Pharmacology of ORAI channels as a tool to understand their physiological functions


Ivan Bogeski, Dalia Al-Ansary, Bin Qu, Barbara A Niemeyer, Markus Hoth and Christine Peinelt†
†Author for correspondence
Department of Biophysics, Saarland University, 66421 Homburg, Germany
tel.: +49 684 1162 6453 fax: +49 684 1162 6060 christine.peinelt@uks.eu

Store-operated Ca\(^{2+}\) entry (SOCE) is a major Ca\(^{2+}\) entry mechanism that is present in most cell types. In immune cells, SOCE is almost exclusively mediated by Ca\(^{2+}\) release-activated Ca\(^{2+}\) (CRAC) channels. Ca\(^{2+}\) entry through these channels and the corresponding cytosolic Ca\(^{2+}\) signals are required for many immune cell functions, including all aspects of T-cell activation. ORAI proteins are the molecular correlates for the CRAC channels. The three human members, ORAI1, ORAI2 and ORAI3, are activated through the stromal interaction molecules (STIM)1 and 2 following depletion of endoplasmic reticulum Ca\(^{2+}\) stores. Different combinations of STIM and ORAI can form different CRAC channels with distinct biophysical properties. In this article, we review and discuss mechanistic and functional implications of two important CRAC/ORAI inhibitors, 2-APB and BTP2, and the antibiotic G418 that has also been reported to interfere with ORAI channel function. The use of pharmacological tools should help to assign distinct physiological and pathophysiological functions to different STIM–ORAI protein complexes.

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**Ca\(^{2+}\) release-activated Ca\(^{2+}\) channels have a central role during Ca\(^{2+}\) signaling**

In all cells, Ca\(^{2+}\) ions play a pivotal role in many signaling cascades. The amplitude and kinetics of Ca\(^{2+}\) signals vary greatly according to cell type and function [1]. Elevations in intracellular Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_{i}\)) have to be tightly controlled in order to generate accurate cellular responses. One cellular strategy to ensure precise Ca\(^{2+}\) signaling is the expression of specific subsets of Ca\(^{2+}\) transport and Ca\(^{2+}\) binding proteins. In order to generate spatially and temporarily distinct Ca\(^{2+}\) signals, their expression pattern may vary even between different regions of the cell [1]. Store-operated Ca\(^{2+}\) entry (SOCE) is a major Ca\(^{2+}\) source in immune cells for ubiquitous cellular functions, such as cell cycle control and gene expression [2,3], as well as for specific immunological functions, such as T-cell activation following formation of the immunological synapse (IS) [4–7]. To initiate SOCE, Ca\(^{2+}\) must be released from the endoplasmic reticulum (ER) upon IP\(_3\) binding to the IP\(_3\) receptor and, subsequently, extracellular Ca\(^{2+}\) enters the cell through specific channels, known as Ca\(^{2+}\) release-activated Ca\(^{2+}\) (CRAC) channels [5,8,9].

The entry of Ca\(^{2+}\) through CRAC channels generates a current (I), known as I\(_{\text{CRAC}}\). Initially described in lymphocytes, I\(_{\text{CRAC}}\) has also been found in many other cell types, including hepatocytes, neurons and vascular smooth muscle cells [10–12], where it is likely to also have a central role for Ca\(^{2+}\) signaling.

The identity of the molecular components of I\(_{\text{CRAC}}\) remained elusive for nearly two decades of research but, by utilizing thorough RNA-silencing screens, stromal interaction molecules (STIM)1 and 2 following depletion of endoplasmic reticulum Ca\(^{2+}\) stores. Different combinations of STIM and ORAI can form different CRAC channels with distinct biophysical properties. In this article, we review and discuss mechanistic and functional implications of two important CRAC/ORAI inhibitors, 2-APB and BTP2, and the antibiotic G418 that has also been reported to interfere with ORAI channel function. The use of pharmacological tools should help to assign distinct physiological and pathophysiological functions to different STIM–ORAI protein complexes.

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Figure 1. Structural features of the human STIM and ORAI proteins. STIM is a single transmembrane phosphoprotein. The N-terminus extends in the lumen of the ER and exhibits an EF and a sterile α-motif (SAM) including N-linked glycosylation site(s). The C-terminus protrudes into the cytosol and contains two lengthy coiled-coil domains overlapping with an ezrin–radixin–moesin (ERM)-like domain, which contains a praline/histidine/glutamic acid-rich region in the case of STIM2. Proline/serine- and lysine-rich domains are located within the proximal part of the STIM1 C-terminal region, with the latter being present in STIM2 as well. The figure also shows domains essential for I_{CRAC} activation (CAD) and modulation (CMD). Human ORAI proteins span the membrane with four transmembrane segments with conserved negatively charged residues within the first and third segments. Three glutamate residues within the C-terminus are involved in fast Ca^{2+}-dependent inactivation of ORAI2 and ORAI3 but not ORAI1, although two of them are conserved. The N-terminus of ORAI1 includes a calmodulin binding domain, an arginine-, a proline- and an arginine/lysine-rich domain. The first extracellular loop of the three proteins contains the negatively charged amino acids thought to be involved in differential ion selectivity (see text).
**STIM proteins**

In humans, the ER Ca^{2+}-sensor STIM has two homologs: STIM1 and STIM2. Whereas STIM1 is activated in IP_3-induced signaling pathways, STIM2 seems to play a role for Ca^{2+} entry under resting conditions. Both, STIM1 and STIM2 have one transmembrane segment and span mainly the ER membrane, although a portion of the protein is also found in the plasma membrane (Figure 1). The luminal N-terminal domain features an EF hand as a Ca^{2+} binding pocket and a sterile α-motif (SAM) domain. The C-terminal domain contains an ezrin–radixin–myosin-like domain, including two coiled-coil regions, a serine/proline-rich region and a polylysine-rich region [26,33,34].

The IP_3-induced decrease in luminal Ca^{2+} concentration leads to a dissociation of Ca^{2+} bound to the EF hand of STIM1 (EC_50 = 200–600 μM) [35]. Subsequently, STIM1 molecules oligomerize and then aggregate in clusters (puncta), localized 10–25 nm beneath the plasma membrane, and activate CRAC channels [31,36,37]. Recent studies started to assign structural features of STIM1 to distinct actions during CRAC channel activation [38,39]. Baba et al. stated that puncta formation requires the N-terminal SAM domain and that the coiled-coil region and a serine/threonine-rich region mediate the constitutive movement of STIM. In another study, the C-terminal coiled-coil region was found to be crucial for puncta formation and the serine/proline-rich region was found to be important for the correct targeting of the STIM1 cluster to the plasma membrane, whereas the polylysine-rich region is characterized by a supportive but not essential function for the targeting [39]. Smyth et al. reported that IP_3-induced Ca^{2+} suppression during mitosis is based on phosphorylation of Ser646 and Ser668 and, possibly, other sites in STIM1 [40].

In contrast to STIM1, STIM2 does not seem to redistribute within the ER after store depletion but may rather be precoupled to plasma membrane CRAC channels [25]. One cytosolic inhibitor of precoupling STIM2/ORAI1 is Ca^{2+} calmodulin [29]. STIM2 activates CRAC ion channels already at smaller decreases of Ca^{2+} concentration in the ER (EC_50 of the EF hand for Ca^{2+} is 500 μM), which leads to a basal Ca^{2+} influx into the cell [27,28]. STIM1 and STIM2 appear to have distinct properties; whereas STIM2-mediated Ca^{2+} influx seems to sustain the basal intracellular Ca^{2+} concentration, IP_3-induced STIM1-mediated Ca^{2+} elevations lead to the activation of a plethora of cellular responses. A recent study utilizing STIM2-knockout mice demonstrated that STIM2 is also a key player for Ca^{2+} signal transduction during hypoxic neurological damage [41].

Before being described as a major component of the SOCE machinery [16,18,42], STIM1 was identified as a tumor suppressor [43–45] located at the plasma membrane (PM) [46]. The latter finding is rather controversial. Several studies failed to confirm cell surface expression of N-terminally tagged STIM1 when rather bulky tags were used [18,36,47]. In a recent paper, Haueter et al. tried to resolve this controversy by using a relatively small His-tag coupled to the C-terminal domain of STIM1 and, again, provide evidence of PM-located STIM1 [48]. However, despite the high homology with STIM1, there is no evidence for localization of STIM2 to the PM.

**STIM1 expression levels** are much higher in the ER membrane than in the PM [26], and there is no clear indication that PM-located STIM1 can activate the ORAI channels.

A very strong argument that PM-localized STIM1 is not critical for activation of I_{CRAC} is provided by Shuttleworth’s group where they prevent PM localization of STIM1 by mutating its glycosylation motifs. These mutant STIM1 proteins do not influence the amplitude of I_{CRAC} in a background where endogenous STIM1 is downregulated [49].

**ORAI proteins**

All three ORAI family members – ORAI1, ORAI2 and ORAI3 – are expressed in various cell types and generate, when co-overexpressed with STIM1, currents with distinct biochemical and biophysical properties. ORAI1 is a plasma membrane protein required for I_{CRAC} [13,45]. ORAI1 has four transmembrane segments, cytoplasmic N- and C-termini, two extracellular and one intracellular loop (Figure 1). Whole-cell recordings from different cell types overexpressing both STIM1 and ORAI1 revealed large inwardly rectifying, store-operated Ca^{2+} currents featuring, not identical but very similar characteristics of I_{CRAC} [13,47,50].

I_{CRAC} is predominantly mediated by multimeric ORAI1 channels [15,51,52]. The finding, that one protein (STIM1) may be able to control both multimerization and activation of pore-forming ORAI1 channel subunits constitutes a novel concept of ion channel activation. Upon activation with the C-terminal domain of STIM1 (see later for detailed discussion and references), two ORAI1-dimers dimerize and assemble the Ca^{2+} selectivity filter and ion-conduction pore-yielding active ORAI1 tetrameric channels [53]. However, Park et al. challenge the view of preformed dimers as they observe discrete ORAI1 particles in electron microscopy that cluster but do not change in size after activation [54].

The human homologs of ORAI1, ORAI2 and ORAI3 (Figure 1) are expressed in various tissues [55]. Whereas some reports vary in their conclusions about the mechanism of activation of ORAI2 and ORAI3 [24,47,56], other studies show that, when coexpressed with the Ca^{2+}-sensor protein STIM1, all three members of the ORAI family activate upon store depletion [21,50]. The characteristics of STIM1/ORAI1-, STIM1/ORAI2- and STIM1/ORAI3-mediated I_{CRAC} differ in activation kinetics, ion conductivity and in the pharmacological profile (see ‘Pharmacology’ section). The most striking difference between STIM1/ORAI1, 2 and 3 is their regulation by intracellular Ca^{2+}. The Ca^{2+}-dependent inactivation of native CRAC currents features a fast and a slow component [57–60]. The fast Ca^{2+}-dependent inactivation of CRAC is most pronounced in STIM1/ORAI3. The slow Ca^{2+}-dependent inactivation by 500-nM free intracellular Ca^{2+} results in a nearly complete block of STIM1/ORAI1, in a 60% reduction for STIM1/ORAI2, whereas STIM1/ORAI3 is not inactivated at all. The concept that ORAI homomers might form heteromultimeric channels with distinct functions [21,24,51,55] was experimentally confirmed by the Romanin group [61]. In addition, ORAI channels might be expressed in more than one splice variant with distinct properties as shown for ORAI2 [62]. Altogether these features point to heterogeneous channel populations with different Ca^{2+} selectivities.
and feedback regulation, which are probably important to generate different Ca\(^{2+}\) signatures resulting in various cellular functions in different cell types.

In the past, several different mechanisms of CRAC channel activation had been proposed, such as the existence of the diffusible Ca\(^{2+}\) influx factor [63–66], the secretion-like coupling mechanism [67,68] and the conformational coupling mechanism [69]. The latter concept of a direct interaction between STIM1 and ORAI1 proteins is supported by a number of studies [24,36,37,53,69–73]. Yuan et al., identified the STIM1/ORAI1-activating region, 344–442 amino acids in length) on STIM1 as sufficient for ORAI1 channel activation [74]. In addition, two further studies describe a similar region on STIM1 as ORAI1-activating domain [75,76]. Additional proof for the conformational coupling model was provided by Lewis and coworkers, who, using a yeast split-ubiquitin interaction system, showed that the amino acid region (342–440), the CRAC activation domain (CAD) directly binds to the N- and C-termini of ORAI1 and, thereby, activates the ORAI1 channels (Figure 1) [54].

**Pinpointing \(I_{\text{CRAC}}\) features to the STIM–ORAI CRAC channels**

Several studies describe the molecular base for certain CRAC channel properties as being confined within the STIM1 protein rather than within the pore-forming ORAI1 subunits. Kawasaki et al. characterized a 31-amino acid region (444–475) next to the CAD as an area responsible for preventing CRAC channel activation when Ca\(^{2+}\) stores are refilled [5]. Another 11-amino acid-long region in the cytosolic region of STIM1 (474–485), the CRACM domain (CMD) (Figure 1), mediates fast Ca\(^{2+}\)-dependent inactivation in ORAI1 and, to a lesser extent, in ORAI3 [77]. Basically, the same 470–491 amino acid region, or several acidic acids within this amino acid sequence (475–485), as well as a calmodulin binding site in the N-terminus of ORAI1 (68–91), were identified by Mullins et al. to be indispensable for Ca\(^{2+}\)-dependent inactivation [78].

Furthermore, Lee et al. assigned the differences in fast Ca\(^{2+}\)-dependent inactivation of ORAI2 and 3 [21] to three conserved glutamate residues (E233, E235 and E236 in ORAI2, and E281, E283 and E284 in ORAI3) [79].

Scrimgeour et al. found that fast Ca\(^{2+}\)-dependent inactivation is dependent on the ratio of STIM1:ORAI1 expression. The higher the STIM1:ORAI1 ratio, the more pronounced the fast Ca\(^{2+}\)-dependent inactivation and the less pronounced the activation at negative potentials [80]. One possible interpretation of these findings might be that the tetrameric ORAI1 channel can couple to a varying number of STIM1 proteins, and that fast Ca\(^{2+}\)-dependent inactivation is more pronounced when more STIM1 CMD domains are present in the complex. A study using fluorescent ORAI1 tandem multimers describes activation of the tetrameric ORAI1 channel by two STIM1 proteins [70].

In addition to the presence of three conserved glutamates in ORAI2 and 3, which are absent in ORAI1, the pronounced fast Ca\(^{2+}\)-dependent inactivation of STIM1–ORAI3 CRAC channels could be based on an altered STIM1 participation in STIM1–ORAI3 compared with STIM1–ORAI1 CRAC channels. A study by Frischauf et al. investigates the interaction sites of STIM1–ORAI2 and STIM1–ORAI3 CRAC channels and, indeed, their activation differs from STIM1–ORAI1 CRAC channel activation [81].

**Pharmacological profile of STIM–ORAI channels**

Immediately after the discovery of CRAC channels [57,82], all known Ca\(^{2+}\) channel blockers were tested on them; however, they were all found to be rather nonspecific [83]. Between 1992 and 2009, many more substances have been discovered that can inhibit CRAC channel activity. An excellent and very comprehensive review about all known CRAC channel blockers was published by Sweeney et al. [84]. In this article, we will focus on two substances that have been most extensively used to discriminate between different CRAC channels and to study the functional importance of CRAC channels: 2-aminoethoxydiphenyl borate (2-APB) and N-(4-[3,5-bis(trifluoromethyl)-1H-pyrazol-1-yl] phenyl)-4-methyl-1,2,3-thiadiazole-5-carboxamide (BTP2, also known as YM-58483). In addition, we will discuss the antibiogenic Geneticin® (G418), which, surprisingly, also interferes with CRAC channel activity (see later) [25,85–89].

2-APB is a promising pharmacological tool to examine different STIM–ORAI CRAC channels

2-aminoethoxydiphenyl borate was originally discovered as a blocker of IP\(_3\) receptors [90]. It affects a number of transport proteins (e.g., the sarcoplasmic/ER Ca\(^{2+}\) ATPase [SERCA]) [91], as well as several TRP channels. 2-APB blocks TRPC3 [92], activates TRPV1–3 [93] and potentiates TRPV6 (GaT1) [94], as well as TRPM6, and has a bimodal action on TRPM7. Whereas several hundred micromolars of 2-APB inhibits TRPM7, concentrations above 1 mM increase TRPM7 currents [95].

2-aminoethoxydiphenyl borate was shown to block SOCE and CRAC currents in several different cell lines, such as Chinese hamster ovary (CHO) cells, rat basophilic leukemia cells (RBL), avian DT40 and Jurkat T cells [86–88,96]. Lewis and coworkers were the first to show that 2-APB has a bimodal effect on CRAC currents when they performed a thorough analysis in Jurkat T cells [87]. 2-APB affects \(I_{\text{CRAC}}\) only when applied extracellularly and not when added inside the patch pipette. At low concentrations (≤5 µM) 2-APB enhances \(I_{\text{CRAC}}\), and at concentrations of at least 10 µM, it not only potentiated but also blocked \(I_{\text{CRAC}}\) in Jurkat T cells. The IC\(_{50}\) value of 2-APB-dependent amplification is approximately 3 µM, whereas the 2-APB-induced block has an IC\(_{50}\) value of approximately 10 µM. In addition, \(I_{\text{CRAC}}\) enhanced by 5 µM 2-APB displayed a more pronounced, fast, Ca\(^{2+}\)-dependent inactivation than nonamplified \(I_{\text{CRAC}}\). In addition to Jurkat T cells, RBL and DT40 cells also display a biphasic current response (amplification and block) upon application of 2-APB. However, the characteristics of the 2-APB response differ slightly with a less pronounced \(I_{\text{CRAC}}\) enhancement (or faster block) in RBL cells and a less effective block (or slower block) in DT40 cells [87].

Several studies have investigated how application of 2-APB affects STIM–ORAI-mediated currents in overexpression systems [20,21,23–25,50,52,97]. Co-overexpression of STIM1–ORAI1 in different cell
types results in a strong enhancement of CRAC current amplitude. Upon application of 50 µM 2-APB, these currents exhibited the biphasic 2-APB response, as seen for $I_{\text{CRAC}}$ in T cells (potentiation up to 193% and nearly complete inhibition) [50,52]. Interestingly, the 2-APB response of STIM1–ORAI2- and STIM1–ORAI3-mediated currents differ from currents carried by STIM1–ORAI1. STIM1–ORAI2-mediated $\text{Ca}^{2+}$ currents feature the biphasic response upon 2-APB, but the current block is incomplete (~40%). STIM1–ORAI3-mediated currents are not blocked but amplified up to eightfold by 2-APB [21]. In 2008, four independent groups published that ORAI1 can be activated by 2-APB independently of STIM1 and/or store depletion. The 2-APB-induced ORAI3 current–voltage relationship (I/V) features an additional large outward component (~1 nA/pF at +130 mV) that is not present in store-operated inwardly rectifying CRAC currents, suggesting a 2-APB-induced change in the ion channel conductivity profile [20,23,24,97]. In addition to ORAI3, a 2-APB-induced, store-independent mode of activation was also found for ORAI1 [20,23]. The 2-APB-induced ORAI1 current features a similar I/V relationship compared with the 2-APB-induced current of ORAI3 but exhibits much smaller current densities (~10 pA/pF at 130 mV) [20].

The store-operated inwardly rectifying CRAC current has a reversal potential of more than +50 mV and an ion selectivity of $\text{Ca}^{2+}$:Na$^+$ of approximately 1000:1, although complete removal of $\text{Ca}^{2+}$ leads to a large monovalent inward current through CRAC channels [57,58,82]. The 2-APB-induced ORAI3 current exhibits reduced $\text{Ca}^{2+}$ selectivity [20,23,24,97]. A Goldman–Hodkin–Katz analysis of the 2-APB-induced shift of reversal potentials (from +50 to ~+30 mV) in STIM1–ORAI3 co-overexpressing cells revealed that 2-APB shifts ion selectivity for the inward current to $\text{Ca}^{2+}$:Na$^+$ of approximately 33:1. The true decrease in ion selectivity is most probably even larger, as this value is calculated under the assumption that transport of different ions is independent of each other, which is not the case for ORAI channels [20]. Schindl et al. performed permeation experiments with differently sized ions (N-methylammonium) and estimated the diameter of STIM1, activated the selectivity filter of ORAI3, to approximately 3.8 Å, whereas for the 2-APB-induced ORAI3, they found an enlarged pore diameter of approximately 5.34 Å [97].

2-APB dose-dependently activates and blocks SOCE in HEK293 cells and HEK293 cells overexpressing STIM1 [23]. In STIM–ORAI1 co-overexpressing HEK293 cells, the EC$_{50}$ values for 2-APB-induced $I_{\text{CRAC}}$ amplification and inhibition were found to be $EC_{\text{amp}}(\text{SiO}) = 4 \mu M$, $EC_{\text{amp}}(\text{SO$_2$}) = 6 \mu M$, $EC_{\text{amp}}(\text{Si}) = 15 \mu M$ (and $EC_{\text{amp}}(\text{O}) = 13.5 \mu M$ in a Ca$^{2+}$ imaging experiment [23]) and $IC_{\text{inh}}(\text{SiO}) = 8 \mu M$ and $IC_{\text{inh}}(\text{SO$_2$}) = 6 \mu M$ [20].

Confocal fluorescence images show that 2-APB reversed the formation of YFP-tagged STIM1 puncta in HEK293 cells [20,23]. This finding suggests that the 2-APB-induced block of CRAC currents might be, at least in part, due to a decoupling of STIM–ORAI proteins. However, coexpression of STIM1 with ORAI1 leads to a decrease of 2-APB-induced puncta reversal. In addition, pretreatment of these cells with 2-APB (50 µM) showed STIM1 accumulation beneath the plasma membrane, despite the fact that SOCE was blocked. These findings argue that the molecular target for 2-APB is on the ORAI1 channel itself. Low concentrations of 2-APB (≤20 µM), did not change the eYFP–STIM1 fluorescence in total internal reflection fluorescence experiments, suggesting that 2-APB-induced current amplification is not due to an increase in the number of STIM1 [23].

The molecular mechanism of the fast Ca$^{2+}$-dependent inactivation of $I_{\text{CRAC}}$ depends on STIM1 [77]. On the other hand, it has been shown that the 2-APB-induced current amplification enhances the same fast Ca$^{2+}$-dependent inactivation [87]. Hence, one could hypothesize that the current amplification might be governed by STIM1. However, two recently published papers suggest that the molecular basis for 2-APB-induced current amplification is within the ORAI1 channels rather than in STIM1 [20,23].

To pinpoint the structures of ORAI1 important for the store-independent mode of activation by 2-APB, a systematic analysis of various ORAI1–3 chimeras has been carried out by Zhang et al. [24]. In one of these chimeras, the second and third transmembrane segments, as well as the intracellular loop of ORAI3, were replaced with the corresponding structures of ORAI1 (ORAI1–TM23–ORAI3). Cells overexpressing ORAI1-TM23–ORAI3 showed a 2-APB-induced Ca$^{2+}$ increase and a 2-APB-induced conductance with an I/V similar to ORAI3. These findings suggest that the TM23–ORAI3 structure is sufficient to mediate ORAI activation by 2-APB.

In native cells, the pharmacological profile of 2-APB is likely to be even more complex if one takes into account that ORAI family members are able to form heteromultimers. Heteromeric channels are likely to show mixed phenotypes that will depend on the ratios of the individual subunits within the tetramer. Mignen et al. suggested that a pentameric ORAI1/ORAI3 multimer is the molecular bases for the store-independently operated arachidonic-acid-regulated Ca$^{2+}$-selective current ($I_{\text{arc}}$) [98]. $I_{\text{arc}}$ is insensitive to 2-APB [99].

Although the molecular mechanisms of 2-APB interacting with STIM–ORAI are not completely understood and need to be further analyzed, the 2-APB phenotype of the different STIM–ORAI combinations allows a functional characterization of diverse STIM–ORAI combinations in various cell types. Concomitantly, different 2-APB responses have been reported in cells of different origin [11,88,100,101]. Further studies are needed to link different STIM–ORAI combinations with specific cellular functions. Given the differential 2-APB effects on different STIM–ORAI combinations, 2-APB has emerged as the most valuable pharmacological tool to discriminate different CRAC channel functions.

In a very recent paper, Goto et al. describe the effects of two new 2-APB analogs on CRAC channels in native and overexpressing systems [102]. The authors show that the compounds named DPB162-AE and DPB163-AE potently inhibit SOCE in DT40, CHO-K1 and HeLa cells, with an IC$_{50}$ in the range of 30–600 nM. Interestingly, the effect of the two novel compounds was dependent on the cell type, suggesting variations of SOCE constituents according to the cell type. Current measurements in cells overexpressing STIM1 and ORAI1 showed an inhibitory effect with an IC$_{50}$ of approximately 80 nM for DPB162-AE.
and 170 nM for DPB163-AE. In addition, the authors show that ORAI2 was only partially inhibited, while ORAI3 was activated by lower concentrations and inhibited by higher concentrations (10 µM) of DBP. The finding that these novel compounds prevent STIM1 clustering suggest that in contrast to 2-APB, the mechanism of inhibition depends on STIM1 instead of ORAI1 [102].

**G418 blocks precoupled STIM2–ORAI1 complexes but does not affect STIM1–ORAI1-mediated Ca2+ signals**

G418 was first described in 1974 as a broad-spectrum antibiotic [103,104]. Today, it is not used as a therapeutic agent but rather as a selection marker for genetically engineered cells containing the gene for neomycin phosphotransferase (e.g., in HEK293 cells stably overexpressing STIM1 or STIM2) [26]. Unlike HEK293 cells expressing STIM1 and ORAI1, expression of STIM2 and ORAI1 in HEK293 cells did not exhibit large Ca2+ currents so long as G418 was present in the cell culture media, indicating that G418 inhibits STIM2–ORAI1 CRAC currents [25]. This G418-induced block of STIM2–ORAI1 could be reversed by either omitting G418 from the cell culture media or by application of 2-APB. Application of 50 µM 2-APB onto cells overexpressing STIM2–ORAI1 generates a large Ca2+ current within seconds, which then immediately inactivates. This mode of activation is absent in STIM1–ORAI1-overexpressing HEK293 cells, suggesting that, unlike STIM1, STIM2 is precoupled to CRAC channels. The time constant of this transient activation of CRAC channels is faster, and the size of the transiently developing current was larger when G418 was absent, suggesting that 2-APB might remove the inhibitory G418 from the precoupled STIM2–ORAI1 and, thus, allow the development of Ca2+ current before 2-APB itself blocks ORAI1 [25].

When used without preincubation but acutely and intracellularly in a patch clamp experiment, G418 is a potent (EC50 = 640 nM) and specific blocker of STIM2/ORAI1. G418 blocks STIM2/ORAI1 only weakly and at much higher concentrations (10 µM) when applied to cells that have already developed a Ca2+ current. This block is rather unspecific and might not be correlated with the specific G418 block of the STIM2–ORAI1 complex, as it was also detected in cells expressing STIM1–ORAI1 [25]. The findings that extracellularly applied high G418 concentrations do not inhibit the Ca2+ currents can be explained in two ways:

- G418 enters cells only slowly and does not show an effect within the time window of application (seconds)
- G418 does not block the preactivated STIM2–ORAI1 complex

It is, therefore, unclear whether G418 can be used to functionally detect STIM2 in native cells. The specific block of STIM2–ORAI1 mediated currents would require a preincubation with G418; however, because G418 also inhibits protein synthesis in mammalian cells, it is likely that cell death of native cells would be induced, or, if applied acutely and intracellularly in a patch-clamp experiment, the size of STIM2-mediated Ca2+ currents may be too small to be resolved with this method. Nevertheless, G418 could turn out to be a valuable tool to discriminate different functions of different STIM–ORAI combinations.

**BTP2 to examine the physiological function & therapeutic use of CRAC inhibition in various cells & animal models**

Also known as YM-58483, BTP2 is another important CRAC channel blocker that has yet to be applied to different STIM–ORAI combinations. BTP2 has been used successfully to analyze CRAC channel function in native human tissue. Initially, Ishikawa et al. found that BTP2 blocks SOCE in Jurkat T cells with an EC50 value of approximately 100 nM. As a consequence, IL-2 production and NFAT1-driven promoter activity were inhibited, and the authors suggested BTP2 as future candidate for the treatment of autoimmune diseases and chronic inflammation [105]. A direct effect of BTP2 (EC50 ~10 nM) upon ICa,CRAC and ICa,CRAC-mediated Ca2+ signaling in Jurkat T cells and in primary human CD4+ T cells has been shown by Zitt et al. [85].

In this study, BTP2 was shown to inhibit not only production of IL-2, but also of IL-5 and IFN-γ, and T-cell proliferation. Both antigen-induced histamine release from an IgE-primed rat basophilic leukemia cell line (RBL-2H3) and IL-5 and IL-13 production in human peripheral blood cells were blocked by BTP2 with an IC50 in the low nanomolar range [106]. BTP2 suppressed ovalbumin-induced airway hyperresponsiveness and bronchoconstriction by inhibiting the ICa,CRAC-induced inflammatory mediator and cytokine production [106]. In addition, BTP2 has been shown to prevent antigen-induced airway eosinophil infiltration and late-phase asthmatic responses, probably by inhibition of ICa,CRAC-induced production of cytokines and inflammatory mediators in Th2 cells [107]. Thus, BTP2 might be a candidate therapeutic agent for bronchial asthma [107]. In a graft-versus-host disease, BTP2 not only reduced the number of donor T-cells, especially donor CD8+ T-cells, but also suppressed donor anti-host cytotoxic T-lymphocyte activity and IFN-γ, suggesting its therapeutic use in autoimmune diseases, such as autoimmune hepatitis and rheumatoid arthritis [108]. BTP2 reduced the superoxide anion production in human neutrophils but did not significantly affect phagocytosis, intraphagosomal radical production or bacterial killing, suggesting a possible application in downregulation of neutrophils in chronic inflammatory disease without compromising antibacterial host defence [109]. While recent studies with BTP2 analyze the physiological function of CRAC and support the idea of BTP2 as a therapeutic agent, the mechanistic effects of BTP2 on the different ORAI channel family members have not been investigated.

**Physiological & pathophysiological roles of STIM & ORAI**

Patients with nonfunctional (mutated) ORAI1 or STIM1 develop severe immunodeficiencies. In addition, they suffer from myopathy, ectodermal dysplasia and several other symptoms [110,111]. The findings obtained not only from humans but also mice with defective CRAC machinery have been summarized recently in several excellent reviews [109,112,113]. Owing to this, we will not focus on the physiological and pathophysiological roles of STIM–ORAI combinations in the immune system, but rather summarize the physiological and pathological roles of ORAI and STIM proteins in other tissues and systems.
There are still relatively few articles describing the specific role of ORAI and STIM in nonimmune tissues. Nevertheless, several recent papers indicate an important role for ORAI1 and STIM1 but, so far, not for the other SOCE constituents, ORAI2 and 3 and STIM2, for vascular and skeletal smooth muscle and myoblast proliferation, differentiation and migration [114–123]. The findings that ORAI1 and/or STIM1 regulate not only the physiological but also the hypertrophic growth of cardiomyocytes [124], the pathogenesis of hypertension [125,126] and that they are involved in arterial thrombosis and ischemic brain infarction [127], as well as in premature platelet activation and bleeding [128], present further evidence for the role of these proteins in the physiology and pathophysiology of the cardiovascular system.

It has been proposed recently that ORAI1 and STIM1 are involved in breast cancer metastasis and that suppression of SOCE leads to a decrease in tumor metastasis in mice [129]. In addition, a recent paper by Richie et al. suggested that the regulation of STIM1 expression by Wilms tumor suppressor 1 and early growth response 1 could underlie the altered Ca2+ signaling in cancer cells [130]. However, the fact that STIM1 was previously characterized as a tumor suppressor should not be neglected [43–45]. Further studies are necessary to elucidate the role of SOCE and its constituents in cancer growth and metastasis.

An involvement of both STIM proteins in the murine nervous system was elegantly demonstrated by the Nieswandt group. The authors showed that mainly STIM1 and, to a lesser degree, also STIM2 are key regulators of autoimmune CNS inflammation and that STIM2 but not STIM1 is responsible for Ca2+ accumulation in neurons during ischemia [131]. The same group also demonstrated that the absence of STIM2 in mice prevents neuronal cell death under hypoxic conditions [131].

The role of SOCE in the pathogenesis of nasal polyposis and inflammatory bowel disease (IBD) was also analyzed recently [132,133]. IBD is a generalized term encompassing both Crohn’s disease and ulcerative colitis, and leads to severe impairment of gastrointestinal structure and function. One of the major causes in the pathogenesis of IBD is hyperactive CD4+ T-helper cells (Figure 2). An increased activation, proliferation and mucosal infiltration of T-helper cells is observed in patients with both Crohn’s disease and ulcerative colitis [134]. T-helper cell infiltration is subsequently followed by increased cytokine production and mucosal inflammation. Mucosal inflammation can be regulated via two distinct, Th1- or Th2-mediated pathways.

While Th1-mediated inflammation is linked with increased secretion of TNF, IFN-γ and IL-12, the Th2-mediated pathway is associated with excessive secretion of IL-4, IL-5 and IL-13 [134]. Given the role of T-helper cells in the pathogenesis of IBD, T-helper cell-targeted immunosuppression is a plausible therapeutic approach. Although the contribution of STIM and ORAI1 was not directly investigated in these studies, specific CRAC channel inhibitors may have therapeutic potential for the treatment of nasal polyposis and IBD.

Suppression of the signaling cascade following IS formation would induce immunosuppression through decreased cytokine secretion and, subsequently, reduce mucosal inflammation. It has already been shown that Ca2+ signaling in T cells from patients with IBD is severely disturbed, and that SOCE in these patients is significantly enlarged compared with healthy individuals (Figure 2) [135,136]. Considering the inhibitory effects of BTP-2 and potentially other pyrazole derivatives as potent inhibitors of CRAC channels and T-cell function with an IC50 in the low nanomolar range [85,105], developing a safe drug derivative has high therapeutic potential.

Figure 2. Hyperactive CD4+ T-helper cells are a major cause for inflammatory bowel disease (IBD). In patients with IBD, an increased mucosal infiltration of hyperactive T-helper cells is observed. These T-helper cells have augmented SOCE and secrete high amounts of cytokines via two distinct, Th1- (TNF, IFN-γ and IL-12) or Th2- (IL-4, IL-5 and IL-13) cell-mediated pathways, subsequently inducing mucosal inflammation. CRAC channel inhibition would, hence, be a plausible therapeutic approach.
Recently, Di Sabatino et al. examined the effect of Synta 66 compound (GSK1349571A), known to inhibit CRAC channels [137], on Ca2+-dependent activation and cytokine secretion in T cells from lamina propria of healthy individuals and patients with IBD. The authors report an inhibitory effect of Synta 66 with an IC50 of approximately 1 µM for ICrAC, SOCE and cytokine secretion [133].

The example of BTP2 effects on cells from patients with IBD showed how specific and potent CRAC channel blockers may be in treating patients with SOCE disorders. Owing to additional unspecific side effects, 2-APB, BTP2 and G418 are very likely not the drugs that will be used in clinical studies; however, novel derivatives may provide subtype-specific inhibition of distinct STIM–ORAI combinations.

Expert commentary
Store-operated Ca2+ signals play a decisive role for various physiological and pathophysiological functions. In nonexcitable cells, they regulate the balance between apoptosis, proliferation and differentiation. The best-known store-operated Ca2+ channel type, the CRAC channel, controls diverse immune tasks in cells, such as neutrophilic granulocytes and T cells. How does one central Ca2+ pathway fulfill so many different tasks?

The recent discovery of the key molecular players of CRAC channels, the ER Ca2+ sensor protein, STIM1, and the homologous STIM2, and the family of ORAI ion channels, mark a milestone in Ca2+-signaling research. Recently, first steps toward an understanding of the physiological and pathophysiological functions of certain STIM–ORAI combinations have been made. Pharmacological tools are very useful to discriminate distinct functions of different STIM–ORAI combinations. While 2-APB, and the antibiotic G418, can be used to selectively interfere with certain STIM–ORAI combinations, the CRAC channel blocker BTP2 has been successfully used to study CRAC channel functions in human T cells from peripheral blood and the lamina propria of the intestine. Structurally similar compounds are promising tools to target Ca2+ signaling in different immune cells to specifically suppress certain immune functions.

Key issues

- In many different cell types, store-operated Ca2+ entry (SOCE) is a major Ca2+ source in various cellular functions. Ca2+ release-activated Ca2+ (CRAC) channels are the best-known SOCE channels.
- Stromal interaction molecule (STIM) and ORAI proteins are the molecular correlate of CRAC channels.
- After Ca2+ store depletion, the Ca2+ sensor proteins STIM in the membrane of the endoplasmic reticulum activate ORAI channels in the plasma membrane and subsequently Ca2+ enters the cell.
- Depending on the subtype STIM (STIM1 or STIM2) and ORAI channel (ORAI1, 2 or 3) expression and distinct Ca2+ signals (kinetics and amplitude) are generated.
- In addition, ORAI proteins can form heteromultimeric Ca2+ channels and may be expressed in more than one, functionally differing splice variant (as has been shown for ORAI2).
- STIM and ORAI proteins are expressed in various cell types and tissues but the specific function of these protein combinations is widely unknown.
- The family members of STIM and ORAI proteins have a differentiated, complex pharmacology.
- Specific inhibitors of STIM and ORAI proteins might serve as tools to characterize the function of these proteins in vivo.
- Hyperactive T cells with increased CRAC channel activity are one of the major causes of inflammatory bowel disease. CRAC channel inhibitors thus present potential treatment against this severe disease and also for other diseases caused by hyperactive immune cells.

Five-year view
We expect that, within the next 5 years, certain cellular functions will be assigned to certain STIM–ORAI combinations. To achieve this, it is necessary to learn more about the molecular mechanisms of STIM–ORAI interactions and about the expression pattern of the different STIMs and ORAIs on the protein level. Furthermore, selective inhibition of specific STIM and ORAI proteins is very much needed. This has already been achieved by knockdown strategies; however, unfortunately, the immune system of men and mice can be quite different. Therefore, selective inhibition of specific human STIM and ORAI proteins is also needed, if possible, in primary human cells. In our opinion there are two main strategies to achieve this: Knockdown of STIM and ORAI proteins by siRNA is one possibility. There are already several reports in this direction. The second possibility is the selective inhibition of STIM and ORAI proteins by small-molecule inhibitors, such as 2-APB and BTP-2. We believe that new small-molecule inhibitors with potentially even more specific profiles will be available within the next 5 years. They should greatly facilitate our understanding of the physiological and pathophysiological importance of distinct STIM–ORAI combinations. We hope that selective inhibition of STIM–ORAI combinations will also allow the selective interference with different immune cell functions, which should, ultimately, help to treat, for instance, autoimmune diseases.

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