2-Aminoethoxydiphenyl Borate Alters Selectivity of Orai3 Channels by Increasing Their Pore Size*

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Stim1 in the endoplasmic reticulum and the three Orai (also termed CRACM) channels in the plasma-membrane are main components of native Ca2+ release-activated Ca2+ channels. A pharmacological hallmark of these channels is their distinct sensitivity to 2-aminoethoxydiphenyl borate (2-APB). Here we report that Orai3 currents can be robustly stimulated by 75 μM 2-APB independent of Stim1, whereas 2-APB at similar concentrations inhibited store-operated Orai1 currents. 2-APB did not only promote currents through Orai3 channels but also dramatically altered ion selectivity of Orai3 channels. This allowed for permeation of monovalent cations both in the inward as well as outward direction, which is in sharp contrast to the high Ca2+ selectivity of store-operated Orai3 currents. An Orai3-R66W mutant, which lacked in analogy to the severe combined immune deficiency mutant Orai1-R91W store-operated activation, was also found to be resistant to 2-APB stimulation. The change in selectivity by 2-APB was associated with an increase in Orai3 minimum pore size from about 3.8 Å to more than 5.34 Å. In line with a potential interaction of 2-APB with the Orai3 pore, among three pore mutants tested, the Orai3 E165Q mutant particularly resembled in its permeation properties those of 2-APB stimulated Orai3 and additionally exhibited a reduced response to 2-APB. In aggregate, stimulation of Orai3 currents by 2-APB occurred along with an alteration of the permeation pathway that represents a unique mechanism for regulating ion channel selectivity by chemical compounds.

A major mechanism for many cell types to maintain long-lasting elevation of intracellular Ca2+ is the use of store-operated Ca2+ influx (1). Initiated by the second messenger inositol 3-phosphate, Ca2+ is released from the endoplasmic reticulum. Subsequently, Stim1 located in the endoplasmic reticulum senses via a luminal EF-hand the Ca2+ signal and activates the Orai1 complex (10). It is proposed that 2-APB displaces the inhibitory calmodulin from the Stim1-Orai1 complex (10), and the Orai3 current is exclusively stimulated by 2-APB (6). We demonstrate in this study that 2-APB stimulates Orai3 currents and concomitantly changes the selectivity of the channel via an increase in the minimal dimension of the pore size, independent of Stim1.

EXPERIMENTAL PROCEDURES

Molecular Cloning and Mutagenesis—Human Orai1 (Orai1; accession number NM_032790) was kindly provided by A. Rao, Harvard Medical School and human Orai3 (Orai3; accession number NM_152288) was kindly provided by L. Birnbaumer, NIEHS, National Institutes of Health. For fluorescence labeling of Orai1 at its N terminus the construct was cloned into the pEYFP-C1 (Clontech) expression vector via its SalI and SmaI restriction sites. Orai3 was cloned into the pEYFP-C1 (Clontech) expression vector via its BamHI and XbaI restriction sites also for N-terminal labeling. For the generation of the R66W mutant pEYFP-C1 Orai3 was used as a template. Suitable primers exchanged the corresponding codon from CGC to TGG (R66W) using the QuikChange XL site-directed mutagenesis kit (Stratagene). pEYFP-C1 Orai3 was also used as template for the generation of the E165D and E165Q mutants. For the E81D mutant pECFP-C1 Orai3 was used as a template. Suitable primers were used for amplification of full-length Orai1 and Orai3 cDNA using the following primers: Orai1 forward (5′-ATGAGATCCATGTGCTCCCTCAGT-3′) and reverse (5′-TCTGTCGACAGGCCTCCTCTG-3′); Orai3 forward (5′-ATGAGATCCATGTGCTCCCTCAGT-3′) and reverse (5′-TCTGTCGACAGGCCTCCTCTG-3′). The PCR products were purified, digested with ScaI and BamHI, and ligated into the pEYFP-C1 vector (Clontech) expression vector via its SalI and SmaI restriction sites. The plasmid was verified by DNA sequencing.

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§§ The abbreviations used are: CRAC, Ca2+ release-activated Ca2+; 2-APB, 2-aminoethoxydiphenyl borate; Stim1, stromal interaction molecule; NaDVF, Na+-based divalent free solution; CsDVF, Cs+-based divalent free solution; siRNA, small interfering RNA; CFP, cyan fluorescent protein; YFP, yellow fluorescent protein.
ers exchanged the corresponding codon from GAA to GAT (E81D and E165D) or from GAA to CAA (E165Q). All point mutations were introduced using the QuickChange XL site-directed mutagenesis kit (Stratagene). Human Stim1 (Stim1; accession number NM_003156) N-terminal CFP-tagged was kindly provided by T. Meyer, Stanford University.

Transfection Procedure—The cells were transfected every 3–4 days and typically used for 12–14 passages. Transient transfection was carried out with 6 μl of Transpass (New England Biolabs) with 2 μg of DNA of CFP- or YFP-tagged Stim1 or 400 nm validated siRNA against human Stim1 (purchased from Ambion) and/or 3 μg of DNA of CFP- or YFP-tagged Orai3 or YFP-Orai3 pore mutants. Transfected cells were identified directly via YFP/CFP-tagged proteins. Measurements were carried out about 24 h following transfection.

Electrophysiology—Electrophysiological experiments were performed at 21–25 °C, using the patch clamp technique in whole cell recording configurations. An Ag/AgCl electrode in combination with a 3 M KCl-filled agar bridge was used as reference electrode. For Stim1- and Orai1/Orai3-mediated currents voltage ramps were applied every 5 s from a holding potential of 0 mV, covering a range of −90 to 90 mV over 1 s. The internal pipette solution designed for passive store depletion of Orai-derived currents contained (in mM): 145 Cs methan sulfonate, 8 NaCl, 3.5 MgCl2, 10 HEPES, 10 EGTA, pH 7.2. Standard extracellular solution consisted of 145 NaCl, 5 CsCl, 1 MgCl2, 10 HEPES, 10 glucose, 10 CaCl2, pH 7.4. Divalent free solution included 150 NaCl, 10 HEPS, 10 EGTA, 10 EDTA. Where indicated, 150 NaCl was substituted by 150 CsCl, methylene HCl, dimethylamine HCl, or trimethylamine HCl. Applied potentials were corrected for a liquid junction of 12 mV resulting from a Cl−-based bath solution and a sulfonate-based pipette solution. All substances were purchased from Sigma.

RESULTS

2-APB Alters Orai3 Selectivity—2-APB Stimulates Orai3 Independent of Stim1—Coexpression of human YFP-Orai3 and CFP-Stim1 in HEK cells yielded activation of inwardly rectifying currents following passive store depletion with 10 mM EGTA in the pipette and a 10 mM Ca2+ containing bath solution (Fig. 1A). Voltage ramps depicted a highly positive reversal potential of >50 mV for store-operated Orai3 currents (Fig. 1C). Application of 2-APB (75 μM) stimulated Orai3 inward currents ~7-fold and was accompanied by a profound change in current-voltage relationships (Fig. 1D). In contrast to inwardly rectifying store-operated Orai3 currents induced by store depletion, currents observed following perfusion with 2-APB (75 μM) displayed a double rectifying current-voltage relationship with a pronounced outward current component (Fig. 1D), suggesting that the selectivity is changed. Overexpression of Orai3 without Stim1, as expected, failed to generate a store-operated conductance. Nevertheless, 75 μM 2-APB induced Orai3 currents to a similar extent as in the presence of Stim1 (Fig. 1, A and B). Moreover, Orai3 current stimulation by 2-APB (75 μM) was similarly obtained when endogenous Stim1 expression was inhibited by siRNA against Stim1 (Fig. 1, A and B). Effectiveness of Stim1-siRNA has been verified by clear suppression of YFP-tagged STIM1 (2). All 2-APB-evoked Orai3 currents exhibited comparable maximal current densities (Fig. 1B), similar current-voltage relationships (Fig. 1D), and reversal potentials (Stim1 + Orai3 coexpression, Vrev = +3.2 ± 1.0 mV; Orai3, Vrev = +3.3 ± 0.7 mV; and Orai3 + siRNA-Stim1, +4 ± 1 mV). On the contrary, 75 μM 2-APB abolished store-operated currents of cells coexpressing Orai1 and Stim1, whereas 10 μM 2-APB transiently stimulated Orai1 currents, yet without altering the inwardly rectifying current-voltage relationship (data not shown). 2-APB-stimulated Orai3 inward as well as outward currents were robustly blocked by addition of 10 μM La3+ (Fig. 1F). Furthermore, we investigated an Orai3-R66W mutant analogous to the Orai1-R91W mutant that is linked to severe combined immune deficiency and was reported to lack any store-operated activation (4). Here we tested whether 2-APB is able to alternatively stimulate Orai3-R66W. Yet Orai3-R66W coexpression with Stim1 neither gave rise to any significant current activation by store depletion nor displayed any sensitivity to stimulation by 2-APB (75 μM) (Fig. 1F), indicating that this mutation prevents permeation through Orai3 channels independent of the mode of activation. These experiments demonstrated that 2-APB is able to stimulate Orai3 currents independent of Stim1.

2-APB Alters Selectivity of Orai3 Currents—Analysis of the current-voltage relationship of Orai3 conductance during 2-APB stimulation revealed that the reversal potential shifted from > +15 mV of initial 2-APB-evoked Orai3 currents (after 5 s) to 3.3 ± 0.7 mV in the presence of 2-APB at maximum Orai3 stimulation (Fig. 2A), suggesting that Ca2+-selectivity of store-operated Orai3 currents was altered. When Ca2+ was omitted from the bath solution, 2-APB-stimulated inward currents of Orai3 were significantly larger than those in the presence of 10 mM Ca2+ (Fig. 2B) and resulted in a more linear current-voltage relationship (Fig. 2C). It is reasonable to assume that Ca2+-ions hinder monovalent Na+-ions to enter the pore at negative potentials in 2-APB-modified Orai3 channels. To further explore the selectivity of 2-APB-stimulated Orai3 currents, after reaching maximum activation, standard Ca2+-bath solution was replaced by a 150 mM Na+-based divalent free solution (Na-DVF). This Na+-based solution resulted in a pronounced current increase of 2-APB-stimulated Orai3 channels that exhibited a linear current-voltage relationship (Fig. 2, D and F) with a reversal potential of −7.5 ± 0.2 mV. In an analogous approach, a Cs+-based DVF solution also increased Orai3 currents (Fig. 2, E and F), with a reversal potential of −2 ± 0.7 mV. In control experiments using a similar protocol in the absence of 2-APB, the store-operated conductance generated by coexpression of Stim1 and Orai3 resulted also in a pronounced current increase when Ca2+-solution was exchanged to Na-DVF, yet maintaining inward rectification with a reversal potential above +50 mV (Fig. 2, G and I). In contrast, store-operated Orai3 currents were robustly suppressed in a Cs-DVF solution (Fig. 2, H and I), suggesting that the selectivity profile of Orai3 channels is dramatically altered in 2-APB-stimulated versus store-operated Orai3 currents. Hence, 2-APB application alters Orai3 permeability from a highly Ca2+-selective to a nonselective current that passes both Na+ as well as Cs+. 
2-APB Increases Minimum Pore Size of Orai3 Channels—To investigate whether the 2-APB-induced change in the permeation/selectivity properties of Orai3 channels is accompanied by a modification/increase in their minimal dimension of pore size, we made use of methylated derivates of ammonium, an approach recently carried out with Orai1 pore mutants (8). The narrowest region of native CRAC and Orai1 channels is only 3.78 Å, dimethylammonium is 4.6 Å, and trimethylammonium is 5.34 Å. We initially focused on Stim1-mediated Orai3 currents after maximum store-depletion activation, as pore size of Orai3 has not yet been addressed. Store-operated currents of coexpressed Stim1 and Orai3 were monitored in a standard 10 mM Ca\(^{2+}\) solution followed by perfusion with a 150 mM methylammonium-DVF solution (Fig. 3A). This resulted in a further increase of Orai3 currents that still exhibited an inwardly rectifying current-voltage relationship (Fig. 3C). The larger dimethylammonium was not anymore permeable for store-operated Orai3, as examined in a similar approach, resulting in completely suppressed currents upon perfusion with 150 mM dimethylammonium-DVF solution (Fig. 3B and C). In contrast, 2-APB (75 μM)-stimulated Orai3 channels showed large currents in 150 mM dimethylammonium-DVF solution, yielding a linear current-voltage relationship (Fig. 3B, D and F). Moreover, 2-APB (75 μM) modified Orai3 currents still increased about 3-fold upon exchange of a 10 mM Ca\(^{2+}\) containing bath solution by 150 mM trimethylammonium-DVF solution (Fig. 3). The current-voltage relationship revealed a more negative reversal potential for trimethylammonium ($V_{rev} = -37.8 \pm 2.1$ mV) than dimethylammonium ($V_{rev} = -16.8 \pm 3.4$ mV) or sodium ($V_{rev} = -7.5 \pm 0.2$ mV), suggesting that selectivity or permeability for larger cations is steadily decreasing (Fig. 3F). These experiments demonstrate that 2-APB is able to increase the effective pore size of Orai3 channels to more than 5.34 Å concomitant with a profound alteration in channel selectivity, whereas the Ca\(^{2+}\) selectivity of store-operated Orai3 channels is associated with an effective pore size of about 3.8 Å. Mechanistically, it is tempting to speculate that 2-APB via its positive charge (14) might interact with the Orai3 selectivity filter that results in the altered permeation properties. This hypothesis was further evaluated by generating Orai3 pore mutants analogously to Orai1 that are expected to display a modified response to 2-APB.

Glutamates in Transmembrane Domains 1 and 3 Contribute to the Selectivity Filter of Orai3—Comparison of 2-APB-activated Orai3 currents with those recently described from store-operated Orai1 reveals similar double-rectifying current-voltage relationships (15). Hence, 2-APB might have modified pore configuration of Orai3 in a way that resembles at least in part that obtained by a mutation in the selectivity filter. We therefore focused on the conserved glutamates in transmembrane domains 1 and 3 that have previously been shown to affect permeation properties of Orai1 (8, 15–17). In brief, mutation of Glu-106 in the TM1 domain to Asp (Orai1-E106D) results in a more linear current-voltage relationship of
Orai1, whereas a similar mutation at position 190 (Orai1-E190D) did not affect Orai1 Ca$^{2+}$/H$^{11001}$ selectivity (15–17). However, substitution of Glu-190 to neutral Gln (Orai1-E190Q) yields a double rectifying current-voltage relationship in 10 mM Ca$^{2+}$/H$^{11001}$ containing bath solution (8, 15–17).

The amino acids that contribute to the selectivity filter of the Orai3 pore have not yet been investigated. Therefore in analogy to Orai1, we mutated the conserved Glu-81 in TM1 to an Asp (Orai3-E81D) that corresponds to E106D in Orai1 and Glu-165 in TM3 to Asp (Orai3-E165D) or Gln (Orai3-E165Q). Coexpression of Orai3-E81D and Stim1 yielded activation of store-operated currents with a linear current-voltage relationship and a reversal potential of 2$^{11006}$1.5 mV in the presence of 10 mM Ca$^{2+}$/H$^{11001}$ containing bath solution (Fig. 4, A and B). Perfusion with 150 mM Cs$^{+}$/H$^{11001}$-containing divalent free solution resulted in a large increase of the current in the inward rather than outward direction and a reversal potential around 0$^{11006}$2 mV (Fig. 4, A and B) similar as previously observed for Orai1-E106D (16). Cs$^{+}$ permeability of the Orai3-E81D has largely increased in comparison to wild-type Orai3, which was almost impermeable for Cs$^{+}$. Furthermore, this Orai3-E81D mutant showed some similarity to 2-APB-modified Orai3 currents in that Cs$^{+}$ permeability was markedly increased, yet with an inwardly rectifying current-voltage relationship.

Next, we examined the Orai3-E165D mutant coexpressed with Stim1. Store-operated currents in 10 mM Ca$^{2+}$/H$^{11001}$ containing bath solution exhibited inwardly rectifying current-voltage relationship with a high reversal potential of $>$50 mV (Fig. 4, C and D) suggesting preservation of high Ca$^{2+}$/H$^{11001}$ selectivity. Exchange of bath solution by a Cs$^{+}$/H$^{11001}$-divalent-free solution substantially decreased currents suggesting minor permeability to Cs$^{+}$ (Fig. 4, C and D). Thus, the Orai3-E165D mutant resembled in its permeation properties the wild-type Orai3.

**FIGURE 2.** 2-APB alters selectivity of Orai3 currents. A, representative IV plot is shown for Orai3 expressing HEK cells 0, 5, 10, and 35 s (maximum current) after 2-APB (75 μM) application in a 10 mM Ca$^{2+}$/H$^{11001}$ containing bath solution. Time course (B) and representative IV plot (C) compares 2-APB (75 μM) evoked currents with either a standard 10 mM Ca$^{2+}$/H$^{11001}$ containing bath solution or without added Ca$^{2+}$/H$^{11001}$ and a pipette solution including 10 mM EGTA. Time course of 2-APB (75 μM)-stimulated Orai3 currents (D and E) or store depletion-activated Stim1 and Orai3 currents (no 2-APB added; G and H) are presented in standard Ca$^{2+}$/H$^{11001}$ solution substituted by 150 mM NaCl (Na-DVF; D and G) or 150 mM CsCl (Cs-DVF; E and H) based divalent free solution. Representative IV characteristics in a Na-DVF or Cs-DVF for 2-APB-stimulated Orai3 expressing cells (F) or coexpression of Stim1 and Orai3 by store depletion (no 2-APB added; I).

**FIGURE 3.** 2-APB increases minimum Orai3 pore size. Time course starts at already fully developed store-operated currents obtained from Stim1 and Orai3 coexpressing cells in a standard Ca$^{2+}$/H$^{11001}$ solution followed by perfusion with (A) a 150 mM methylammonium-DVF or (B) 150 mM dimethylammonium-DVF solution. D, time course of 2-APB-stimulated Orai3 currents in a dimethylammonium-DVF solution. E, time course starts at 100 s with already developed maximum 2-APB-stimulated Orai3 currents in a 10 mM Ca$^{2+}$/H$^{11001}$ containing bath solution followed by perfusion with a 150 mM trimethylammonium-DVF solution. C, representative IV characteristics in a methylammonium-DVF or dimethylammonium-DVF solution are shown for store depletion-activated currents of Stim1 and Orai3 expressing cells, and F, in dimethylammonium-DVF and trimethylammonium-DVF solutions for 2-APB-stimulated Orai3 expressing cells.
channel. In accordance, Prakriya et al. (16) have previously reported similar permeation properties of the analogue Orai1-E190D mutant in comparison to wild-type Orai1.

In contrast to the Asp substitution, Orai3-E165Q coexpressed with Stim1 yielded store-operated conductance in the 10 mM Ca\(^{2+}\)/H\(^{110}\) buffer solution with a more linear current-voltage relationship displaying some outward rectification (\(V_{rev}/H^{110} = 8.5\) mV). Exchange of buffer solution by a Cs\(^{-}\)/H\(^{110}\)-divalent free solution resulted in a clear increase in both inward and outward currents with a linear current-voltage relationship and a reversal potential of \(-3\) mV. Thereby, the Orai3 E165Q mutation mimicked the conductance phenotype of the 2-APB-stimulated Orai3.

All three Orai3 pore mutants failed to activate detectable store-operated currents in the absence of Stim1 (data not shown). Stim1 and Orai3 pore mutant coexpressing cells showed a similar delay in activation by passive store depletion with 10 mM EGTA and comparable inward current densities in 10 mM Ca\(^{2+}\) solution as wild-type Orai3, suggesting comparable coupling to Stim1, similar as reported for Orai1-E106A (18).

**The Orai3-E165Q Mutant Almost Lacked 2-APB Stimulation**—We hypothesized that Orai3 pore mutants upon store depletion probably adopted a similar pore configuration as wild-type Orai3 following 2-APB stimulation. Therefore, addition of 2-APB to such pore mutants might not be able to further stimulate these channels. We initially monitored maximum store depletion-activated currents derived from coexpression of Stim1 and Orai3-E81D in a 10 mM containing Ca\(^{2+}\) solution followed by addition of 75 \(\mu M\) 2-APB (Fig. 5A). 2-APB induced an increase in both inward and outward currents with a linear current-voltage relationship that reversed at \(-4.4\) mV (Fig. 5B), and was distinct to that of 2-APB evoked double-rectifying currents from wild-type Orai3. Following a subsequent exchange of bath solution by a 150 mM Cs\(^{-}\)/H\(^{110}\)-DVF solution, the current-voltage relationship remained almost unchanged with a slight, yet not significant rightward shift of the reversal potential to \(-2.3\) \(\pm\) 1.6 mV (Fig. 5, A and B). As the Orai3-E81D mutant was still amenable to stimulation by 2-APB and failed to show a subsequent increase in Cs\(^{+}\) conductance,
its behavior does not mimic that of Orai3 in the presence of 2-APB.

Next we examined the Orai3-E165D mutant coexpressed with Stim1 that exhibited similar store-operated currents as wild-type Orai3 in a 10 mM Ca\(^{2+}\) bath solution. Following full activation of the highly Ca\(^{2+}\)-selective store-operated Orai3-E165D currents, application of 2-APB (75 \(\mu M\)) produced a double rectifying current-voltage relationship with a leftward shift in the reversal potential to +20 ± 3 mV (Fig. 5, C and D). Perfusion of a Cs\(^+\)-DVF solution resulted in further increased linear currents reversing at +0.6 ± 2 mV. Qualitatively, the response of this mutant to 2-APB in both Ca\(^{2+}\) and Cs\(^+\) containing solution was similar to wild-type Orai3. Hence this Orai3-E165D mutation is suggested to barely affect pore properties and behaved as expected similar to wild-type Orai3.

Finally, the Orai3-E165Q mutant in coexpression with Stim1 was monitored in the standard Ca\(^{2+}\) bath solution. When store-operated Orai3-E165Q currents were fully developed, addition of 2-APB (75 \(\mu M\)) failed to elicit a clear stimulating response yielding a double-rectifying current-voltage relationship with a reversal potential of about 24 ± 2.7 mV (Fig. 5, E and F). Moreover, closer inspection of the time course upon application of 2-APB revealed a transient inhibition of Orai3-E165Q currents (Fig. 5G) that was followed by a modest recovery. In contrast to the dramatic 2-APB stimulation of store-operated wild-type Orai3 currents, the response to 2-APB of the Orai3-E165Q mutant was almost abolished. Subsequent application of the Cs\(^+\)-DVF solution in the continuous presence of 2-APB increased Orai3-E165Q currents, leading to a linear current-voltage relationship with a reversal potential of -2.6 ± 1 mV (Fig. 5, E and F) as previously observed with this mutant in the absence of 2-APB (see Fig. 4F). The transient inhibition following 2-APB application (Fig. 5G) probably occurred analogously to the known inhibitory effect of 75 \(\mu M\) 2-APB on Orai1 that is masked in wild-type Orai3 and the other pore mutants by a more rapid onset and stronger 2-APB stimulation.

Thus, the Orai3-E165Q mutation qualitatively mimicked the 2-APB-stimulated wild-type Orai3 channel, with similar Cs\(^+\)-permeation properties. Moreover, the stimulatory effect of 2-APB in the Orai3-E165Q mutant was almost abolished. This is further underlined in Fig. 5H displaying similar normalized current-voltage relationships in a 10 mM Ca\(^{2+}\) bath solution for store-operated activated Orai3-E165Q and, following stimulation by 2-APB, for Orai3, Orai3-E106D, and Orai3-E165Q.

**DISCUSSION**

The membrane-permeable 2-APB is a well characterized inhibitor for native store-operated channels and has been demonstrated to block Orai1 and Orai2 (6, 9, 12, 19), sarco/endoplasmic reticulum Ca\(^{2+}\)-ATPase pumps (20), and the mitochondrial permeability transition pore (21). 2-APB has also been shown to activate TRPV1, TRPV2, and TRPV3 (22) and at low concentrations stimulates Orai1 and CRAC currents (9). The stimulatory effect on native CRAC currents has been suggested to be based on an increase in the number of active CRAC channels (12).

Here we demonstrate that the effect of 2-APB to stimulate Orai3 currents is associated with an alteration of the channels’ permeability from highly Ca\(^{2+}\)-selective to a non-differentiating monovalent one. While this paper was under review, consistent results have been reported from Peinelt et al. (19) and Zhang et al. (23) that also show stimulation of Orai3 by 50 \(\mu M\) 2-APB in the absence of coexpressed Stim1. Even in the absence of endogenous Stim1, as shown here utilizing Stim1-siRNA, comparable stimulation of Orai3 by 2-APB was obtained that is characterized by a leftward shift in reversal potential and a double rectifying current-voltage relationship, in accordance with Refs. 19 and 23. This 2-APB-induced stimulation of Orai3 channels comes along with an increase in Orai3 channels’ pore size and represents a unique mechanism. We suggest that 2-APB binds directly to Orai3, as neither coexpression of Stim1 nor siRNA interference against Stim1 affected the 2-APB-dependent Orai3 stimulation. One possibility is that 2-APB mimics the store-induced coupling of Stim1 with Orai3. Recently we have identified a putative coiled-coil motif in the C terminus of Orai1 that interacts with Stim1 (2). A similar C-terminal coiled-coil domain is also present in Orai2 and Orai3. Yet, if 2-APB interacts with the coiled-coil domain of Orai3, one would not expect an alteration in the permeability profile and current-voltage relationship as observed here. Alternatively direct interaction of 2-APB with the Orai3 pore that affects both gating and pore architecture might be responsible for the observed stimulation and increase in pore diameter. As 2-APB is to a high extent positively charged in an aqueous environment at pH 7.4 (14), access to the pore might enable partial electrostatic interaction with one of the negatively charged glutamates/aspartates that contribute to the selectivity filter resulting in partial charge compensation and alteration of pore geometry. Therefore we generated single point mutations of conserved amino acids Glu-81 and Glu-165 corresponding to Glu-106 and Glu-190 in the selectivity filter of Orai1. A reasonable hypothesis would imply a reduced 2-APB response of pore mutants that mimic pore properties of 2-APB-stimulated Orai3. Indeed one of these mutations in the 3rd transmembrane domain, Orai3-E165Q, not only resulted in an altered I/V relationship following store-operated activation similar to that obtained by 2-APB stimulation of wild-type Orai3, but in addition almost completely abolished the stimulatory 2-APB effect. The potential involvement of Glu-165 in the 3rd transmembrane domain of Orai3 is consistent with a very recently published report (23) showing that the structural determinants mediating 2-APB stimulation of Orai3, as judged from a series of Orai1-Orai3 chimeras, are located within the sequence ranging from the 2nd to the 3rd transmembrane domain of Orai3.

Another Orai3-E81D mutant resembled the 2-APB-stimulated Orai3 only to a minor extent in that this mutation rendered the channel permeable to Cs\(^+\) upon store-depletion, however, yet retained the inwardly rectifying current-voltage relationship. More importantly this mutant exhibited a clear stimulatory response to 2-APB.

The properties of a third Orai3-E165D pore mutant were barely different from wild-type Orai3. Furthermore, 2-APB besides affecting pore properties is able to alter gating of Orai3 channels as evident from their activation independent of store depletion. Although 2-APB stimulation of wild-type Orai3 and Orai3-E165Q currents shows functional similarities, one main
difference is that this E165Q mutant still requires store depletion to get activated. It is yet unresolved if the gate and the selectivity filter of Orai3 represent overlapping or rather separated structures of the channel. However, it is conceivable that a modification of the pore by 2-APB directly leads to gating of the Orai3 channel. Based on these experiments, it is obvious that a further domain is essential for 2-APB stimulation as the glutamates at positions 81 and 165 are conserved within all three Orai3 channels.

We have previously shown that an Orai1 mutation, Orai-R91W, that causes non-functional Ca\(^{2+}\)/H\(_{\text{11001}}\) signaling in severe combined immune deficiency T-lymphocytes, couples normally to Stim1 and is able to homomultimerize (2). The analogue Orai3-R66W mutant failed to develop currents both upon store-depletion or 2-APB application, suggesting a more general, severe defect in gating/permeation. In summary, we have conclusively shown that the chemical compound 2-APB is able to modify Orai3 channels’ selectivity by interaction with the pore domain concomitant with an increase in pore size.

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