EXPERT REVIEWS

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

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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.

KEYWORDS: 2-aminoethoxydiphenyl borate • BTP2 • Ca^{2+} release-activated Ca^{2+} channels • G418 • immune cells • ion channel pharmacology • ORAI1, 2 and 3 • STIM1 and 2

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 ($[Ca]_i$) 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 temporary 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_{CRAC}. Initially described in lymphocytes, I_{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_{CRAC} remained elusive for nearly two decades of research but, by utilizing thorough RNA-silencing screens, stromal interaction molecule (STIM) and ORAI proteins (in Greek mythology, this means 'keepers of the gates of heaven'), also known as CRAC modulator (CRACM) proteins, were identified as the molecular key players for I_{CRAC}-mediated Ca^{2+} signaling in 2005 and 2006 [13–18]. Accordingly, STIM and ORAI deficiencies cause defects in various cellular functions in human and mice [19].

Recently, functional differences between members of the STIM and ORAI protein family have been investigated [20–32]. This review focuses on the pharmacological profiles of different STIM–ORAI combinations and how pharmacological studies may help to identify their physiological functions *in vivo*.

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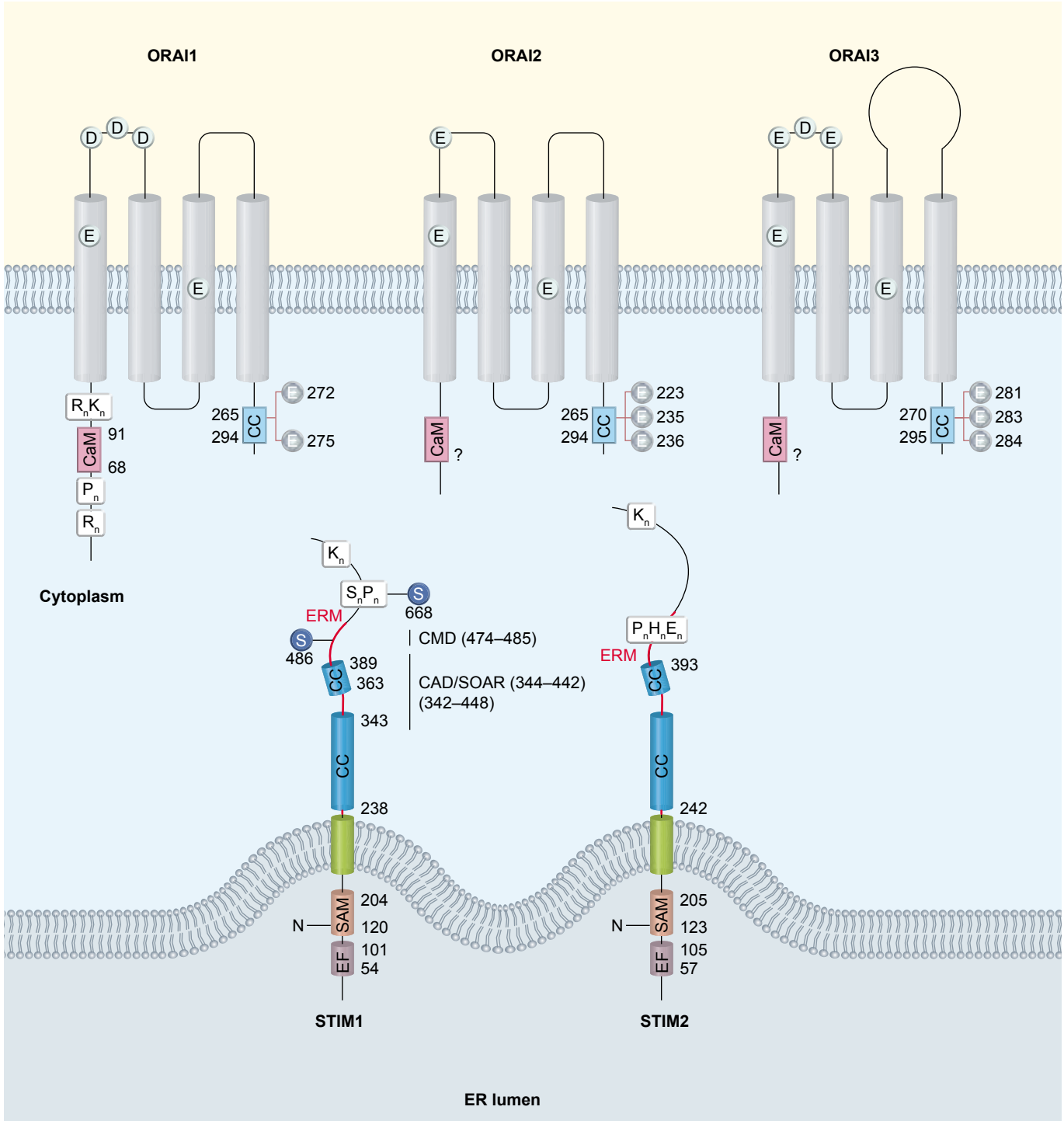

Figure 1.Structural features of the human STIM and ORAI proteins. STIM is a single transmembrane phosphoprotein. The N-terminus extends in the lumen of the ER and exhibits an EF and a sterile α-motif (SAM) including N-linked glycosylation site(s). The C-terminus protrudes into the cytosol and contains 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 I_{CRAC} suppression during mitosis is based on phosphorylation of Ser486 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 [25]. 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, Hauser *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} [13,14,17]. ORAI1 has four transmembrane segments, cytoplasmatic 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 ORAI 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 ORAI 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/ORAI-activating region, 344–442 amino acids in length) on STIM1 as sufficient for ORAI channel activation [74]. In addition, two further studies describe a similar region on STIM1 as ORAI-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_{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 [75]. 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 antibiotic 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 (CaT1) [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_{CRAC} only when applied extracellularly and not when added inside the patch pipette. At low concentrations (≤5 μM) 2-APB enhances I_{CRAC}, and at concentrations of at least 10 μM, it not only potentiated but also blocked I_{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_{CRAC} enhanced by 5 μM 2-APB displayed a more pronounced, fast, Ca^{2+}-dependent inactivation than nonamplified I_{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_{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_{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 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 ORAI3 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 Ca^{2+}:Na^{+} of approximately 1000:1, although complete removal of Ca^{2+} leads to a large monovalent inward current through CRAC channels [57,58,82]. The 2-APB-induced ORAI3 current exhibits reduced 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 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–ORAI co-overexpressing HEK293 cells, the EC_{50} values for 2-APB-induced I_{CRAC} amplification and inhibition were found to be $EC_{amp/S1O1}$ = 4 µM, $EC_{amp/S1O2}$ = 6 µM, $EC_{amp/S1O3}$ = 15 µM (and $EC_{amp/O3}$ = 13.5 µM in a Ca^{2+} imaging experiment [23]) and $IC_{inh/S1O1}$ = 8 µM and $IC_{inh/S1O2}$ = 6 µM [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_{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 ORAI channels rather than in STIM1 [20,23].

To pinpoint the structures of ORAI3 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 a 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 arachidonate-regulated Ca^{2+}-selective current (I_{arc}) [98]. I_{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 Ca^{2+} 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 Ca^{2+} 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 Ca^{2+} 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 Ca^{2+} 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 (EC_{50} = 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 Ca^{2+} 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 Ca^{2+} 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 Ca^{2+} 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 EC_{50} value of approximately 100 nM. As a consequence, IL-2 production and NFAT-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 (EC_{50} ~10 nM) upon I_{CRAC} and I_{CRAC}-mediated Ca^{2+} 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 IC_{50} in the low nanomolar range [106]. BTP2 suppressed ovalbumin-induced airway hyperresponsiveness and bronchoconstriction by inhibiting the I_{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 I_{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 therapeutical 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 [19,112,113]. 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 Ca^{2+} 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 Ca^{2+} 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 ORAI 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 Ca^{2+} 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 IC_{50} in the low nanomolar range [85,105], developing a safe drug derivative has high therapeutic potential.

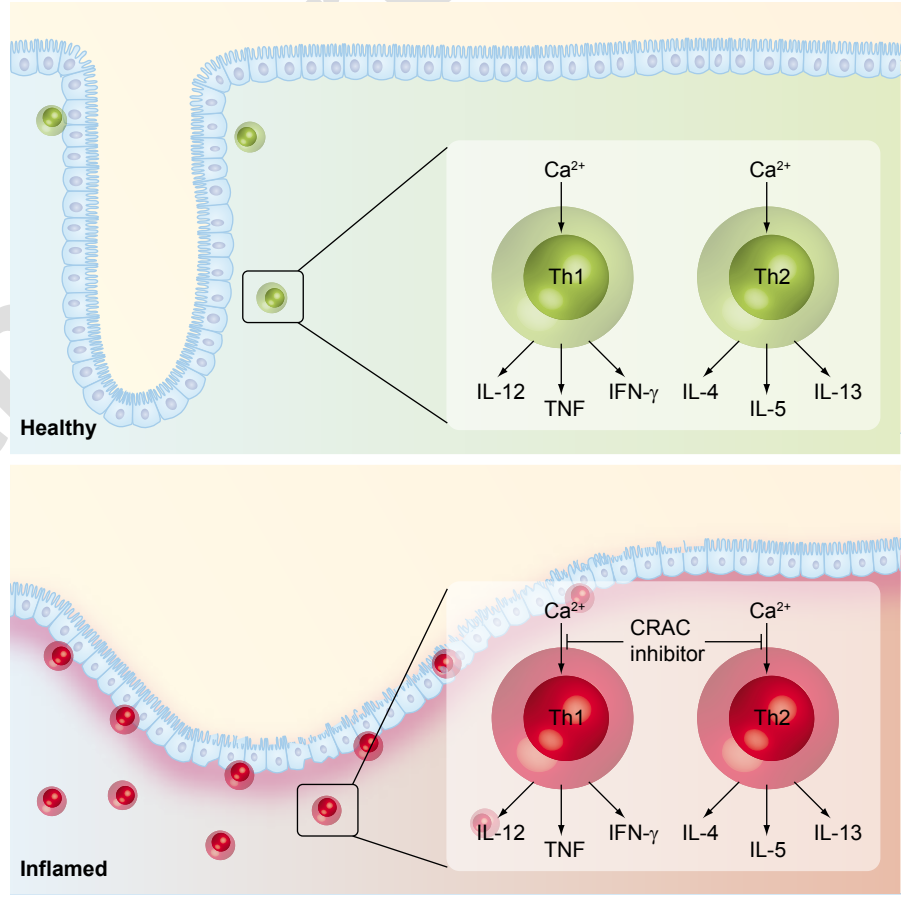

Figure 2. Hyperactive $CD4^+$ T-helper cells are a major cause for inflammatory bowel disease (IBD). In patients with IBD, an increased mucosal infiltration of hyperactive T-helper cells is observed. These T-helper cells have augmented SOCE and secrete high amounts of cytokines via two distinct, Th1- (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 Ca^{2+}-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 IC_{50} of approximately 1 µM for I_{CRAC}, 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 Ca^{2+} 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 Ca^{2+} channel type, the CRAC channel, controls diverse immune tasks in cells, such as neutrophilic granulocytes and T cells. How does one central Ca^{2+} pathway fulfil so many different tasks?

The recent discovery of the key molecular players of CRAC channels, the ER Ca^{2+} sensor protein, STIM1, and the homologous STIM2, and the family of ORAI ion channels, mark a milestone in Ca^{2+}-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 Ca^{2+} signaling in different immune cells to specifically suppress certain immune functions.

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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Key issues

- In many different cell types, store-operated Ca^{2+} entry (SOCE) is a major Ca^{2+} source in various cellular functions. Ca^{2+} release-activated Ca^{2+} (CRAC) channels are the best-known SOCE channels.
- Stromal interaction molecule (STIM) and ORAI proteins are the molecular correlate of CRAC channels.
- After Ca^{2+} store depletion, the Ca^{2+} sensor proteins STIM in the membrane of the endoplasmic reticulum activate ORAI channels in the plasma membrane and subsequently Ca^{2+} enters the cell.
- Depending on the subtype STIM (STIM1 or STIM2) and ORAI channel (ORAI1, 2 or 3) expression and distinct Ca^{2+} signals (kinetics and amplitude) are generated.
- In addition, ORAI proteins can form heteromultimeric Ca^{2+} 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.

References

Papers of special note have been highlighted as:
• of interest
•• of considerable interest

1 Berridge MJ, Lipp P, Bootman MD. The versatility and universality of calcium signalling. *Nat. Rev. Mol. Cell Biol.* 1(1), 11–21 (2000).

2 Tani D, Monteilh-Zoller MK, Fleig A, Penner R. Cell cycle-dependent regulation of store-operated I(CRAC) and Mg^{2+}-nucleotide-regulated MagNuM (TRPM7) currents. *Cell Calcium.* 41(3), 249–260 (2007).

3 Feske S, Giltnane J, Dolmetsch R, Staudt LM, Rao A. Gene regulation mediated by calcium signals in T lymphocytes. *Nat. Immunol.* 2(4), 316–324 (2001).

4 Barr VA, Bernot KM, Srikanth S *et al.* Dynamic movement of the calcium sensor STIM1 and the calcium channel Orai1 in activated T-cells: puncta and distal caps. *Mol. Biol. Cell.* 19(7), 2802–2817 (2008).

5 Cahalan MD, Chandy KG. The functional network of ion channels in T lymphocytes. *Immunol. Rev.* 231(1), 59–87 (2009).

• **Excellent review about Ca^{2+} signaling in T cells.**

6 Kummerow C, Junker C, Kruse K, Rieger H, Quintana A, Hoth M. The immunological synapse controls local and global calcium signals in T lymphocytes. *Immunol. Rev.* 231(1), 132–147 (2009).

• **Excellent review about Ca^{2+} signaling following formation of an immunological synapse.**

7 Lioudyno MI, Kozak JA, Penna A *et al.* Orai1 and STIM1 move to the immunological synapse and are up-regulated during T cell activation. *Proc. Natl Acad. Sci. USA* 105(6), 2011–2016 (2008).

8 Parekh AB, Putney JW Jr. Store-operated calcium channels. *Physiol. Rev.* 85(2), 757–810 (2005).

9 Lewis RS. The molecular choreography of a store-operated calcium channel. *Nature.* 446(7133), 284–287 (2007).

10 Rychkov GY, Litjens T, Roberts ML, Barritt GJ. ATP and vasopressin activate a single type of store-operated Ca^{2+} channel, identified by patch-clamp recording, in rat hepatocytes. *Cell Calcium.* 37(2), 183–191 (2005).

11 Kachoei BA, Knox RJ, Uthuza D, Levy S, Kaczmarek LK, Magoski NS. A store-operated $Ca^{(2+)}$ influx pathway in the bag cell neurons of Aplysia. *J. Neurophysiol.* 96(5), 2688–2698 (2006).

12 Potier M, Gonzalez JC, Motiani RK *et al.* Evidence for STIM1- and Orai1-dependent store-operated calcium influx through ICRAC in vascular smooth muscle cells: role in proliferation and migration. *FASEB J.* DOI: 10.1096/fj.09-131128 (2009) (Epub ahead of print).

13 Zhang SL, Yeromin AV, Zhang XH *et al.* Genome-wide RNAi screen of $Ca^{(2+)}$ influx identifies genes that regulate $Ca^{(2+)}$ release-activated $Ca^{(2+)}$ channel activity. *Proc. Natl Acad. Sci. USA* 103(24), 9357–9362 (2006).

14 Vig M, Peinelt C, Beck A *et al.* CRACM1 is a plasma membrane protein essential for store-operated Ca^{2+} entry. *Science* 312(5777), 1220–1223 (2006).

15 Prakriya M, Feske S, Gwack Y, Srikanth S, Rao A, Hogan PG. Orai1 is an essential pore subunit of the CRAC channel. *Nature* 443(7108), 230–233 (2006).

16 Roos J, DiGregorio PJ, Yeromin AV *et al.* STIM1, an essential and conserved component of store-operated Ca^{2+} channel function. *J. Cell Biol.* 169(3), 435–445 (2005).

•• **First paper to describe the endoplasmic reticulum (ER) Ca^{2+} sensor STIM1.**

17 Feske S, Gwack Y, Prakriya M *et al.* A mutation in Orai1 causes immune deficiency by abrogating CRAC channel function. *Nature* 441(7090), 179–185 (2006).

•• **First paper to describe the molecular identity of CRAC channels. A mutation in the ORAI1 CRAC channel causes severe combined immunodeficiency in patients.**

18 Liou J, Kim ML, Heo WD *et al.* STIM is a Ca^{2+} sensor essential for Ca^{2+}-store-depletion-triggered Ca^{2+} influx. *Curr. Biol.* 15(13), 1235–1241 (2005).

19 Feske S. ORAI1 and STIM1 deficiency in human and mice: roles of store-operated Ca^{2+} entry in the immune system and beyond. *Immunol. Rev.* 231(1), 189–209 (2009).

20 Peinelt C, Lis A, Beck A, Fleig A, Penner R. 2-aminoethoxydiphenyl borate directly facilitates and indirectly inhibits STIM1-dependent gating of CRAC channels. *J. Physiol.* 586(13), 3061–3073 (2008).

21 Lis A, Peinelt C, Beck A *et al.* CRACM1, CRACM2, and CRACM3 are store-operated Ca^{2+} channels with distinct functional properties. *Curr. Biol.* 17(9), 794–800 (2007).

22 DeHaven WI, Smyth JT, Boyles RR, Putney JW Jr. Calcium inhibition and calcium potentiation of ORAI1, ORAI2, and ORAI3 calcium release-activated calcium channels. *J. Biol. Chem.* 282(24), 17548–17556 (2007).

23 DeHaven WI, Smyth JT, Boyles RR, Bird GS, Putney JW Jr. Complex actions of 2-aminoethyldiphenyl borate on store-operated calcium entry. *J. Biol. Chem.* 283(28), 19265–19273 (2008).

24 Zhang SL, Kozak JA, Jiang W *et al.* Store-dependent and -independent modes regulating Ca^{2+} release-activated Ca^{2+} channel activity of human ORAI1 and ORAI3. *J. Biol. Chem.* 283(25), 17662–17671 (2008).

25 Parvez S, Beck A, Peinelt C *et al.* STIM2 protein mediates distinct store-dependent and store-independent modes of CRAC channel activation. *FASEB J.* 22(3), 752–761 (2008).

26 Soboloff J, Spassova MA, Hewavitharana T *et al.* STIM2 is an inhibitor of STIM1-mediated store-operated Ca^{2+} entry. *Curr. Biol.* 16(14), 1465–1470 (2006).

27 Brandman O, Liou J, Park WS, Meyer T. STIM2 is a feedback regulator that stabilizes basal cytosolic and endoplasmic reticulum Ca^{2+} levels. *Cell* 131(7), 1327–1339 (2007).

28 Zheng L, Stathopulos PB, Li GY, Ikura M. Biophysical characterization of the EF-hand and SAM domain containing Ca^{2+} sensory region of STIM1 and STIM2. *Biochem. Biophys. Res. Commun.* 369(1), 240–246 (2008).

29 Frischauf I, Schindl R, Derler I, Bergsmann J, Fahrner M, Romanin C. The STIM/ORAI coupling machinery. *Channels (Austin)* 2(4), 261–268 (2008).

30 Zhou Y, Meraner P, Kwon HT *et al.* STIM1 gates the store-operated calcium channel ORAI1 *in vitro*. *Nat. Struct. Mol. Biol.* 17(1), 112–116 (2010).

31 Lur G, Haynes LP, Prior IA *et al.* Ribosome-free terminals of rough ER allow formation of STIM1 puncta and segregation of STIM1 from IP(3) receptors. *Curr. Biol.* 19(19), 1648–1653 (2009).

32 Walsh CM, Chvanov M, Haynes LP, Petersen OH, Tepikin AV, Burgoyne RD. Role of phosphoinositides in STIM1 dynamics and store-operated calcium entry. *Biochem. J.* 425(1), 159–168 (2010).

33 Soboloff J, Spassova MA, Tang XD, Hewavitharana T, Xu W, Gill DL. ORAI1 and STIM reconstitute store-operated calcium channel function. *J. Biol. Chem.* 281(30), 20661–20665 (2006).

34 Feske S. Calcium signalling in lymphocyte activation and disease. *Nat. Rev. Immunol.* 7(9), 690–702 (2007).

35 Stathopulos PB, Li GY, Plevin MJ, Ames JB, Ikura M. Stored Ca^{2+} depletion-induced oligomerization of stromal interaction molecule 1 (STIM1) via the EF-SAM region: an initiation mechanism for capacitive Ca^{2+} entry. *J. Biol. Chem.* 281(47), 35855–35862 (2006).

36 Wu MM, Buchanan J, Luik RM, Lewis RS. Ca^{2+} store depletion causes STIM1 to accumulate in ER regions closely associated with the plasma membrane. *J. Cell Biol.* 174(6), 803–813 (2006).

37 Luik RM, Wu MM, Buchanan J, Lewis RS. The elementary unit of store-operated Ca^{2+} entry: local activation of CRAC channels by STIM1 at ER-plasma membrane junctions. *J. Cell Biol.* 174(6), 815–825 (2006).

38 Baba Y, Nishida K, Fujii Y, Hirano T, Hikida M, Kurosaki T. Essential function for the calcium sensor STIM1 in mast cell activation and anaphylactic responses. *Nat. Immunol.* 9(1), 81–88 (2008).

39 Li Z, Lu J, Xu P, Xie X, Chen L, Xu T. Mapping the interacting domains of STIM1 and Orai1 in Ca^{2+} release-activated Ca^{2+} channel activation. *J. Biol. Chem.* 282(40), 29448–29456 (2007).

40 Smyth JT, Petranka JG, Boyles RR *et al.* Phosphorylation of STIM1 underlies suppression of store-operated calcium entry during mitosis. *Nat. Cell Biol.* (2009).

41 Berna-Erro A, Braun A, Kraft R *et al.* STIM2 regulates capacitive Ca^{2+} entry in neurons and plays a key role in hypoxic neuronal cell death. *Sci. Signal.* 2(93), ra67 (2009).

42 Zhang SL, Yu Y, Roos J *et al.* STIM1 is a Ca^{2+} sensor that activates CRAC channels and migrates from the Ca^{2+} store to the plasma membrane. *Nature* 437(7060), 902–905 (2005).

43 Parker NJ, Begley CG, Smith PJ, Fox RM. Molecular cloning of a novel human gene (*D11S4896E*) at chromosomal region 11p15.5. *Genomics* 37(2), 253–256 (1996).

44 Sabbioni S, Barbanti-Brodano G, Croce CM, Negrini M. *GOK*: a gene at 11p15 involved in rhabdomyosarcoma and rhabdoid tumor development. *Cancer Res.* 57(20), 4493–4497 (1997).

45 Sabbioni S, Veronese A, Trubia M *et al.* Exon structure and promoter identification of STIM1 (alias *GOK*), a human gene causing growth arrest of the human tumor cell lines G401 and RD. *Cytogenet. Cell Genet.* 86(3–4), 214–218 (1999).

46 Manji SS, Parker NJ, Williams RT *et al.* STIM1: a novel phosphoprotein located at the cell surface. *Biochim. Biophys. Acta* 1481(1), 147–155 (2000).

47 Mercer JC, Dehaven WI, Smyth JT *et al.* Large store-operated calcium selective currents due to co-expression of ORAI1 or ORAI2 with the intracellular calcium sensor, STIM1. *J. Biol. Chem.* 281(34), 24979–24990 (2006).

48 Hauser CT, Tsien RY. A hexahistidine-Zn^{2+}-dye label reveals STIM1 surface exposure. *Proc. Natl Acad. Sci. USA* 104(10), 3693–3697 (2007).

49 Mignen O, Thompson JL, Shuttleworth TJ. STIM1 regulates Ca^{2+} entry via arachidonate-regulated Ca^{2+}-selective (ARC) channels without store depletion or translocation to the plasma membrane. *J. Physiol.* 579(Pt 3), 703–715 (2007).

50 Peinelt C, Vig M, Koomoa DL *et al.* Amplification of CRAC current by STIM1 and CRACM1 (Orai1). *Nat. Cell Biol.* 8(7), 771–773 (2006).

- **STIM1 and ORAI1 are both essential for $Ca^{2=}$ release-activated Ca^{2+} current.**

51 Vig M, Beck A, Billingsley JM *et al.* CRACM1 multimers form the ion-selective pore of the CRAC channel. *Curr. Biol.* 16(20), 2073–2079 (2006).

52 Yeromin AV, Zhang SL, Jiang W, Yu Y, Safrina O, Cahalan MD. Molecular identification of the CRAC channel by altered ion selectivity in a mutant of ORAI. *Nature* 443(7108), 226–229 (2006).

53 Penna A, Demuro A, Yeromin AV *et al.* The CRAC channel consists of a tetramer formed by STIM-induced dimerization of ORAI dimers. *Nature* 456(7218), 116–120 (2008).

54 Park CY, Hoover PJ, Mullins FM *et al.* STIM1 clusters and activates CRAC channels via direct binding of a cytosolic domain to ORAI1. *Cell* 136(5), 876–890 (2009).

- **Proof of direct interaction of STIM1 and ORAI1 in STIM1-dependent ORAI1 activation.**

55 Gwack Y, Srikanth S, Feske S *et al.* Biochemical and functional characterization of Orai proteins. *J. Biol. Chem.* 282(22), 16232–16243 (2007).

56 Shuttleworth TJ, Thompson JL, Mignen O. STIM1 and the noncapacitative ARC channels. *Cell Calcium.* 42(2), 183–191 (2007).

57 Hoth M, Penner R. Depletion of intracellular calcium stores activates a calcium current in mast cells. *Nature* 355(6358), 353–356 (1992).

58 Hoth M, Penner R. Calcium release-activated calcium current in rat mast cells. *J. Physiol.* 465, 359–386 (1993).

59 Zweifach A, Lewis RS. Slow calcium-dependent inactivation of depletion-activated calcium current. Store-dependent and -independent mechanisms. *J. Biol. Chem.* 270(24), 14445–14451 (1995).

60 Fierro L, Parekh AB. Fast calcium-dependent inactivation of calcium release-activated calcium current (CRAC) in RBL-1 cells. *J. Membr. Biol.* 168(1), 9–17 (1999).

61 Schindl R, Frischauf I, Bergsmann J *et al.* Plasticity in Ca^{2+} selectivity of Orai1/Orai3 heteromeric channel. *Proc. Natl Acad. Sci. USA* 106(46), 19623–19628 (2009).

62 Gross SA, Wissenbach U, Philipp SE, Freichel M, Cavalie A, Flockerzi V. Murine ORAI2 splice variants form functional Ca^{2+} release-activated Ca^{2+} (CRAC) channels. *J. Biol. Chem.* 282(27), 19375–19384 (2007).

63 Randriamampita C, Tsien RY. Degradation of a calcium influx factor (CIF) can be blocked by phosphatase inhibitors or chelation of Ca^{2+}. *J. Biol. Chem.* 270(1), 29–32 (1995).

64 Randriamampita C, Tsien RY. Emptying of intracellular Ca^{2+} stores releases a novel small messenger that stimulates Ca^{2+} influx. *Nature* 364(6440), 809–814 (1993).

65 Thomas D, Kim HY, Hanley MR. Regulation of inositol trisphosphate-induced membrane currents in *Xenopus* oocytes by a Jurkat cell calcium influx factor. *Biochem. J.* 318(Pt 2), 649–656 (1996).

66 Thomas D, Hanley MR. Evaluation of calcium influx factors from stimulated Jurkat T-lymphocytes by microinjection into *Xenopus* oocytes. *J. Biol. Chem.* 270(12), 6429–6432 (1995).

67 Patterson RL, van Rossum DB, Gill DL. Store-operated Ca^{2+} entry: evidence for a secretion-like coupling model. *Cell* 98(4), 487–499 (1999).

68 Yao Y, Ferrer-Montiel AV, Montal M, Tsien RY. Activation of store-operated Ca^{2+} current in *Xenopus* oocytes requires SNAP-25 but not a diffusible messenger. *Cell* 98(4), 475–485 (1999).

69 Berridge MJ. Capacitative calcium entry. *Biochem. J.* 312(Pt 1), 1–11 (1995).

70 Ji W, Xu P, Li Z *et al.* Functional

stoichiometry of the unitary calcium-release-activated calcium channel. *Proc. Natl Acad. Sci. USA* 105(36), 13668–13673 (2008).

71 Muik M, Frischauf I, Derler I *et al*. Dynamic coupling of the putative coiled-coil domain of ORAI1 with STIM1 mediates ORAI1 channel activation. *J. Biol. Chem.* 283(12), 8014–8022 (2008).

72 Huang GN, Zeng W, Kim JY *et al*. STIM1 carboxyl-terminus activates native SOC, $I_{(CRAC)}$ and TRPC1 channels. *Nat. Cell Biol.* 8(9), 1003–1010 (2006).

73 Muik M, Fahrner M, Derler I *et al*. A cytosolic homomerization and a modulatory domain within STIM1 C terminus determine coupling to ORAI1 channels. *J. Biol. Chem.* 284(13), 8421–8426 (2009).

74 Navarro-Borelly L, Somasundaram A, Yamashita M, Ren D, Miller RJ, Prakriya M. STIM1–ORAI1 interactions and ORAI1 conformational changes revealed by live-cell FRET microscopy. *J. Physiol.* 586(Pt 22), 5383–5401 (2008).

75 Yuan JP, Zeng W, Dorwart MR, Choi YJ, Worley PF, Muallem S. SOAR and the polybasic STIM1 domains gate and regulate ORAI channels. *Nat. Cell Biol.* 11(3), 337–343 (2009).

76 Kawasaki T, Lange I, Feske S. A minimal regulatory domain in the C terminus of STIM1 binds to and activates ORAI1 CRAC channels. *Biochem. Biophys. Res. Commun.* 385(1), 49–54 (2009).

77 Derler I, Fahrner M, Muik M *et al*. A CRAC modulatory domain (CMD) within STIM1 mediates fast Ca^{2+}-dependent inactivation of ORAI1 channels. *J. Biol. Chem.* DOI: 10.1074/jbc.C109.024083 (2009) (Epub ahead of print).

78 Mullins FM, Park CY, Dolmetsch RE, Lewis RS. STIM1 and calmodulin interact with Orai1 to induce Ca^{2+}-dependent inactivation of CRAC channels. *Proc. Natl Acad. Sci. USA* 106(36), 15495–15500 (2009).

79 Lee KP, Yuan JP, Zeng W, So I, Worley PF, Muallem S. Molecular determinants of fast Ca^{2+}-dependent inactivation and gating of the Orai channels. *Proc. Natl Acad. Sci. USA* 106(34), 14687–14692 (2009).

80 Scrimgeour N, Litjens T, Ma L, Barritt GJ, Rychkov GY. Properties of ORAI1 mediated store-operated current depend on the expression levels of STIM1 and ORAI1 proteins. *J. Physiol.* DOI: 10.1113/jphysiol.2009.170662 (2009) (Epub ahead of print).

81 Frischauf I, Muik M, Derler I *et al*. Molecular determinants of the coupling between STIM1 and ORAI channels: differential activation of ORAI1–3 channels by a STIM1 coiled-coil mutant. *J. Biol. Chem.* 284(32), 21696–21706 (2009).

82 Zweifach A, Lewis RS. Mitogen-regulated Ca^{2+} current of T lymphocytes is activated by depletion of intracellular Ca^{2+} stores. *Proc. Natl Acad. Sci. USA* 90(13), 6295–6299 (1993).

83 Franzius D, Hoth M, Penner R. Non-specific effects of calcium entry antagonists in mast cells. *Pflugers Arch.* 428(5–6), 433–438 (1994).

84 Sweeney ZK, Minatti A, Button DC, Patrick S. Small-molecule inhibitors of store-operated calcium entry. *Chem. Med. Chem.* 4(5), 706–718 (2009).

•• **Excellent and comprehensive review focusing on inhibitors of store-operated Ca^{2+} entry.**

85 Zitt C, Strauss B, Schwarz EC *et al*. Potent inhibition of Ca^{2+} release-activated Ca^{2+} channels and T-lymphocyte activation by the pyrazole derivative BTP2. *J. Biol. Chem.* 279(13), 12427–12437 (2004).

86 Kukkonen JP, Lund PE, Akerman KE. 2-aminoethoxydiphenyl borate reveals heterogeneity in receptor-activated $Ca^{(2+)}$ discharge and store-operated $Ca^{(2+)}$ influx. *Cell Calcium* 30(2), 117–129 (2001).

87 Prakriya M, Lewis RS. Potentiation and inhibition of $Ca^{(2+)}$ release-activated $Ca^{(2+)}$ channels by 2-aminoethyldiphenyl borate (2-APB) occurs independently of IP(3) receptors. *J. Physiol.* 536(Pt 1), 3–19 (2001).

88 Bakowski D, Glitsch MD, Parekh AB. An examination of the secretion-like coupling model for the activation of the Ca^{2+} release-activated Ca^{2+} current $I_{(CRAC)}$ in RBL-1 cells. *J. Physiol.* 532(Pt 1), 55–71 (2001).

89 Iwasaki H, Mori Y, Hara Y, Uchida K, Zhou H, Mikoshiba K. 2-aminoethoxydiphenyl borate (2-APB) inhibits capacitative calcium entry independently of the function of inositol 1,4,5-trisphosphate receptors. *Receptors Channels* 7(6), 429–439 (2001).

90 Maruyama T, Kanaji T, Nakade S, Kanno T, Mikoshiba K. 2APB, 2-aminoethoxydiphenyl borate, a membrane-penetrable modulator of Ins(1,4,5)P3-induced Ca^{2+} release. *J. Biochem.* 122(3), 498–505 (1997).

91 Missiaen L, Callewaert G, De Smedt H, Parys JB. 2-aminoethoxydiphenyl borate affects the inositol 1,4,5-trisphosphate receptor, the intracellular Ca^{2+} pump and the non-specific Ca^{2+} leak from the non-mitochondrial Ca^{2+} stores in permeabilized A7r5 cells. *Cell Calcium* 29(2), 111–116 (2001).

92 Trebak M, Bird GS, McKay RR, Putney JW Jr. Comparison of human TRPC3 channels in receptor-activated and store-operated modes. Differential sensitivity to channel blockers suggests fundamental differences in channel composition. *J. Biol. Chem.* 277(24), 21617–21623 (2002).

93 Hu HZ, Gu Q, Wang C *et al*. 2-aminoethoxydiphenyl borate is a common activator of TRPV1, TRPV2, and TRPV3. *J. Biol. Chem.* 279(34), 35741–35748 (2004).

94 Voets T, Prenen J, Fleig A *et al*. CaT1 and the calcium release-activated calcium channel manifest distinct pore properties. *J. Biol. Chem.* 276(51), 47767–47770 (2001).

95 Li M, Jiang J, Yue L. Functional characterization of homo- and heteromeric channel kinases TRPM6 and TRPM7. *J. Gen. Physiol.* 127(5), 525–537 (2006).

96 Ma HT, Beaven MA. Regulation of Ca^{2+} signaling with particular focus on mast cells. *Crit. Rev. Immunol.* 29(2), 155–186 (2009).

97 Schindl R, Bergsmann J, Frischauf I *et al*. 2-aminoethoxydiphenyl borate alters selectivity of ORAI3 channels by increasing their pore size. *J. Biol. Chem.* 283(29), 20261–20267 (2008).

98 Mignen O, Thompson JL, Shuttleworth TJ. The molecular architecture of the arachidonate-regulated Ca^{2+}-selective ARC channel is a pentameric assembly of ORAI1 and ORAI3 subunits. *J. Physiol.* 587(Pt 17), 4181–4197 (2009).

99 Mignen O, Thompson JL, Shuttleworth TJ. Ca^{2+} selectivity and fatty acid specificity of the noncapacitative, arachidonate-regulated Ca^{2+} (ARC) channels. *J. Biol. Chem.* 278(12), 10174–10181 (2003).

100 Vanden Abeele F, Shuba Y, Roudbaraki M *et al*. Store-operated Ca^{2+} channels in prostate cancer epithelial cells: function, regulation, and role in carcinogenesis. *Cell Calcium.* 33(5–6), 357–373 (2003).

101 Abad E, Lorente G, Gavara N, Morales M, Gual A, Gasull X. Activation of store-operated $Ca^{(2+)}$ channels in trabecular meshwork cells. *Invest. Ophthalmol. Vis. Sci.* 49(2), 677–686 (2008).

102 Goto JI, Suzuki AZ, Ozaki S *et al*. Two novel 2-aminoethyl diphenylborinate (2-APB) analogues differentially activate and inhibit store-operated $Ca^{(2+)}$ entry via STIM proteins. *Cell Calcium* 47(1), 1–10 (2009).

• **Novel 2-aminoethoxydiphenyl borate analogues to analyze STIM and ORAI proteins in different cell types.**

103 Wagman GH, Testa RT, Marquez JA, Weinstein MJ. Antibiotic G-418, a new *Micromonospora*-produced aminoglycoside with activity against protozoa and helminths: fermentation, isolation, and preliminary characterization. *Antimicrob. Agents Chemother.* 6(2), 144–149 (1974).

104 Waitz JA, Sabatelli F, Menzel F, Moss EL Jr. Biological activity of antibiotic G-418, a new micromonospora-produced aminoglycoside with activity against protozoa and helminths. *Antimicrob. Agents Chemother.* 6(5), 579–581 (1974).

105 Ishikawa J, Ohga K, Yoshino T *et al*. A pyrazole derivative, YM-58483, potently inhibits store-operated sustained Ca^{2+} influx and IL-2 production in T lymphocytes. *J. Immunol.* 170(9), 4441–4449 (2003).

106 Ohga K, Takezawa R, Yoshino T, Yamada T, Shimizu Y, Ishikawa J. The suppressive effects of YM-58483/BTP-2, a store-operated Ca^{2+} entry blocker, on inflammatory mediator release *in vitro* and airway responses *in vivo*. *Pulm. Pharmacol. Ther.* 21(2), 360–369 (2008).

107 Yoshino T, Ishikawa J, Ohga K *et al*. YM-58483, a selective CRAC channel inhibitor, prevents antigen-induced airway eosinophilia and late phase asthmatic responses via Th2 cytokine inhibition in animal models. *Eur. J. Pharmacol.* 560(2–3), 225–233 (2007).

108 Ohga K, Takezawa R, Arakida Y, Shimizu Y, Ishikawa J. Characterization of YM-58483/BTP2, a novel store-operated Ca^{2+} entry blocker, on T cell-mediated immune responses *in vivo*. *Int. Immunopharmacol.* 8(13–14), 1787–1792 (2008).

109 Steinckwich N, Frippiat JP, Stasia MJ *et al*. Potent inhibition of store-operated Ca^{2+} influx and superoxide production in HL60 cells and polymorphonuclear neutrophils by the pyrazole derivative BTP2. *J. Leukoc. Biol.* 81(4), 1054–1064 (2007).

110 McCarl CA, Picard C, Khalil S *et al*. ORAI1 deficiency and lack of store-operated Ca^{2+} entry cause immunodeficiency, myopathy, and ectodermal dysplasia. *J. Allergy Clin. Immunol.* 124(6), 1311–1318.e7 (2009).

111 Picard C, McCarl CA, Papolos A *et al*. STIM1 mutation associated with a syndrome of immunodeficiency and autoimmunity. *N. Engl. J. Med.* 360(19), 1971–1980 (2009).

• **Describes the immune defects of STIM1 mutation in patients.**

112 Feske S. CRAC channelopathies. *Pflugers Arch.* DOI: 10.1007/s00424-009-0777-5 (2010) (Epub ahead of print).

113 Feske S, Picard C, Fischer A. Immunodeficiency due to mutations in ORAI1 and STIM1. *Clin. Immunol.* DOI: 10.1016/j.clim.2010.01.011 (2010) (Epub ahead of print).

114 Abdullaev IF, Bisaillon JM, Potier M, Gonzalez JC, Motiani RK, Trebak M. STIM1 and ORAI1 mediate CRAC currents and store-operated calcium entry important for endothelial cell proliferation. *Circ. Res.* 103(11), 1289–1299 (2008).

115 Bisaillon JM, Motiani RK, Gonzalez-Cobos JC *et al*. Essential role for STIM1/ORAI1-mediated calcium influx in PDGF-induced smooth muscle migration. *Am. J. Physiol. Cell. Physiol.* DOI:10.1152/ajpcell.00325.2009 (2010) (Epub ahead of print).

116 Darbellay B, Arnaudeau S, Konig S *et al*. STIM1- and ORAI1-dependent store-operated calcium entry regulates human myoblast differentiation. *J. Biol. Chem.* 284(8), 5370–5380 (2009).

117 Potier M, Gonzalez JC, Motiani RK *et al*. Evidence for STIM1- and ORAI1-dependent store-operated calcium influx through ICRAC in vascular smooth muscle cells: role in proliferation and migration. *FASEB J.* 23(8), 2425–2437 (2009).

118 Zhao X, Weisleder N, Thornton A *et al*. Compromised store-operated Ca^{2+} entry in aged skeletal muscle. *Aging Cell* 7(4), 561–568 (2008).

119 Guo RW, Wang H, Gao P *et al*. An essential role for stromal interaction molecule 1 in neointima formation following arterial injury. *Cardiovasc. Res.* 81(4), 660–668 (2009).

120 Aubart FC, Sassi Y, Coulombe A *et al*. RNA interference targeting STIM1 suppresses vascular smooth muscle cell proliferation and neointima formation in the rat. *Mol. Ther.* 17(3), 455–462 (2009).

121 Dietrich A, Kalwa H, Storch U *et al*. Pressure-induced and store-operated cation influx in vascular smooth muscle cells is independent of TRPC1. *Pflugers Arch.* 455(3), 465–477 (2007).

122 Stiber J, Hawkins A, Zhang ZS *et al*. STIM1 signalling controls store-operated calcium entry required for development and contractile function in skeletal muscle. *Nat. Cell Biol.* 10(6), 688–697 (2008).

123 Takahashi Y, Watanabe H, Murakami M *et al*. Functional role of stromal interaction molecule 1 (STIM1) in vascular smooth muscle cells. *Biochem. Biophys. Res. Commun.* 361(4), 934–940 (2007).

124 Voelkers M, Salz M, Herzog N *et al*. ORAI1 and STIM1 regulate normal and hypertrophic growth in cardiomyocytes. *J. Mol. Cell. Cardiol.* (2010).

125 Giachini FR, Webb RC, Tostes RC. STIM and ORAI proteins: players in sexual differences in hypertension-associated vascular dysfunction? *Clin. Sci. (Lond.)* 118(6), 391–396 (2010).

126 Giachini FR, Chiao CW, Carneiro FS *et al*. Increased activation of stromal interaction molecule-1/ORAI-1 in aorta from hypertensive rats: a novel insight into vascular dysfunction. *Hypertension* 53(2), 409–416 (2009).

127 Varga-Szabo D, Braun A, Kleinschnitz C *et al*. The calcium sensor STIM1 is an essential mediator of arterial thrombosis and ischemic brain infarction. *J. Exp. Med.* 205(7), 1583–1591 (2008).

128 Grosse J, Braun A, Varga-Szabo D *et al*. An EF hand mutation in STIM1 causes premature platelet activation and bleeding in mice. *J. Clin. Invest.* 117(11), 3540–3550 (2007).

129 Yang S, Zhang JJ, Huang XY. ORAI1 and STIM1 are critical for breast tumor cell migration and metastasis. *Cancer. Cell.* 15(2), 124–134 (2009).

130 Ritchie MF, Yue C, Zhou Y, Houghton PJ, Soboloff J. Wilms tumor suppressor 1 and early growth response 1 are regulators of STIM1 expression. *J. Biol. Chem.* DOI: 10.1074/jbc.M109.083493 (2010) (Epub ahead of print).

131 Berna-Erro A, Braun A, Kraft R *et al*. STIM2 regulates capacitive Ca^{2+} entry in neurons and plays a key role in hypoxic neuronal cell death. *Sci. Signal.* 2(93), ra67 (2009).

132 Di Capite J, Nelson C, Bates G, Parekh AB. Targeting Ca^{2+} release-activated Ca^{2+} channel channels and leukotriene receptors provides a novel combination strategy for treating nasal polyposis. *J. Allergy Clin. Immunol.* 124(5), 1014–21.e1–3 (2009).

133 Di Sabatino A, Rovedatti L, Kaur R *et al*. Targeting gut T cell Ca^{2+} release-activated Ca^{2+} channels inhibits T cell cytokine

production and T-box transcription factor T-Bet in inflammatory bowel disease. *J. Immunol.* 3(1), S3–S3 (2009).

- **CRAC channel inhibition as a therapeutical strategy against inflammatory bowel disease.**

134 Bouma G, Strober W. The immunological and genetic basis of inflammatory bowel disease. *Nat. Rev. Immunol.* 3(7), 521–533 (2003).

135 Schwarz A, Tutsch E, Ludwig B, Schwarz EC, Stallmach A, Hoth M. Ca^{2+} signaling in identified T-lymphocytes from human intestinal mucosa. Relation to hyporeactivity, proliferation, and inflammatory bowel disease. *J. Biol. Chem.* 279(7), 5641–5647 (2004).

136 Tutsch E, Griesemer D, Schwarz A, Stallmach A, Hoth M. Two-photon analysis of calcium signals in T lymphocytes of intact lamina propria from human intestine. *Eur. J. Immunol.* 34(12), 3477–3484 (2004).

137 Ng SW, di Capite J, Singaravelu K, Parekh AB. Sustained activation of the tyrosine kinase Syk by antigen in mast cells requires local Ca^{2+} influx through Ca^{2+} release-activated Ca^{2+} channels. *J. Biol. Chem.* 283(46), 31348–31355 (2008).