DVF protocol was used to assess the reduction of store-operated currents by 100 mM Orai1 siRNA treatment, as well as the ability of Stim1 alone (G), or Stim1 co-expressed with Orai1 (H), or Orai1 (E) to rescue Orai1 knockdown. These results were compared with siGLO EYFP controls (A), as described previously (7). G, scatter plot of the peak currents developed under DVF conditions for siGLO YFP (−3.17 ± 0.33 pA/pF; n = 9), Orai1 siRNA (−0.63 ± 0.11 pA/pF; n = 6), and Orai1 siRNA-treated cells rescued with Stim1 alone (−0.64 ± 0.22 pA/pF; n = 5), Orai3 alone (−2.26 ± 0.61 pA/pF; n = 6), or Stim1 co-expressed with Orai1 (−2.14 ± 0.26 pA/pF; n = 13). Orai2 (−16.77 ± 2.03 pA/pF; n = 11), or Orai3 (−6.05 ± 0.49 pA/pF; n = 13). Analysis of variance performed on all conditions showed significant increase in currents between siGLO controls and Orai1 + Stim1 rescue (p < 0.001) or Orai2 + Stim1 rescue (p < 0.0001). A further analysis of variance test on all conditions excluding Orai1 + Stim1 and Orai2 + Stim1 rescue revealed a significant reduction in Na\(^+\) I\(_{\text{crac}}\) in siRNA-treated cells (p < 0.01), as well as Orai1 siRNA cells expressing Stim1 (p < 0.01). Under this analysis, Orai3 + Stim1 rescued currents were significantly larger than siGLO controls (p < 0.001), but there was no significant difference between siGLO and Orai3 alone rescued currents. H, scatter plot of the inactivation indices for Na\(^+\)-CRAC currents in HEK293 treated with Orai1 siRNA, followed by rescue with Orai3 alone (0.26 ± 0.04), or Stim1 co-expressed with Orai3 (0.65 ± 0.03), or Orai2 alone (0.70 ± 0.05), or Orai3 alone (0.30 ± 0.03). Orai3 alone and co-expressed with Stim1 inactivation indices from Orai1 RNAi rescue experiments were significantly less than the Orai1 + Stim1 and Orai2 + Stim1 counterparts (p < 0.001).

Inward currents recorded at −100 mV but did not completely eliminate them. In both Orai1-Stim1- and Orai2-Stim1-expressing cells, which show significant Ca\(^{2+}\) currents, only ~20 and ~10% (normalized to the Ca\(^{2+}\) currents) of the inward currents remained after switching the extracellular solution from a Ca\(^{2+}\) -containing buffer to one containing Ba\(^{2+}\) or Mg\(^{2+}\), respectively.

**FIGURE 5.** Relative abilities of Ca\(^{2+}\), Ba\(^{2+}\), and Mg\(^{2+}\) to potentiate store-operated Na\(^{+}\) currents in Orai1-, Orai2-, or Orai3-expressing cells. Representative time courses of the transient currents that occur under divalent free extracellular conditions in HEK293 cells expressing Orai1 + Stim1 (A), Orai2 + Stim1 (B), and Orai3 + Stim1 (C) currents, after cells were exposed to either 2 mM Ba\(^{2+}\), Mg\(^{2+}\), or Ca\(^{2+}\). Inward currents were recorded at −100 mV but did not completely eliminate them. In both Orai1-Stim1- and Orai2-Stim1-expressing cells, which show significant Ca\(^{2+}\) currents, only ~20 and ~10% (normalized to the Ca\(^{2+}\) currents) of the inward currents remained after switching the extracellular solution from a Ca\(^{2+}\) -containing buffer to one containing Ba\(^{2+}\) or Mg\(^{2+}\), respectively.

Fig. 5D illustrates that the rank order for the potentiating effects of the different cations on store-operated Na\(^{+}\) currents for all three Orai was Ca\(^{2+}\) > Ba\(^{2+}\) > Mg\(^{2+}\). However, the differences in potentiation in the Orai3 cells were less than with the other two Orai. Pre-exposure of Orai1 and Orai2 cells to external Ba\(^{2+}\) or Mg\(^{2+}\) resulted in subsequent DVF currents that were ~60 or ~45% of those seen following pre-exposure to Ca\(^{2+}\), respectively. The DVF currents following Mg\(^{2+}\) pre-exposure did not appear to depotentiate and did not appear appreciably larger than the fully depotentiated DVF currents, indicating that for Orai1 and -2, Mg\(^{2+}\) produces little or no potentiation. However, for Orai3 cells DVF currents of 90% (Ba\(^{2+}\)) and 80% (Mg\(^{2+}\)) of those with Ca\(^{2+}\) were observed. Finally, for Orai1 and -2, switching from DVF to Ca\(^{2+}\)-containing solution invariably resulted in currents that showed slow potentiation, and this was not observed for Orai3 (see also Fig. 6, A and B).

We next examined Orai1, Orai2, and Orai3 selectivity for monovalent cations (Fig. 6). We first activated the store-operated inward currents under the presence of 2 mM extracellular Ca\(^{2+}\). After inward Ca\(^{2+}\) current stabilization (recorded from −100 mV voltage steps), divalent free solutions containing either Na\(^{+}\), Li\(^+\), Rb\(^+\), Cs\(^+\), TEA, or NMDG were added, with Ca\(^{2+}\) added between the different monovalent solutions (Fig. 6, A–C). Using this protocol, peak monovalent currents were normalized to peak Na\(^{+}\) currents, and the rank order for monovalent permeability through all Orai was Na\(^+\) > Li\(^+\) > Rb\(^+\) > Cs\(^+\) > TEA and NMDG (Fig. 6E). This sequence is similar to that reported previously for native I\(_{\text{crac}}\) (23, 24). Furthermore, the representative I-V of an Orai3 + Stim1-overexpressing
forms by examining the sensitivity of Na\(^+\) currents to Ca\(^{2+}\) block, presumably a measure of the affinity of the selectivity pore for Ca\(^{2+}\).

To compare the Ca\(^{2+}\) block of store-operated Na\(^+\) currents through Orai1, Orai2, and Orai3 channels, whole-cell electrophysiological experiments were performed in HEK293 cells co-expressing Orai1, Orai2, or Orai3 with Stim1. A–E in Fig. 7 depict representative voltage clamp experiments (−100 mV) carried out on wild type HEK293 cells (A, YFP control), as well as cells expressing Stim1 alone (B), and Stim1 with Orai1 (C), Orai2 (D), or Orai3 (E). To determine the Ca\(^{2+}\) inhibition of the Na\(^+\) currents, cells were initially perfused with a solution containing 2 mM Ca\(^{2+}\), but they were switched using a focal applicator to extracellular solutions containing either DVF solution or micromolar levels of Ca\(^{2+}\) (as indicated in figure), determined by Maxchelator software (see “Materials and Methods”). Extracellular solutions containing different concentrations of Ca\(^{2+}\) were applied using both increasing and decreasing concentrations of extracellular Ca\(^{2+}\), as well as random concentration applications (see Fig. 7, C and D). Fig. 6F shows a summary of the data for Ca\(^{2+}\) inhibition of store-operated Na\(^+\) current in wild type HEK293 cells and cells ectopically expressing Orai and Stim1 proteins. The plots for each expression condition were fitted to the Hill equation shown in the Fig. 7 legend. There was no significant difference among the concentrations of Ca\(^{2+}\) producing half-maximal block of YFP (18.2 ± 2.7 μM), Stim1 (20.4 ± 2.8 μM), Orai1 + Stim1 (20.5 ± 4.9 μM), Orai2 + Stim1 (21.1 ± 4.1 μM), or Orai3 + Stim1 (21.6 ± 6.7 μM). Importantly, similar results were collected in HEK293 cells treated with Orai1 siRNA and then rescued with either Orai1, Orai2, or Orai3 (data not shown). These data reveal marked similarity between the sensitivity of native CRAC channels and expressed Orai channels to Ca\(^{2+}\) block. The value of −20 μM Ca\(^{2+}\) is similar to previously published values for native channel in hematopoietic cells (4, 13, 14, 24), and Orai1 rescue of I\(_{crac}\) in SCID T-cells also has a half-maximal block of Na\(^+\)-CRAC currents −20 μM Ca\(^{2+}\) (4).

HEK293 cell shows that Cs\(^+\) does not appear to pass through the channels at all, and TEA and NMDG actually reduce inward leak taken at −100 mV. Identical I-V relationships were seen for Orai1 and Orai2 co-expressed with EYFP-Stim1.

The results to this point indicate similarities between all three Orai forms but reveal a fundamental difference in Ca\(^{2+}\) regulation of Orai3 channels. A candidate site for Ca\(^{2+}\) potentiation is the channel pore itself. Thus, we next investigated Ca\(^{2+}\)-binding properties of the channel pore with these three
**DISCUSSION**

The discovery of Orai1 (or CRACM1) as a key component of CRAC channels (4–6) has provided a molecular tool to understand the function and regulation of these physiologically important store-operated channels. Feske et al. (4) demonstrated that a mutation in Orai1 eliminates $I_{\text{crac}}$ in human T-cells. Similarly, RNAi knockdown of Orai1 in T-cell lines essentially eliminates $I_{\text{crac}}$. Co-expression of Orai with the endoplasmic reticulum Ca$^{2+}$ sensor, Stim1, results in the appearance of huge $I_{\text{crac}}$-like currents (6, 7, 17, 18). Subtle mutation of a critical residue between the first and second transmembrane segments alters the selectivity of Orai indicating that it is likely a pore-forming subunit of the CRAC channel (20, 25, 26).

In addition to Orai1, Feske et al. (4) described two other highly similar genes, which they designated Orai2 and Orai3. It seems likely, therefore, that proteins encoded by these genes may constitute or contribute to store-operated channels in specific cell types. Thus, it is important to delineate the properties of the channels formed by these different Orai forms in order to understand possible differences in biophysical properties or modes of regulation of different types of native store-operated channels.

Mercer et al. (7) reported that a combination of Orai2 and Stim1 produced currents that appeared similar to those with Orai1; however, no currents were observed with Orai3, although Orai3 could rescue store-operated entry. It is perhaps not surprising that these currents would not be detected, because the endogenous currents in HEK293 cells are also below the level of reliable detection. Thus, in this study we utilized a novel approach that should be generally applicable to investigating store-operated currents in cells with small, difficult to see currents. Switching from Ca$^{2+}$-containing to DVF solutions following Ca$^{2+}$-store depletion revealed robust and reproducible currents in cells with small, difficult to see currents. Switching from Ca$^{2+}$-containing to DVF solutions following Ca$^{2+}$-store depletion revealed robust and reproducible currents in wild type HEK293 cells. Previously, we reported that the effects of overexpressing Stim1 in HEK293 cells were variable (7); others have reported increases in Ca$^{2+}$ entry (2, 27). With the DVF switching technique, a clear augmentation of store-operated Na$^{+}$ current was readily apparent. Finally, with the DVF switching technique, a clear

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**FIGURE 7. Calcium inhibition of Na$^{+}$-$I_{\text{crac}}$ in HEK293 cells co-expressing Orai and Stim1 proteins.** Representative time courses of dose-dependent inhibition of Na$^{+}$ CRAC currents by extracellular Ca$^{2+}$ in HEK293 cells expressing YFP (A), Stim1 (B), Orai1 + Stim1 (C), Orai2 + Stim1 (D), and Orai3 + Stim1 (E), after stores were depleted using IP$_3$ and BAPTA in the pipette. All experiments were performed in 2 mM Ca$^{2+}$-containing solutions were carried out in both increasing and decreasing Ca$^{2+}$ concentrations to avoid protocol-dependent artifacts. F, concentration dependence of Na$^{+}$-CRAC current block in HEK293 cells expressing YFP (n = 8), Stim1 (n = 13), Orai1 + Stim1 (n = 11), Orai2 + Stim1 (n = 9), and Orai3 + Stim1 (n = 8). S.E. bars were omitted for clarity but were on average <10% of the mean values. The dose-response data were fitted to the Hill equation: $i = i_{\text{max}}/(1 + [\text{Ca}^{2+}]^n)$, where $i$ represents the store-operated Na$^{+}$ current in the presence of various [Ca$^{2+}$]; $i_{\text{max}}$ is the maximal Na$^{+}$-$I_{\text{crac}}$; $K_i$ is the dissociation constant; [Ca$^{2+}$] is the Ca$^{2+}$ concentration, and $n$ is the Hill coefficient. There was no significant difference between the half-maximal blocks of YFP (18.2 ± 2.7 μM), Stim1 (20.4 ± 2.8 μM), Orai1 + Stim1 (20.5 ± 4.9 μM), Orai2 + Stim1 (21.1 ± 4.1 μM), or Orai3 + Stim1 (21.6 ± 6.7 μM).
increase in store-operated Na\(^{+}\) current was seen in Orai3-transfected cells.

Using this technique, we were able to investigate a number of fundamental properties of CRAC channels formed by overexpressing each of the three Orai forms. We confirmed with current measurements the observation made previously from fura-2 measurements that overexpression of Orai1 and -2, but not -3, inhibited store-operated Ca\(^{2+}\) entry and that Orai3 can rescue \(I_{\text{CRAC}}\) after knockdown of Orai1. Under our expression conditions, Orai1, -2 and -3 were indistinguishable with regard to the sensitivity of the Na\(^{+}\) currents to Ca\(^{2+}\) block and showed indistinguishable selectivity for monovalent cations. This is consistent with current views as to the location of key selectivity filter-forming residues (20, 25, 26), as these regions are highly conserved among the three forms. This raises the question of the biological roles of these three distinct genes. Aside from their activation by Stim1, little is known of other regulatory mechanisms for these channels. \(I_{\text{CRAC}}\) is reported to be sensitive to inhibition by protein kinase C in RBL cells (28) but not in HEK293 cells (29). Thus, it is possible that different Orai are regulated by cross-talk from other signaling pathways in different ways. The expected advent of engineered organisms lacking each or combinations of these channel genes may shed light on this question.

We also examined the process of depotentiation that occurs following removal of extracellular divalent cations (11, 22). Depotentiation is believed to reflect the dissociation of Ca\(^{2+}\) from an external site where its binding is required for optimal CRAC channel function (13). The extent and kinetics of depotentiation were similar for Na\(^{+}\) currents in wild type HEK293 cells, and Orai1- and -2-expressing cells. However, Orai3-expressing cells were markedly different in this respect. Three observations indicate that these channels are considerably less sensitive to the process of depotentiation. First, the rate and extent of decline in Na\(^{+}\) current following removal of divalent cations is less for Orai3 channels than for Orai1 or -2. Second, the magnitude of Na\(^{+}\) currents following pre-exposure to Ba\(^{2+}\), Mg\(^{2+}\), or Ca\(^{2+}\)-containing solutions was similar. Third, the size of the Orai3 Na\(^{+}\) current is much larger than would be predicted from its very small, marginally detectable Ca\(^{2+}\) current. The physiological significance of the process of depotentiation is not clear; it is not expected that under physiological conditions, such a site will ever be unoccupied by Ca\(^{2+}\). One possibility is that Ca\(^{2+}\) binding stabilizes the channel structure, and subtle differences in the sequence and structure of Orai3 obviate this requirement to some degree. Interestingly, modification of Orai3 structure by addition of a fluorescent tag to the N terminus resulted in loss of this distinction. Further analysis of the structural similarities and differences between Orai3 and its congeners, and experimental manipulations of these differences may eventually shed light on the structural basis of depotentiation. At the very least, this strikingly different behavior of Orai3 channels may serve as a marker for identification of such channels in native cells and tissues.

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