Store-operated Ca\(^{2+}\) entry (SOCE) is likely the most common mode of regulated influx of Ca\(^{2+}\) into cells. However, only a limited number of pharmacological agents have been shown to modulate this process. 2-Aminoethylidiphenyl borate (2-APB) is a widely used experimental tool that activates and then inhibits SOCE and the underlying calcium release-activated Ca\(^{2+}\) current (\(I_{\text{CRAC}}\)). The mechanism by which depleted stores activates SOCE involves complex cellular movements of an endoplasmic reticulum Ca\(^{2+}\) sensor, STIM1, which redistributes to puncta near the plasma membrane and, in some manner, activates plasma membrane channels comprising Orai1, -2, and -3 subunits. We show here that 2-APB blocks puncta formation of fluorescently tagged STIM1 in HEK293 cells. Accordingly, 2-APB also inhibited SOCE and \(I_{\text{CRAC}}\)-like currents in cells co-expressing STIM1 with the CRAC channel subunit, Orai1, with similar potency. However, 2-APB inhibited STIM1 puncta formation less well in cells co-expressing Orai1, indicating that the inhibitory effects of 2-APB are not solely dependent upon STIM1 reversal. Further, 2-APB only partially inhibited SOCE and current in cells co-expressing STIM1 and Orai2 and activated sustained currents in HEK293 cells expressing Orai3 and STIM1. Interestingly, the Orai3-dependent currents activated by 2-APB showed large outward currents at potentials greater than +50 mV. Finally, Orai3, and to a lesser extent Orai1, could be directly activated by 2-APB, independently of internal Ca\(^{2+}\) stores and STIM1. These data reveal novel and complex actions of 2-APB effects on SOCE that can be attributed to effects on both STIM1 as well as Orai channel subunits.

In many cell types, the activation of phospholipase C through G protein-coupled receptors liberates Ca\(^{2+}\) from the lumen of the endoplasmic reticulum (ER).\(^2\) This depletion of ER Ca\(^{2+}\) stores results in activation of a process called capacitative or store-operated Ca\(^{2+}\) entry (SOCE) whereby extracellular Ca\(^{2+}\) enters the cell via plasma membrane channels (1, 2). The best characterized SOCE current is the Ca\(^{2+}\) release-activated Ca\(^{2+}\) current (\(I_{\text{CRAC}}\)), first described in mast cells (3) and since recorded in several cell types (4). Until recently, the mechanism by which \(I_{\text{CRAC}}\) is activated by store depletion, as well as the channels themselves, was unknown. However, the discoveries of both STIM1 (5, 6) and Orai1 (CRACM1) (7–9) have revealed two key molecular components of the \(I_{\text{CRAC}}\)-signaling pathway. It is now clear that STIM1 functions as the Ca\(^{2+}\) sensor within the ER, whereas members of the family of Orai proteins (including Orai1, -2, and -3) function as pore-forming subunits of CRAC channels in the plasma membrane. When intracellular Ca\(^{2+}\) stores are depleted, STIM1 rearranges from a fibrillar localization that depends on microtubules to discrete punctate structures near the plasma membrane (6, 10–12). Remarkably, Orai1 channels also rearrange into punctate structures, in response to store depletion, that coincide with those formed by STIM1 (13–15). Thus, highly orchestrated molecular rearrangements underlie \(I_{\text{CRAC}}\) activation.

Overexpression of Orai1 together with STIM1 in HEK293 cells produces unusually large currents with biophysical properties similar to \(I_{\text{CRAC}}\) (9, 16–18), suggesting that either these two proteins are sufficient to completely recapitulate \(I_{\text{CRAC}}\) or that any additional proteins required must be present in excess within the cell (19). Orai2 and Orai3 also are highly calcium-selective when co-expressed with STIM1, although the currents are somewhat smaller and the biophysical and pharmacological properties differ somewhat (20, 21).

Knowledge of the key molecular players in the SOCE pathway has allowed, for the first time, investigation of the cellular and molecular mechanisms of pharmacological modulators. Of the limited number of SOCE inhibitors that have been described, perhaps 2-aminoethylidiphenylborate (2-APB) has been the most extensively utilized. This drug was originally described as a membrane-permeant inhibitor of the inositol 1,4,5-triphosphate (IP\(_3\)) receptor, and the inhibition of SOCE by 2-APB was taken as evidence for direct IP\(_3\) receptor activation of CRAC channels (22) in a process called conformational coupling (23). However, more recent work has shown that 2-APB inhibition of \(I_{\text{CRAC}}\) is independent of IP\(_3\) receptors (24–30). It has been reported that Orai1, Orai2 and Orai3 differ in embryonic kidney; BPATA, 1,2-bis(2-aminoethoxy)ethane-N,N,N',N'-tetraacetic acid; WT, wild type; TG, thapsigargin; DVF, divalent free external solution; siRNA, small interfering RNA.
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their responses to 2-APB (20). However, the cellular or molecular mechanism underlying these differing effects has not been investigated.

Ideally, one would like to investigate the actions of pharmacological agents on ion channels expressed in their native environments. However, to date, functional expression of only native Orai1 channels has been documented, whereas the functions of native Orai2 or Orai3 channels have not been clearly demonstrated in any specific cell type. We have thus investigated the actions of 2-APB on HEK293 cells transiently transfected with plasmids encoding Orai1, -2, or -3, N-terminally tagged with a cyan fluorescent protein (CFP-Orai1, -Orai2, and -Orai3), in most instances together with an eYFP-STIM1 construct. Our results indicate a complex array of effects of 2-APB that differ among the three Orai subtypes. We also describe for the first time striking effects of 2-APB on the function of the Ca\(^{2+}\) sensor, STIM1. Thus, the effects of 2-APB on STIM1 and Orai proteins are extremely complex and may at some point reveal details of the molecular actions of these important proteins. However, the complexity of 2-APB actions also brings into question the utility of this drug as a specific modulator of SOCE.

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% CO\(_2\). All experiments were carried out on HEK293 cells plated onto 30-mm round glass coverslips mounted in a Teflon chamber.

Plasmids and Transfections—N-terminal tagged eYFP-STIM1 was obtained from the laboratory of Tobias Meyer at Stanford University. CFP-tagged Orai plasmids were constructed using human Orai1, -2, and -3, purchased from Stanford University. CFP-tagged Orai plasmids were constructed using human Orai1, -2, and -3, purchased from Invitrogen in the pENTR\textsuperscript{TM}221 vector and the Gateway system. STIM1 was obtained from the laboratory of Tobias Meyer at Stanford University.

Transfections were carried out using Lipofectamine 2000 (2 µl/well; Invitrogen) as described previously (18). Briefly, HEK293 cells were plated in a 6-well plate. On the following day, cells were transfected with CFP-Orai1 (1 µg/well), CFP-Orai2 (3 µg/well; in some experiments, shown in Figs. 5 and 6, an untagged version was used), CFP-Orai3 (3 µg/well), or eYFP-STIM1 (1 µg/well) cDNA. Six hours later, the medium bathing the cells was replaced with complete Dulbecco’s modified Eagle’s medium, and the cells were maintained in culture overnight. The next morning transfected cells were transferred to 30-mm glass coverslips in preparation for experiments. In some experiments, STIM1 expression was reduced by RNA interference, as described previously (31).

Intracellular Ca\(^{2+}\) Measurements—Intracellular Ca\(^{2+}\) measurements were carried out as described previously (18). Briefly, cells were loaded with 1 µM Fura-5F/AM (Invitrogen) for 25 min at 37 °C. Fura-5F fluorescence was measured when cells were excited alternately at 340 and 380 nm, and Ca\(^{2+}\) concentrations are reported as the ratio of fluorescence emission at the two excitation wavelengths. Cells transfected with eYFP-STIM1 were chosen based on their fluorescence when excited at 477 nm. Generally, 20–30 eYFP-positive cells were measured on a single coverslip in each experiment. Ratio values were corrected for contributions by autofluorescence, which was measured after treating cells with 4 µM ionomycin and 20 mM MnCl\(_2\) after the experiments had ended.

Live Cell Confocal and TIRFM Imaging—Cells were maintained in HEPES-buffered saline solution (HBSS) (in mM: 120 NaCl, 5.4 KCl, 1.8 CaCl\(_2\), 0.8 MgCl\(_2\), 11 glucose, and 20 HEPES, pH 7.4) at room temperature. Confocal imaging was carried out using a Zeiss LSM 510 laser scanning system, and either a 40× water immersion (NA 1.2) or a 63× oil immersion (NA 1.4) objective was used. All confocal images were collected with the pinhole set at 1 airy unit. For eYFP-STIM1, 488 or 514 nm illumination was provided by an argon laser, and emission was selected with a 530–600-nm bandpass filter. TIRFM was carried out essentially as described previously (32). For fluorescence intensity profiles, data are represented as the fluorescence intensity at each time point divided by the fluorescence intensity at the start of the experiment (IF\(_0\)). Fluorescence intensities were collected from regions of interest encompassing the visible footprints of single cells and were background-subtracted.

Electrophysiology—Whole-cell currents were measured at room temperature in HEK293 cells using the patch clamp technique in the whole-cell configuration. The standard HEPES-buffered saline solution for these experiments contained (mm): 145 NaCl, 3 KCl, 10 CsCl, 1.2 MgCl\(_2\), 10.0 CaCl\(_2\), 10 glucose, and 10 HEPES (pH to 7.4 with NaOH). Fire-polished pipettes fabricated from borosilicate glass capillaries (World Precision Instruments, Sarasota, FL) with 3–5 megohms resistance were filled with (in mm): 145 cesium methanesulfonate, 20 BAPTA, 10 HEPES, and 8 MgCl\(_2\) (pH to 7.2 with CsOH). For experiments in Figs. 8 and 9, Ca\(^{2+}\) was added to the internal solution to yield a final concentration of 100 µM free Ca\(^{2+}\) as determined using Maxchelator software; this prevents passive store depletion of the internal Ca\(^{2+}\) stores. In the indicated experiments, the pipette also contained 20 µM IP\(_3\) (Sigma) to actively deplete intracellular Ca\(^{2+}\) pools. 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 determined by taking an initial ramp current before I\(_{\text{CRAC}}\) developed, and this was subtracted 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).

RESULTS

2-APB Dose-dependently Activates and Inhibits SOCE—Fig. 1 shows Fura-5F-mediated calcium imaging experiments on wild-type (WT) HEK293 cells (Fig. 1A) or in cells expressing eYFP-STIM1 (Fig. 1C). Under nominally Ca\(^{2+}\)-free conditions (HBSS), internal Ca\(^{2+}\) stores were depleted with the SERCA pump inhibitor, thapsigargin (TG), and extracellular Ca\(^{2+}\) was...
restored revealing SOCE. Five minutes after the addition of Ca²⁺, various concentrations (3–50 μM) of the drug 2-APB were added in the continued presence of extracellular Ca²⁺. In wild-type HEK293 cells, SOCE was nearly completely blocked by 2-APB at concentrations greater than 30 μM (Fig. 1B). However, lower 2-APB concentrations (3 and 10 μM shown and up to 20 μM 2-APB, not shown) appeared to potentiate SOCE. Often, inhibition by high 2-APB concentrations was preceded by a brief potentiation, as evident for 30 μM 2-APB in Fig. 1A. Expression of eYFP-STIM1 (Fig. 1, C and D) did not appear to influence the potentiating or inhibitory effects of 2-APB on SOCE.

2-APB Reverses or Prevents STIM1 Localization in Near Plasma Membrane Puncta—Upon store depletion, STIM1 rearranges into puncta in close proximity to the plasma membrane, and this rearrangement of STIM1 appears necessary for SOCE and I_{CRAC} development. Thus, we considered whether the bimodal actions of 2-APB on SOCE could result from enhancement or disruption of STIM1 puncta formation. Fig. 2A shows a representative experiment using confocal microscopy with HEK293 cells expressing eYFP-STIM1. Shown is the distribution of eYFP-STIM1 before and after TG treatment, confirming that STIM1 clearly rearranges into puncta in response to ER Ca²⁺ store depletion. After the addition of 50 μM 2-APB, the punctate structures were largely dispersed, and the eYFP-STIM1 arrangement more closely resembled that seen when Ca²⁺ stores are filled (before TG) than when stores are depleted (after TG).

To more quantitatively assess the effect of 2-APB on STIM1, TIRFM experiments were carried out using the eYFP-STIM1 construct. Fig. 2B shows that TG treatment caused a marked increase in near plasma membrane eYFP-STIM1 fluorescence. However, after the addition of 50 μM 2-APB, eYFP-STIM1 near membrane fluorescence intensity was significantly reversed (Fig. 2, B and C). Furthermore, as shown in Fig. 2, D and E, pretreatment of cells with 2-APB completely prevented eYFP-STIM1 from migrating to near membrane regions in response to store depletion. Note that the application of 2-APB prior to store depletion generally decreased the TIRF intensity, as shown by the small decrease in F/F_0 in Fig. 2D after the application of 2-APB. Finally, at lower micromolar concentrations (≤20 μM) of 2-APB, where potentiation is seen in the Ca²⁺ imaging (Fig. 1) and electrophysiological (see below) experiments, no enhancement of near plasma membrane eYFP-STIM1 was detected (data not shown). Note that 5 μM Gd³⁺, a specific channel inhibitor of SOCE and I_{CRAC} (33), had no effect on the rearrangement of eYFP-STIM1 measured by TIRFM, despite significant inhibition of SOCE. Thus, channel block alone is not sufficient to reverse the punctate, near plasma membrane localization of STIM1. These data suggest that the...
inhibition of SOCE by 2-APB may, at least in part, be mediated by the reversal of STIM1 puncta formation. However, the potentiation appears to be independent of STIM1 relocalization, at least within the resolution of our methods.

2-APB Activates and Inhibits \( I_{\text{CRAC}} \) in HEK293 Cells Co-expressing STIM1 and Orai1—Fig. 3 shows recordings of \( I_{\text{CRAC}} \) from HEK293 cells co-expressing CFP-Orai1 and eYFP-STIM1. In these cells, 2-APB increased \( I_{\text{CRAC}} \) in a sustained manner at concentrations less than or equal to 20 \( \mu M \), whereas higher concentrations of 2-APB (≥30 \( \mu M \)) strongly inhibited the currents (Fig. 3, A and B, not shown). Shown in Fig. 3A is a focal application of 20 \( \mu M \) followed by 50 \( \mu M \) 2-APB onto the same cell after active store depletion with IP₃ and BAPTA in the patch pipette. Currents were recorded from voltage ramps (−100 to +100 mV) taken every 2 s from a 0 mV holding potential. At 20 \( \mu M \), 2-APB consistently increased \( I_{\text{CRAC}} \) in a sustained fashion, whereas 50 \( \mu M \) 2-APB strongly inhibited the currents. Inset, graph shows transient increase followed by near complete block of \( I_{\text{CRAC}} \) by the focal application of 50 \( \mu M \) 2-APB. B, bar graph of data shown in A represented as the current developed under different 2-APB concentrations (0, 20, 50 \( \mu M \)) minus the leak current, divided by the \( \Delta I/\Delta V_{\text{Ca}^{2+}} \). Data are represented as means ± S.E. (20 \( \mu M \); n = 6; 50 \( \mu M \); n = 10).

C, current-voltage (I-V) relationship from recording shown in A.

Orai1 Expression Reduces 2-APB Inhibition of STIM1 Rearrangement—The results shown in Fig. 3 indicate that the effects of 2-APB on \( I_{\text{CRAC}} \) in Orai1 and STIM1 co-expressing cells is similar to the effects of 2-APB on SOCE in WT cells or in cells overexpressing only STIM1. Thus, we assessed the effects of 2-APB on SOCE and STIM1 movements in TIRFM experiments with CFP-Orai1 expressed in conjunction with eYFP-STIM1. Parallel experiments were carried out using single-cell \( \text{Ca}^{2+} \) imaging to track the effects of 2-APB on SOCE under the same conditions. Fig. 4 shows TIRFM and \( \text{Ca}^{2+} \) imaging experiments in which 50 \( \mu M \) 2-APB much less effectively (compared with eYFP-STIM1 alone; see Fig. 2) reversed store depletion-dependent eYFP-STIM1 rearrangement (Fig. 4, A and C), yet...
nearly completely blocked SOCE (B and D). Increasing the concentration of 2-APB to 200 μM did not cause any significantly additional block (F/F₀ = 57.8 ± 2.9% of control, n = 9, as compared with 65.6 ± 6.0%, n = 16, for 50 μM). Furthermore, unlike for HEK293 cells expressing eYFP-STIM1 alone, cells co-expressing eYFP-STIM1 with CFP-Orai1 and pretreated with 50 μM 2-APB for 5 min showed significant responses to TIRFM after TG treatment (Fig. 4, E and G). However, pretreatment with 2-APB completely blocked SOCE from developing (Fig. 4, F and H). Thus, although expression of Orai1 in combination with STIM1 does not disrupt the ability of 2-APB to inhibit I\textsubscript{CRAC} and SOCE, it does significantly reduce the ability of 2-APB to interrupt STIM1 reorganization in response to store depletion. This indicates that the inhibitory effects of 2-APB must, at least in part, represent additional actions on the Orai1 channel or the activation mechanism.

We suspect that the effect of overexpressed Orai1 to reduce the effects of 2-APB on STIM1 movements may result from stabilization of STIM1 at plasma membrane puncta when Orai1 is present in stoichiometrically similar amounts. Thus, knowing that under our conditions, Orai2 and -3 would express at considerably lower levels than Orai1 (21), we examined their effects on the reversal and inhibition of STIM1 movements. As predicted, Orai2 and -3 were indeed considerably less effective in either inhibiting or reversing STIM1 movements than was Orai1 (supplemental Fig. 1).

Responses to 2-APB Differ in Cells Co-expressing STIM1 with Orai2 and Orai3—It was reported previously that Orai1, Orai2, and Orai3, when co-expressed with STIM1, differ in their responses to 2-APB (20). Fig. 5, A and B, shows that 2-APB inhibited store-operated currents in Orai2- and eYFP-STIM1-expressing cells in a manner qualitatively different from its action in cells expressing Orai1 and eYFP-STIM1 (see Fig. 3A). 20 μM 2-APB caused a transient activation in some, but not all cells, followed by a slow decline in current. Increasing the concentration to 50 μM caused a small, abrupt drop in current but did not appear to hasten the slow decline in current. Although these experiments were not carried out for longer times, we assume the inhibition by 2-APB would eventually be complete because we showed previously that pretreatment with 2-APB completely blocks SOCE in Orai2- and STIM1-expressing cells (18). In all instances the current-voltage relationships were inwardly rectifying, reminiscent of I\textsubscript{CRAC} (Fig. 5B).

Even more interesting were the results obtained with cells expressing CFP-Orai3 plus eYFP-STIM1. Although 20 μM 2-APB caused a small potentiation of Orai3 current, the focal application of 50 μM 2-APB caused the activation of a biphasic current that initially resembled I\textsubscript{CRAC}\textsuperscript{2APB}, however, longer exposure to 2-APB caused much larger currents to develop, which were no longer solely inwardly rectifying (Fig. 5, C and D). Over a period of minutes, the initially inwardly rectifying current changed to one that appeared doubly rectifying, with large inward and outward currents at the most negative and positive potentials. The inward currents recorded at −100 mV were eight times larger after prolonged 2-APB exposure when compared with the Ca\textsuperscript{2+} currents after store depletion (data not shown). A larger overall current with the development of an outward current suggests that 2-APB causes changes in the Ca\textsuperscript{2+} selectivity of channels formed by ectopically expressed Orai3. Similar currents were not seen in cells expressing eYFP or eYFP-STIM1 alone or in conjunction with CFP-Orai1 or CFP-Orai2 in response to these concentrations of 2-APB (data not shown).

Direct Activation of Orai3 by 2-APB—The data from Fig. 5 show that following Ca\textsuperscript{2+} store depletion, 2-APB increases whole-cell currents in cells expressing STIM1 plus Orai3. To determine whether the potentiating effects of 2-APB depend on store depletion and activation by STIM1, single-cell calcium imaging experiments were carried out using HEK293 cells overexpressing CFP-Orai1, -2, or -3 without exogenous STIM1 expression or after RNA interference knockdown of endogenous STIM1 (as in Ref. 31). In HEK293 cells that were not transfected with any of the three Orai subunits, 50 μM 2-APB had no effect on the basal cytoplasmic Ca\textsuperscript{2+} concentration (not shown). In cells expressing CFP-Orai1, a small, transient, but reproducible Ca\textsuperscript{2+} response was detected, which was identical in cells in which STIM1 had been reduced by RNA interference (Fig. 6, A and D). No such responses were seen in Orai2-expressing cells (Fig. 6, B and D). However, in Orai3-expressing cells 2-APB activated considerable Ca\textsuperscript{2+} influx, and as for Orai1, this entry was unaffected by knockdown of endogenous STIM1 (Fig. 6, C and D). Fig. 6, E and F, shows the concentration-dependent activation of Orai3 by 2-APB from which the half-
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FIGURE 6. Activation of Orai1 and Orai3 by 2-APB does not require STIM1 or store depletion. A–D, Ca\textsuperscript{2+} imaging experiments showing the effects of 50 \mu M and then 200 \mu M 2-APB on cytoplasmic Ca\textsuperscript{2+} levels in control cells (black) or STIM1 siRNA-treated cells (gray) transfected with Orai1 (A), Orai2 (B), or Orai3 (C). Results similar to those seen in Orai2-expressing cells (D) were also seen in YFP alone expressing control and STIM1 siRNA-treated cells (data not shown). D, peak changes in cytoplasmic Ca\textsuperscript{2+} detected in control (black) or STIM1 siRNA-treated (gray) HEK293 cells expressing Orai1, Orai2, or Orai3 and treated with 50 \mu M 2-APB. Under all conditions, the data are the mean \pm S.E. of three coverslips, and each coverslip is the mean of at least 25 cells. E, mean of four independent Ca\textsuperscript{2+} imaging experiments (\textpm 25 cells/experiment) looking at the effects of several concentrations of 2-APB (0–50 \mu M) on cytoplasmic Ca\textsuperscript{2+} concentration in cells expressing CFP-Orai3. F, concentration-response curve showing the direct activation of Orai3 by 2-APB. NCF, nominally Ca\textsuperscript{2+}-free.

maximal concentration was calculated to be 13.5 \pm 4.6 \mu M 2-APB using a least squares fit. Interestingly, the concentration range of 2-APB for inhibition of entry in WT HEK293 cells, or in WT HEK293 cells expressing STIM1 alone or in conjunction with Orai1 (see Figs. 1 and 3), is similar to the concentration range at which 2-APB activates Orai3.

Single-cell Ca\textsuperscript{2+} imaging experiments were also carried out on HEK293 cells expressing CFP-Orai3 alone, in which stores were depleted first with TG. Ca\textsuperscript{2+} was added back to assess SOCE, and then 50 \mu M 2-APB was applied. The purpose of these experiments was to further assess whether endogenous STIM1 or store depletion might play a role in the activation of Orai3 by 2-APB. Fig. 7, B and C, shows that store depletion and endogenous STIM1 had little to no effect on 2-APB activation of Orai3 when compared with the effects of 2-APB on Orai3 without store depletion (see Fig. 6). Although not directly relevant to this study, it should be noted that this is the first time that it has been shown that exogenously expressed Orai3 also suppresses SOCE (Fig. 6B), when not expressed with STIM1, in a manner similar to Orai1 and Orai2 (18) as compared with control WT HEK293 cells (Fig. 6A).

To determine the biophysical properties of the calcium entry seen after 2-APB application in Orai1- and Orai3-expressing cells, whole-cell electrophysiological experiments were carried out in which the internal calcium was clamped to 100 nM to prevent store depletion. In the presence of 2 mM external calcium, 50 \mu M 2-APB was applied, and whole-cell inward currents were recorded in the same fashion as in Figs. 3 and 5 (0 mV holding potential; \textpm 100 to +100 mV ramps every 2 s). Fig. 8A shows a representative recording in which 2-APB directly activated Orai3-dependent currents independently of STIM1 or store depletion. Interestingly, the maximal currents recorded from CFP-Orai3-expressing cells lacking exogenous STIM1 expression appeared identical to the maximal currents recorded when exogenous STIM1 was present and stores were depleted, both in terms of magnitude and current-voltage relationship (Fig. 8, B and D). In a manner similar to the Ca\textsuperscript{2+} imaging experiments shown in Fig. 6, cells expressing CFP-Orai1 also developed transient inward currents in response to 50 \mu M 2-APB, independently of STIM1 or store depletion (Fig. 8A). Unlike Orai3 currents, the currents seen in these cells exhibited inward rectification reminiscent of I\textsubscript{CRAC} (Fig. 8B). In Orai2-expressing cells, no direct activation of current by focal application of 2-APB was ever detected. These results are summarized in the bar graph shown in Fig. 8C. We believe that the Ca\textsuperscript{2+} imaging and electrophysiological data taken together suggest, at least in the case of Orai3 and to a lesser degree Orai1, that 2-APB directly activates these channels independently of store depletion and STIM1.
2-APB Increases Cs⁺ Permeability through Orai3 Channels—

The presence of the outward currents seen in the Orai3-expressing cells after 50 μM 2-APB application suggested that the compound altered the channel properties, now allowing Cs⁺ to permeate. We previously reported that similar to Orai1, Orai3 (and Orai2) does not permeate Cs⁺ well (21). The outward currents also suggest that Ca²⁺ and/or Mg²⁺ is less effective at blocking monovalent conductance through the channels when 2-APB is present. To further investigate these possibilities, whole-cell patch clamp experiments were carried out on HEK293 cells expressing CFP-Orai3. Cells were selected based on CFP fluorescence. Fig. 9A shows a representative recording depicting the effects of extracellular Ca²⁺ on the 2-APB-activated Orai3 whole-cell currents. As seen in Fig. 9, A and B, increasing extracellular Ca²⁺ from 2 to 20 mM reduces the outward currents recorded at +100 mV, shifts the reversal potential rightward, and initially decreases, then slightly increases, the inward currents recorded at −100 mV. Like endogenous $I_{\text{CRAC}}$, Na⁺ permeated through the 2-APB-activated Orai3 channels, and the inward Na⁺ conductance was significantly inhibited by 2 mM extracellular Mg²⁺ in the nominally Ca²⁺ free (NCF) external solution (Fig. 9, A and C). However, this block is not complete (Fig. 9C), whereas for store-operated $I_{\text{CRAC}}$ and for store-operated Orai3 channels (20) the block is complete. There was a leftward shift in the reversal potential seen in the peak Na⁺-DVF currents (Fig. 9C) when compared with the Na⁺ currents seen in Orai3 channels activated by STIM1 and store-depletion (panel G). Thus, the Ca²⁺ selectivity

FIGURE 8. 2-APB activates Orai1 and Orai3 independently of STIM1 and internal Ca²⁺ store depletion. A, whole-cell currents recorded from HEK293 cells expressing Orai1, Orai2, or Orai3 before, during, and after focal application of 50 μM 2-APB. Cytoplasmic Ca²⁺ concentrations were buffered using BAPTA and millimolar concentrations of Ca²⁺ (calculated using Maxchelator software) in order to avoid passive store depletion. 2 mM extracellular Ca²⁺ was present throughout these experiments. B, current-voltage relationships from the recordings shown in A. C, bar graph showing the change in current densities (peak 2-APB current minus leak current) recorded from voltage ramps in cells expressing Orai1 (O1), Orai2 (O2), and Orai3 (O3). Data were collected in studies identical to those shown in A (Orai1: n = 4; Orai2: n = 3; Orai3: n = 6). D, comparison of the peak currents recorded in HEK293 cells expressing Orai3 alone (stores replete; n = 6 (A)), or Orai3 expressed with STIM1 and stores actively depleted with IP₃ and BAPTA in the pipette (Fig. 5; n = 7).

2-APB-activated Orai3 currents have increased Cs⁺ permeability compared with Orai3-mediated CRAC currents activated by store depletion. A, whole-cell patch clamp recording taken at −100 and +100 mV from a HEK293 cell expressing Orai3. The internal pipette solution contained Ca²⁺-“clamped” to 100 mM Ca²⁺. Voltage ramps (−100 to +100 mV) were applied every 2 s from a holding potential of 0 mV. External solutions were applied as indicated by the bars above the recording. NCF, nominally Ca²⁺-free external solution. B, current-voltage relationships taken from the recording shown in A, when either 2 mM (black trace) or 20 mM (gray trace) extracellular Ca²⁺ was present in the bathing solution. C, current-voltage relationships also taken from A, showing that 2 mM Mg²⁺ present in the nominally Ca²⁺-free (gray trace) external solution is sufficient to block nearly all Na⁺ current through the 2-APB-activated Orai3 channels. Black arrows indicate the time points at which the i-V traces were taken. D, representative recording taken under conditions similar to those in A; however, a cesium-DVF solution was applied after activation of Orai3 with 2-APB. E, current-voltage relationships of the Ca²⁺ and Cs⁺ currents seen in these HEK293 cells expressing Orai3 and activated directly with 2-APB. F, representative whole-cell recording taken from a HEK293 cell co-expressing Orai3 and STIM1, in which ER Ca²⁺ stores were actively depleted with 20 μM IP₃ and 20 mM BAPTA in the patch pipette. The extracellular bathing solution was switched from a 2 mM Ca²⁺-containing solution to one lacking all divalent cations and containing either the monovalent cation Na⁺ (black) or Cs⁺ (gray) as the charge carrier. Unlike in 2-APB-activated Orai3 recordings, Cs⁺ does not permeate well through STIM1 and store-depletion-activated Orai3 channels. G, current-voltage relationships from the peak Cs⁺ and Na⁺ currents seen in the Orai3 + STIM1-expressing HEK293 cells after active ER Ca²⁺ store depletion. All traces are representatives of at least three similar recordings.

FIGURE 9. 2-APB-activated Orai3 currents have increased Cs⁺ permeability compared with Orai3-mediated CRAC currents activated by store depletion.
ity of Orai3 channels is also reduced by 2-APB. An additional major difference in pore properties of 2-APB-activated Orai3 channels was revealed by experiments examining Cs\(^+\) conductance through 2-APB-activated Orai3 channels (Fig. 9, D and E). After activating Orai3 by 2-APB under 2 mM Ca\(^{2+}\) conditions, switching to a Cs\(^+\)-DVF extracellular solution revealed a large inward Cs\(^+\) current in these cells, which is not present in Orai3 plus STIM1 co-expressing, store-depleted controls (Fig. 9, F and G). Taken together, these results indicate that 2-APB directly activates Orai3 and alters Orai3 channel properties, reducing the ability of extracellular divalent cations to block monovalent permeation and increasing the Cs\(^+\)/Na\(^+\) permeability ratio (Fig. 9, E and G).

**DISCUSSION**

2-APB was originally described as an IP\(_3\) receptor antagonist and was later shown to potentiate and inhibit SOCE and \(I_{\text{CRAC}}\) dose-dependently, independently of IP\(_3\) receptor inhibition. With the recent discoveries of the STIM and Orai proteins, we sought to investigate the bimodal effects of 2-APB on store-operated currents and Ca\(^{2+}\) entry in cells expressing these proteins to gain insight into the molecular mechanism of action of this extensively employed pharmacological tool. We show here that 2-APB prevents, as well as reverses, eYFP-STIM1 reorganization in response to ER Ca\(^{2+}\) store depletion independently of store refilling. It has previously been shown that the rearrangement of STIM1 in the ER is essential for activation of SOCE (6, 10, 18). Thus, this effect on STIM1 localization could account for the inhibitory effects of 2-APB on SOCE and \(I_{\text{CRAC}}\) development. However, co-expression of STIM1 with the CRAC channel pore-forming subunit, Orai1, significantly reduced the ability of 2-APB to prevent or reverse store depletion-dependent STIM1 movements while having no effect on its ability to inhibit SOCE or current.

The Orai1 homologs, Orai2 and Orai3, respond differently to inhibitory concentrations of 2-APB, yet the activation of all three depends on STIM1. Whereas Orai2-mediated current was only slowly inhibited compared with Orai1, Orai3 was activated by 2-APB and the Orai3-forming channels became permeable to monovalent ions. Finally, we show that exogenously expressed Orai3, and to a lesser extent Orai1, can be directly activated by 2-APB independently of STIM1 and store depletion. Taken together, these data reveal a complex mechanism of action of 2-APB on SOCE and store-operated currents, in which 2-APB influences both STIM1 and Orai proteins located in the ER and plasma membranes, respectively. Given that at least some of the inhibitory effects on Orai channels occur downstream of STIM1 movements, and that the degree of inhibition of STIM1 movements depends on the relative levels of STIM1 and Orai, it is not possible to ascertain the degree to which inhibition of STIM1 movement contributes to channel inhibition in native cells. Nonetheless, these findings are significant given that 2-APB is widely used in the investigation of SOCE and \(I_{\text{CRAC}}\).

A better understanding of the effects of 2-APB on STIM1 movements and SOCE can be gained by comparing its effects with another inhibitor of SOCE and \(I_{\text{CRAC}}\), ML-9. We recently reported that ML-9 completely inhibits endogenous SOCE and \(I_{\text{CRAC}}\) by inhibiting store depletion-induced reorganization of STIM1 into near plasma membrane puncta, an action similar to that reported here for 2-APB (15). However, in the case of ML-9, exogenous expression of STIM1 caused a rightward shift of the inhibition curve when compared with WT HEK293 cells. This partial “rescue” of the ML-9 inhibition effect suggested that the primary inhibitory action of ML-9 on SOCE is mediated through STIM1. Unlike ML-9, expression of STIM1 did not influence the concentration of 2-APB required to block SOCE. Furthermore, in HEK293 cells co-expressing STIM1 and Orai1, ML-9 is much less potent at preventing store depletion-dependent STIM1 puncta formation, a finding similar to that shown here for 2-APB (supplemental Fig. 2A). However, in these co-expressing cells, ML-9 also is much less effective at blocking SOCE and store-operated currents (supplemental Fig. 2, B and C), again consistent with the notion that ML-9 inhibition of SOCE and \(I_{\text{CRAC}}\) reflects inhibition of STIM1 puncta formation. Again, this is unlike 2-APB, which blocks \(I_{\text{CRAC}}\) in WT and Orai1 plus STIM1-expressing cells with essentially equal potency despite differences in its inhibition of STIM1 puncta formation. Although the reason is not yet fully understood, we find it intriguing that expression of Orai1 can significantly reduce the reversal of eYFP-STIM1 puncta by 2-APB and ML-9, and we are currently investigating why this is the case.

Although Orai1 and Orai2 (albeit less than Orai1) were both inhibited by high concentrations of 2-APB, Orai3 was activated by the compound. Initially the currents that developed in these cells resembled \(I_{\text{CRAC}}\); however, large outward currents that were permeable to Cs\(^+\) at potentials greater than +50 mV subsequently developed. These currents reached a peak after several minutes, and were sustained in the continued presence of 2-APB. The outwardly rectifying 2-APB-activated currents were present only in Orai3-expressing cells and did not appear to result from the activation of a secondary Ca\(^{2+}\)-dependent conductance. To our knowledge, this is the first report of a pharmacological agent that can alter the selectivity of an \(I_{\text{CRAC}}\)-like store-operated channel. Site-directed mutagenesis directed toward an acidic residue on TM3 (E190Q) of Orai1 alters the selectivity of Orai1-mediated \(I_{\text{CRAC}}\), and the resultant current-voltage relationship recorded from HEK293 expressing this mutated Orai1 looks strikingly similar to the current-voltage relationship seen in Orai3-expressing cells after 2-APB (34) (but see also Ref. 35).

At subinhibitory concentrations, 2-APB strongly potentiates \(I_{\text{CRAC}}\) in HEK293 cells co-expressing Orai1 and STIM1 by up to 50%. Similar results have been described for endogenous \(I_{\text{CRAC}}\) in which low concentrations of 2-APB can increase current amplitude by up to 5-fold (26). Previous results (26, 36) suggest that 2-APB potentiates \(I_{\text{CRAC}}\) only after stores are depleted and does not directly activate the currents independently of STIM1 and store depletion. Nonetheless, results presented here using exogenously expressed Orai1 and Orai3 suggest that 2-APB can elicit direct activation of the CRAC or CRAC-like channels. The reason for the distinct findings is not clear, but they may reflect differences in the stoichiometry of Orai and STIM proteins to one another, or to other proteins, in cells transfected with cDNAs driven by strong viral promoters.
In summary, the results presented here indicate that 2-APB has complex inhibitory and activating mechanisms on Orai channels. The inhibitory action appears to involve at least two effects, one involving alteration of movement of STIM1 into puncta and one at a downstream site involving either a direct effect on the channel or on the ability of STIM1 to activate the channels. The inhibitory actions of 2-APB differed markedly, both quantitatively and qualitatively, among the three Orai species, consistent with but not proving a direct effect on the channels themselves. Earlier findings from a number of laboratories suggest an extracellular site of action of 2-APB on ICa _{CRAC} (24, 26, 27), which is further consistent with a direct action on the channel. Both Orai1 and -3 could also be activated by 2-APB, and Orai3 channels additionally showed altered ion selectivity when activated by 2-APB. These different effects of 2-APB on Orai1, Orai2, and Orai3 could possibly be used as diagnostic criteria for detecting the expression patterns of Orai isoforms in various tissues. However, as indicated in the Introduction, the lack of known models for studying native Orai2 or -3 channels has necessitated the use of exogenously expressed proteins, usually supported by increased expression levels of STIM1. Thus, it cannot be assured that these channels will behave identically at more physiological levels of expression. It is encouraging that the actions of 2-APB on exogenously expressed Orai1 match well those previously described for effects on ICa _{CRAC} in hematopoietic cells, where Orai1 is apparently expressed in its native environment. Future work should focus on identifying physiological sites of expression of all three channel proteins in order to further evaluate the similarities and differences in their biophysical and pharmacological properties.\footnote{While this paper was under revision, two reports appeared with findings similar to some of those reported here (37, 38).}

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REFERENCES

1. Putney, J. W. (1986) Cell Calcium 7, 1–12
2. Smyth, J. T., DeHaven, W. I., Jones, B. F., Mercer, J. C., Trebak, M., Vazquez, G., and Putney, J. W. (2006) Biochim. Biophys. Acta 1763, 1147–1160
3. Hoth, M., and Penner, R. (1992) Nature 355, 353–355
4. Parekh, A. B., and Putney, J. W. (2005) Physiol. Rev. 85, 757–810
5. Roos, P. J., DiGregorio, P. I., Yeromin, A. V., Ohlsen, K., Lioudyno, M., Zhang, S., Safirina, O., Kozak, J. A., Wagner, S. L., Cahalan, M. D., Velicelebi, G., and Stauderman, K. A. (2005) J. Cell Biol. 169, 435–445
6. Liu, J., Kim, M. L., Heo, W. D., Jones, J. T., Myers, J. W., Ferrill, E. J., Jr., and Meyer, T. (2005) Curr. Biol. 15, 1235–1241
7. Feske, S., Gwack, Y., Prakriya, M., Srikantan, S., Puppel, S. H., Tanasa, B., Hogan, P. G., Lewis, R. S., Daly, M., and Rao, A. (2006) Nature 441, 179–185
8. Vign, M., Peinelt, C., Beck, A., Koomoa, D. L., Rabah, D., Koblan-Huberson, M., Kraft, S., Turner, H., Fleig, A., Penner, R., and Kinet, J. P. (2006) Science 312, 1220–1223
9. Zhang, S. L., Yeromin, A. V., Zhang, X. H., Yu, Y., Safirina, O., Penna, A., Roos, P. J., Stauderman, K. A., and Cahalan, M. D. (2006) Proc. Natl. Acad. Sci. U. S. A. 103, 9357–9362
10. Zhang, S. L., Yu, Y., Roos, J., Kozak, J. A., Deerinck, T. J., Ellisman, M. H., Stauderman, K. A., and Cahalan, M. D. (2005) Nature 437, 902–905
11. Baba, Y., Hayashi, K., Fujii, Y., Mizushima, A., Watarai, H., Wakamori, M., Numaga, T., Mori, Y., Ino, M., Hikida, M., and Kurosaki, T. (2006) Proc Natl. Acad. Sci. U. S. A. 103, 16704–16709
12. Smyth, J. T., DeHaven, W. I., Bird, G. S., and Putney, J. W., Jr. (2007) J. Cell Sci. 120, 3762–3771
13. Luik, R. M., Wu, M. M., Buchanan, J., and Lewis, R. S. (2006) J. Cell Biol. 174, 815–825
14. Xu, P., Lu, J., Li, Z., Yu, X., Chen, L., and Xu, T. (2006) Biochem. Biophys. Res. Commun. 350, 969–976
15. Smyth, J. T., DeHaven, W. I., and Putney, J. W., Jr. (2008) J. Cell Sci. 121, 762–772
16. Peinelt, C., Vign, M., Koomoa, D. L., Beck, A., Nadler, M. J. S., Koblan-Huberson, M., Lis, A., Fleig, A., Penner, R., and Kinet, J. P. (2006) Nat. Cell Biol. 8, 771–777
17. Soboloff, J., Spassova, M. A., Tang, X. D., Hewavitharana, T., Xu, W., and Gill, D. L. (2006) J. Biol. Chem. 281, 20661–20665
18. Mercer, J. C., DeHaven, W. I., Smyth, J. T., Wedel, B., Boysle, R. R., Bird, G. S., and Putney, J. W. (2006) J. Biol. Chem. 281, 24979–24990
19. Putney, J. W., Jr. (2007) J. Cell Sci. 120, 1959–1965
20. Lis, A., Peinelt, C., Beck, A., Parvez, S., Montedil-Zoller, M., Fleig, A., and Penner, R. (2007) Curr. Biol. 17, 794–800
21. DeHaven, W. I., Smyth, J. T., Boysle, R. R., and Putney, J. W. (2007) J. Biol. Chem. 282, 17548–17556
22. Ma, H. T., Patterson, R. L., van Rossum, D. B., Birnbaumer, L., Mikoshiba, K., and Gill, D. L. (2000) Science 287, 1647–1651
23. Berridge, M. J. (1995) Biochem. J. 312, 1–11
24. Braun, F. I., Broad, L. M., Armstrong, D. L., and Putney, J. W. (2001) J. Biol. Chem. 276, 1063–1070
25. Broad, L. M., Braun, F. I., Li€evremont, J. P., Bird, G. S., and Putney, J. W. (2001) J. Biol. Chem. 276, 15945–15952
26. Prakriya, M., and Lewis, R. S. (2001) J. Physiol. (Lond.) 536, 3–19
27. Bakowski, D., Glitsch, M. D., and Parekh, A. B. (2001) J. Physiol. (Lond.) 532, 55–71
28. Ma, H. T., Venkataramal, K., Li, H. S., Montell, C., Kurosaki, T., Patterson, R. L., and Gill, D. L. (2001) J. Biol. Chem. 276, 18888–18896
29. Iwasaki, H., Mori, Y., Hara, Y., Uchida, K., Zhou, H., and Mikoshiba, K. (2001) Recept. Channels 7, 429–439
30. Bird, G. S., and Putney, J. W. (2005) J. Physiol. 562, 697–706
31. Wedel, B., Boysle, R. R., Putney, J. W., and Bird, G. S. (2007) J. Physiol. 579, 679–689
32. Smyth, J. T., Lemonnier, L., Vazquez, G., Bird, G. S., and Putney, J. W. (2005) J. Biol. Chem. 281, 11712–11720
33. Broad, L. M., Cannon, T. R., and Taylor, C. W. (1999) J. Physiol. (Lond.) 517, 121–134
34. Vign, M., Beck, A., Billingsley, I. M., Lis, A., Parvez, S., Peinelt, C., Koomoa, D. L., Soboloff, J., Gill, D. L., Fleig, A., Kinet, J. P., and Penner, R. (2006) Curr. Biol. 16, 2073–2079
35. Prakriya, M., Feske, S., and Lewis, R. S. (2000) Nature 403, 230–233
36. Ma, H. T., Venkataramal, K., Parys, J. B., and Gill, D. L. (2002) J. Biol. Chem. 277, 6915–6922
37. Peinelt, C., Lis, A., Beck, A., Fleig, A., and Penner, R. (2008) J. Physiol., in press
38. Zhang, S. L., Kozak, J. A., Ji, W., Yeromin, A. V., Chen, J., Yu, Y., Penna, A., Shen, W., Chi, V., and Cahalan, M. D. (2008) J. Biol. Chem. 283, 17662–17671