Activation and Regulation of Store-operated Calcium Entry

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Summary

The process of store-operated Ca^{2+} entry (SOCE), whereby Ca^{2+} influx across the plasma membrane is activated in response to depletion of intracellular Ca^{2+} stores in the endoplasmic reticulum (ER), has been under investigation for greater than 25 years; however, only in the past five years have we come to understand this mechanism at the molecular level. A surge of recent experimentation indicates that STIM molecules function as Ca^{2+} sensors within the ER that, upon Ca^{2+} store depletion, rearrange to sites very near to the plasma membrane. At these plasma membrane-ER junctions, STIM interacts with and activates SOCE channels of the Orai family. The molecular and biophysical data that have led to these findings are discussed in this review, as are several controversies within this rapidly-expanding field.

Introduction

The calcium ion is arguably the most ubiquitous, and certainly one of the most diverse signaling entities in the cell [1]. Changes in the intracellular Ca^{2+} concentration regulate a wide variety of cellular processes that run the gamut from the mitotic birth of a new cell to apoptosis. Elevations in intracellular Ca^{2+} occur when cells tap into two primary sources: the extracellular medium and intracellular stores, most notably the endoplasmic reticulum (ER). In nonexcitable cells the most common route of Ca^{2+} signal generation results from the activation of cell surface receptors, such as those for various growth factors, hormones, and neurotransmitters, which leads to the generation of the second messenger inositol 1,4,5-trisphosphate (IP_3). IP_3 production results in cytoplasmic Ca^{2+} elevation that can be separated into two distinct phases. In the first phase, Ca^{2+} is released from the endoplasmic reticulum (ER) via the IP_3 receptor (IP_3R). In the second phase, the decrease in ER Ca^{2+} content that results from IP_3R activation signals influx of extracellular Ca^{2+} via plasma membrane Ca^{2+} channels in a process known as capacitative or store-operated Ca^{2+} entry (SOCE) [2]. It should be noted that any reduction in ER Ca^{2+} content, whether the result of IP_3R activation or not, can serve as a stimulus of SOCE; this is, in fact, the defining property of the SOCE mechanism.

The existence of SOCE was first postulated in 1986 [3] and experimental evidence for this concept accrued shortly thereafter [4;5]. Subsequently, a membrane current that underlies SOCE was described; this current is referred to as Ca^{2+} release-activated Ca^{2+} current (I_{CRAC}) [6]. While I_{CRAC} is certainly the best-characterized SOCE current, it remains arguable whether I_{CRAC} is the only current that underlies SOCE. This review will focus primarily on the SOCE pathway that is mediated by I_{CRAC}.

Conflict of Interest The authors confirm that there are no conflicts of interest.

Since the discoveries of SOCE and I_{CRAC}, intense research from numerous laboratories has focused on defining the molecular components of the pathway. The last five years have witnessed incredible breakthroughs in this endeavor. It is now evident that SOCE requires members of two families of proteins: the STIM molecules (STIM1 and STIM2), which function as Ca^{2+} sensors in the ER, and the Orais (Orai1, Orai2, and Orai3) which function as pore-forming subunits of SOCE channels. These molecules cooperate in an elegant signaling mechanism whereby a decrease in ER Ca^{2+} store content causes the ER-resident STIM molecules to reposition themselves very near to the plasma membrane where they activate Orai Ca^{2+} influx channels.

I_{CRAC}

Our understanding of SOCE has been greatly improved by the biophysical characterization of the underlying I_{CRAC} current [6;7]. I_{CRAC} is notable for its exceptional Ca^{2+} selectivity and extremely low single channel conductance [2]. Like voltage-gated Ca^{2+} channels, CRAC channels achieve their high Ca^{2+} selectivity through electrostatic repulsion; however, unlike the voltage gated Ca^{2+} channels, CRAC single channel conductance cannot be measured directly. Approximations of the single CRAC channel conductance have been made using a noise analysis approach, and estimates from Lewis' lab suggest a Ca^{2+} conductance of 20–30 fS [8;9] and a Na^+ conductance of 0.7 pS [9]. CRAC channels are also both positively and negatively regulated by Ca^{2+} itself in a complex manner [7;10–14].

The biophysical characterization of I_{CRAC} by many laboratories has provided a unique fingerprint of the store-operated channels that aided in the correct molecular identification of the pore forming subunits of I_{CRAC}, now known as the Orai family of proteins, of which there are three mammalian homologs (Orai1, Orai2 and Orai3). The molecular structures of the Orai proteins are very different from other known Ca^{2+} channels, which was predictable based on the unique biophysical properties of I_{CRAC}.

Orais

Utilizing gene mapping of a family with a severe combined immunodeficiency (SCID) attributed to a loss of I_{CRAC}, as well as a whole genome RNAi screen in *Drosophila* S2 cells, Feske *et al* discovered a unique family of genes (human: FLJ14466 (Orai1), C7orf19 (Orai2), and MGC13024 (Orai3); drosophila: olf186-F (d-Orai)). The *Drosophila* gene was absolutely required for I_{CRAC}, and they called the protein encoded by this gene Orai (keepers of the gates of heaven [15]). Shortly thereafter, two other independent laboratories also reported similar results using RNAi in *drosophila* S2 cells, in which *olf186-F* (called CRACM1 by Vig *et al.*) was shown to be essential for I_{CRAC} [16;17]. In the SCID patients, it was determined that the underlying defect was a single missense mutation in Orai1 (R91W) [15], which increases the hydrophobicity at the transition between the N terminal cytoplasmic portion and the first transmembrane region [18]. This mutation results in a loss of function, but does not affect interactions with STIM1.

The exogenous expression of Orai1 (and Orai2 or Orai3) alone generally causes a suppression of SOCE and I_{CRAC}, presumably due to inappropriate stoichiometry with endogenous STIM1 [19;20]. However, the co-expression of STIM1 and Orai1 generates very large CRAC currents that are virtually indistinguishable biophysically from native I_{CRAC} [17;19;21;22]. Specifically, these CRAC-like currents are store-operated, are highly Ca^{2+} selective, show inwardly rectifying I–V relationships, have low permeability to Cs^+, have undetectable single channel events, and are inhibited by low concentrations of lanthanides and 2-APB (except for Orai3, see below) [19;20;23–27]. The ability to completely recapitulate I_{CRAC} by co-expressing STIM1 with Orai1 suggests that these two

proteins are necessary and sufficient for SOCE, or that any other factor that is required exists in great excess within the cell [28;29].

The Orai proteins are predicted to have four transmembrane domains, with both the N- and C- termini located on the cytosolic side of the plasma membrane [15;30]. Orai1 has an N glycosylation site located on the extracellular loop between TM3 and TM4; however, mutations that prevent glycosylation apparently have no deleterious effects on SOCE [31]. That Orai1 is a pore forming subunit of CRAC channels was demonstrated by several laboratories by mutating specific conserved acidic residues near the beginning of the first extracellular loop. Mutating a glutamate at position 106 (which is conserved in Orai 1, 2 and 3) to alanine results in complete loss of I_{CRAC}, while the conservative mutation to aspartate (E106D) results in a current that is much less Ca^{2+} selective [30;32;33]. Three additional acidic residues located in the extracellular loop between TM1 and TM2 (D110, D112, and D114) in Orai1 have also been shown to influence Ca^{2+} selectivity of CRAC channels as well as sensitivity to the CRAC channel blocker Gd^{3+} [30;33;34]. Increased monovalent permeation seen in the combined mutation of these three residues in Orai1 suggests that they play an important role in the formation of the channel selectivity filter. Unlike E106, these residues differ among Orai1, Orai2 (E84, Q86, and Q88) and Orai3 (E85, D87, and E89).

Recent studies have begun to map the Orai1 domains critical for its activation. The C-termini of all three mammalian Orai homologs contain putative coiled-coil domains. In Orai1 this region has been shown to be critical for the re-arrangement and co-localization of Orai1 with STIM1 in response to ER Ca^{2+} store depletion [25;35]. A conserved region of the N terminus just prior to the first transmembrane domain is also essential for SOCE [35], and the N terminus also might play a role in regulating CRAC channel gating properties [36]. For example, Orai2 chimeras containing the N terminal proline/arginine region unique to Orai1 show increased Ca^{2+} entry compared to wildtype Orai2 [37]. Recent studies identified a minimal domain of STIM1 required to activate CRAC channels (named SOAR for STIM1-Orai activating region). Interestingly, while full length STIM1 could not effectively activate an N terminal truncation mutant of Orai1 (in this case Orai1Δ1–73), the STIM1 SOAR domain could activate the mutated Orai1 [38]. The authors concluded that the Orai1 N terminus interacts with STIM1 in a region that normally inhibits the action of the SOAR domain. Park *et al* reported a similar domain structure (which they called CAD) and concluded that STIM1 functionally interacts not only with the C terminus, but also with the N terminus of Orai1 at the amino acid residues just before TM1 [39]. Interestingly, in a short period of time, four different laboratories all reported minimal Orai-activating domains of STIM1; the amino acid sequences and proposed nomenclature are summarized in Table 1. For the most part, the activating sequences are conserved among Orai homologs. While these and other studies have greatly enhanced our understanding of Orai channel activation by STIM1, much more remains to be learned about these unique Ca^{2+} channels.

Orai1 proteins are thought to require multimerization in order to form CRAC channels. Biochemical studies have revealed that Orai1 proteins are capable of forming higher order multimeric structures [31;32]. Several recent studies have concluded that the functional CRAC channel pore is made up of Orai1 tetramers [17;40;41]; however, it is not clear if these tetramers are constitutively formed, or if STIM1 brings the subunits together. While Penna *et al* suggest that functional CRAC channels consist of tetramers formed by STIM1-evoked dimerization of Orai dimers [42], negative stain electron microscopy data do not support this conclusion [39]. Further potential complexity arises from the abilities of different Orai subunits to form hetero-multimeric channels. Interestingly, Orai2 and Orai3 also form store-operated Ca^{2+} channels with similar, but not identical biophysical and pharmacological properties to Orai1 (see below). Overexpressed Orai2 and Orai3 have also been shown to co-immunoprecipitate with overexpressed Orai1, and the pore dead mutant of

Orai1 has been shown to significantly reduce I_{CRAC} in HEK293 cells expressing STIM1 with either Orai2 or Orai3, suggesting the different Orais might functionally interact [23;31].

Perhaps the strongest evidence to date suggesting Orai2 or Orai3 form functional CRAC channels comes from studies in Orai1 (in this case called CRACM1) knockout mice that were created using gene trap technology [43]. Ca^{2+} imaging experiments on mast cells isolated from these Orai1 deficient mice revealed a residual Ca^{2+} entry pathway that was activated by the addition of thapsigargin to deplete ER Ca^{2+} stores. Importantly, the currents remaining in these Orai1 knockout mast cells were I_{CRAC}-like, with strong inward rectification. Quantitative RT-PCR suggested that the residual CRAC-like currents in these cells might be mediated by Orai2 and/or Orai3. Incomplete reduction of SOCE and I_{crac} was also observed in T and B cells isolated from Orai1 knockout mice created by the Rao lab, with a similar conclusion that Orai2 and/Orai3 may be responsible for the residual SOCE activity [44].

In vitro studies on over-expressed proteins have shown that Orai2 and Orai3 (as well as Orai2 splice variants Orai2L and Orai2S) can also form CRAC channels that require the depletion of internal Ca^{2+} stores in order to open [19;20;23;24;45]. Like Orai1, these channels are also highly Ca^{2+} selective, with a strongly inwardly rectifying current-voltage relationship. Further, the Ca^{2+} concentrations required to half-maximally block Na^+ conductances of Orai2 and Orai3 are similar to that for Orai1, and none of the Orais permeates Cs^+ well when expressed as homomeric channels [23–25;30;34] The current densities of the Orai2 and Orai3 Ca^{2+} currents are several times smaller than the Orai1 CRAC currents in these over-expression assays. This difference in current size is presumably a consequence of expression levels, and also possibly single channel properties. While Orai3-mediated Ca^{2+} currents are significantly smaller than Orai1 Ca^{2+} currents, the Na^+ currents from Orai3 are much larger in magnitude than Orai1 Na^+ currents [23;24]. It was this difference in Na^+ permeation that initially facilitated recording of Orai3-mediated SOC currents, despite being unable to record Ca^{2+} currents [24]. Orai1, Orai2 and Orai3 apparently show differences in Ca^{2+} dependent regulatory processes, including fast and slow inactivation [23;24]. The Orai homologs also differ in their responses to the pharmacological agent 2-APB. While both Orai1- and Orai2- evoked SOCE and CRAC currents are inhibited by 2-APB (albeit Orai2 appears to be somewhat less sensitive to 2-APB), Orai3 is directly activated by the compound [20;26;46;47;47]. Further, 2-APB-activated Orai3 currents are less Ca^{2+} selective than I_{CRAC}, showing increased outward Cs^+ currents. Whether or not Orai2 and/or Orai3 form endogenous CRAC channels themselves, or in some hetero-multimeric structure with Orai1, is yet to be determined. However, the differences seen with 2-APB on Orais might provide clues to the functions of different Orais in different cells types, and may shed light on the question of whether or not they function as homomeric or heteromeric channel subunits. Our understanding of Orai2 and Orai3 will also greatly be enhanced by the genetic engineering of knockout mice.

STIM1

STIM1 was first cloned in 1996 [48], but it was not until 2005 that its role in SOCE was first realized. A limited RNAi screen of *Drosophila* S2 cells identified *Drosophila* STIM as having an essential role in SOCE activation [49], and a similar conclusion was reached almost simultaneously for human STIM1 from a human RNAi screen [50]. Numerous studies since have confirmed the obligate role of STIM1 in SOCE in a variety of cell systems. Substantial molecular and functional analyses have revealed that STIM1 functions as a Ca^{2+} sensor in the ER that is responsible for communicating depletion of ER Ca^{2+} stores to Orai channels in the plasma membrane [51].

STIM1 is predicted to be a single-pass transmembrane protein that can localize both to the plasma membrane [52;53] and the ER membrane [50;54]. Early evidence suggested that STIM1 is localized within or translocated to the plasma membrane, and that this plays a role in SOCE regulation [53;55]. However, most subsequent studies have concluded that only ER-localized STIM1 is required [19;50;54]. When localized to the ER membrane, STIM1 is oriented such that its N-terminus resides within the ER lumen and its C-terminus in the cytoplasm. The protein is comprised of several identifiable functional motifs, including an EF-hand Ca^{2+} binding domain and a sterile-α motif (SAM) in the luminal N-terminus and a pair of coiled-coil domains, a serine/proline rich region, and a poly-basic region in the cytoplasmic C-terminus [56]. The SOAR domain, key to activation of Orai channels, is located within the coiled-coil domains [38;39;57;58]. Localization of STIM1 is critical to its SOCE function: when Ca^{2+} stores are full STIM1 is localized in tubular structures throughout the ER membrane, but when stores are depleted it moves to discrete punctate structures at sites where the ER is closely apposed to the plasma membrane [50;54;59] (Figure 1). It is this relocalization of STIM1 within the ER network toward the plasma membrane that allows it to directly or indirectly interact with and activate Orai channels [60].

The sequence of molecular reactions and the functional domains of STIM1 that translate changes in ER Ca^{2+} stores to STIM1 localization and activation of SOCE are becoming clear. Loss of ER Ca^{2+} results in an initial oligomerization of STIM1 molecules, which is followed by bulk movement of STIM1 oligomers to ER-plasma membrane junctional sites [61]. The most important domain for the Ca^{2+} sensing function of STIM1 is the EF-hand motif that is localized within the lumen of the ER. The STIM1 EF-hand binds Ca^{2+} with a Kd of 0.2–0.6 mM [62], well within the range of the physiological ER Ca^{2+} concentration. The role of Ca^{2+} binding within the EF-hand was confirmed by experiments demonstrating that mutations of putative Ca^{2+} binding acidic residues within the domain result in constitutive near plasma membrane STIM1 puncta and constitutive SOCE activity [19;50;53;55]. The EF-hand and nearby SAM domains appear to function in concert in response to changes in ER Ca^{2+}; dissociation of Ca^{2+} from this region induces a destabilization of the EF-hand/SAM structure, resulting in oligomerization of multiple STIM1 molecules [63]. Elegant experiments from the Lewis lab further demonstrated that oligomerization via the EF-hand/SAM region is sufficient for STIM1 puncta formation and activation of SOCE [64].

Redistribution of STIM1 oligomers to ER-plasma membrane junctional sites appears to depend largely on functional domains within the cytoplasmic C-terminus of the molecule. Removal of the poly-basic region was shown to prevent STIM1 puncta formation at the plasma membrane [39;61] without interfering with STIM1 oligomerization in response to Ca^{2+} store depletion [61]. It was proposed that interaction of these basic residues with plasma membrane phospholipids, particularly PIP_2, may account for targeting of STIM1 oligomers to the plasma membrane [61]. However, in a separate report, depletion of plasma membrane PIP_2 was found to have no effect on STIM1 redistribution [65;66], although it is possible that PIP may play a role [66;67]. More recently it was reported that the poly-basic region is dispensable for redistribution of STIM1 in the context of over-expressed Orai1, suggesting that interaction of STIM1 with Orai1 may contribute to STIM1 puncta formation [39]. However, the question of whether STIM1 directly interacts with Orai channels has been controversial. Several reports have demonstrated FRET between expressed fluorescently-tagged STIM1 and Orai1 proteins in response to Ca^{2+} store depletion [25;34;68], and co-immunoprecipitation has also been reported ([16;33] but see [31]). However, neither of these techniques can unequivocally differentiate between direct or indirect interactions. In light of this, Varnai *et al.* utilized chemical linkers between the ER and plasma membrane to estimate that Orai1 is part of a molecular complex that protrudes

11–14 nm into the cytoplasm, which is longer than the predicted length of the STIM1 cytoplasmic domain [65]. This result suggests that an intermediary molecule may bridge this gap between STIM1 and Orai1. It has been suggested that iPLA$_2$β may function as such a linker between STIM1 and Orai1 [69;70] and although a role for iPLA$_2$β in SOCE activation has been substantiated by molecular suppression studies [70], its function as a molecular link between STIM1 and Orai1 has not been proven. It has also been suggested that the primary SOCE-activating function of STIM1 may be to generate a soluble product known as Ca^{2+} influx factor (CIF) which then directly activates Orai1 channels [70]. Full analysis of the physiological role of CIF in the SOCE activation pathway still awaits full biochemical purification of the compound.

Despite these suggestions that the functional interaction between STIM1 and Orai1 is indirect, the case for direct coupling between these molecules has been significantly bolstered by four nearly simultaneous studies reporting that a minimal sequence of the STIM1 C-terminus that encompasses the putative coiled-coil domain and part of the ERM domain is sufficient to constitutively activate Orai1 (see Table 1 for exact sequences) [38;39;57;58]. This minimal STIM1 domain exhibits a peripheral localization near the plasma membrane only when co-expressed with Orai1 [38;39;57;58], suggesting that Orai1 influences the localization of the minimal STIM1 domain. Store-independent colocalization [38;57;58] and FRET [57], as well as co-immunoprecipitation [38;39;58] with Orai1 were also demonstrated, suggesting interaction with Orai1. Evidence that this interaction is direct in nature was provided by *in vitro* biochemical analysis of purified proteins as well as by a split-ubiquitin assay in yeast cells [39]. Interestingly, extension of the minimal STIM1 domain in the C-terminal direction results in a construct that is less effective at activating Orai1-dependent SOCE [57;58], and in fact direct perfusion of cells with a peptide comprising the 31 amino acids immediately following the minimal sequence (amino acids 339–475) inhibited I_{CRAC} [58]. This suggests that a C-terminal sequence of STIM1 interacts with and inhibits the Orai1-interacting and/or activating domain of STIM1; presumably, Ca^{2+} dissociation and/or oligomerization of STIM1 relieves this intramolecular interaction, allowing full association with and activation of Orai1 channels. Interestingly, recently three laboratories have reported that a short acidic sequence downstream of the activation domain appears to be involved in Ca^{2+}-dependent inactivation of Orai channels [71–73]. This result was somewhat surprising since it might have been expected that Ca^{2+} would regulate Orai channels by interacting with the channels themselves.

SOCE is a reversible process that terminates in response to Ca^{2+} store refilling. Several studies have now revealed that reversal of near-plasma membrane STIM1 puncta in response to store refilling is responsible for termination of SOCE.[50;65;74]. Reversal of STIM1 puncta by pharmacological agents has also now been demonstrated. In the first such study, the myosin light chain kinase (MLCK) inhibitor ML-9, which was previously shown to inhibit SOCE and I_{CRAC} [75–77] dose-dependently and reversibly inhibited store depletion-induced formation of STIM1 puncta. Furthermore, pre-existing STIM1 puncta, whether formed due to store depletion or due to EF-hand mutations, were also rapidly reversed by the agent. Importantly, it was demonstrated that reversal of STIM1 puncta by ML-9 is responsible for SOCE/ I_{CRAC} inhibition by the agent, which represents the first delineation of a molecular mechanism for pharmacological inhibition of SOCE. It should be noted, however, that the direct target of ML-9 responsible for SOCE inhibition appears not to be MLCK and remains unknown [74]. A similar reversal of STIM1 puncta was also observed with 2-APB, one of the most commonly employed I_{CRAC} inhibitors [20;26;78]. However, in contrast to the case with ML-9, reversal of STIM1 puncta does not appear to be the primary, or at least the only, cause of inhibition of I_{CRAC} [20]. Instead, 2-APB exerts a strong inhibitory effect on the Orai1 channel itself, and this inhibition appears to be independent of STIM1 localization. As discussed previously, the effects of 2-APB on Orai

channels are quite complex. Interestingly, in the case of both ML-9 and 2-APB, co-expression of STIM1 with Orai1 significantly attenuates the ability of the drugs to reverse STIM1 puncta [20]. The reason for this is not clear, but could reflect a strong interaction between STIM1 and Orai1 that is accentuated when both molecules are co-expressed in excess.

It was first documented in 1988 [79], and subsequently confirmed [80–82], that SOCE is strongly suppressed during cell division. This represents one of the only known physiological situations in which depletion of Ca^{2+} stores is dissociated from SOCE activation, and therefore represents an unparalleled system in which to study signaling events that regulate SOCE. Furthermore, the physiological significance of SOCE suppression during mitosis and meiosis is unknown, and delineation of the mechanism by which this suppression occurs will facilitate analysis of broader questions concerning the role of Ca^{2+} signaling during cell division. A recent study has now demonstrated that during mitosis, STIM1 phosphorylation is upregulated and rearrangement of STIM1 into near-plasma membrane puncta in response to Ca^{2+} store depletion is suppressed [83]. In support of the hypothesis that mitosis-specific phosphorylation of STIM1 prevents puncta formation and therefore SOCE activation, expression of STIM1 in which all detectable mitosis-specific phosphorylations were removed by truncation, in combination with Orai1 expression, rescued puncta formation and SOCE in mitotic cells (Figure 1). These cells in which mitotic SOCE was functional exhibited a slower growth rate and an increased accumulation at the G2/M stage of the cell cycle, consistent with the hypothesis that SOCE negatively impacts mitosis and therefore must be suppressed to ensure the fidelity of cell division [83]. However, it remains possible that phosphorylation may also regulate a novel, mitosis-specific function of STIM1 that is distinct from SOCE. It was also recently demonstrated that suppression of SOCE during meiosis in *Xenopus* eggs is mediated primarily by Orai1 internalization [84], and decreases in Orai1 protein expression during mitosis have also been noted [83;85]. Thus, modifications of both STIM1 and Orai1 may contribute to cell cycle-dependent regulation of SOCE. It should also be noted that several mitosis-independent STIM1 phosphorylation sites have been identified [83], suggesting that differential patterns of STIM1 phosphorylation may regulate STIM1 functions in complex ways. Accordingly, it is likely that the C-terminal serine/proline-rich region of STIM1, which contains all STIM1 phosphorylation sites identified to this point, functions as a regulatory domain that modulates STIM1 function according to its state of phosphorylation.

Elucidation of the role of STIM1 in the SOCE signaling pathway has understandably generated a flurry of excitement in the Ca^{2+} signaling field; however, it is equally exciting to note that STIM1 may be a multi-functional protein with additional roles independent of SOCE. Prior to the realization of its role in SOCE, STIM1 was identified as a surface-expressed protein involved in stromal adhesion of pre-B cells [86]. This function of STIM1 has not been explored further, but interestingly STIM1 was also attributed a tumor-suppressive function, with loss of STIM1 expression associated with several malignancies, most notably embryonic rhabdomyosarcoma [87]. More recently, STIM1 was also shown to be a necessary factor in the activation of the arachidonate-regulated Ca^{2+} current (I_{ARC}), which is similarly activated downstream of PLC-coupled receptors but independently of Ca^{2+} store depletion. In contrast to I_{CRAC}, I_{ARC} appears to depend entirely on STIM1 that is localized in the plasma membrane and does not rely on redistribution of ER-resident STIM1 [88]. Production of cyclic AMP (cAMP), another second messenger that is generated in response to G protein-coupled receptor stimulation, has also been shown to be regulated by STIM1 in a store-dependent manner, but in this case independently of Ca^{2+} influx that occurs as a result of activation of SOCE [89]. And finally, several studies that made use of fluorescently-tagged STIM1 constructs have noted constitutive, comet-like movements of STIM1, as well as strong colocalization of STIM1 with microtubules [74;90;91]. These

observations have now been attributed to a novel function whereby STIM1 acts as a link between growing microtubules and the ER and in so doing, regulates microtubule-dependent extension of the ER at the cell periphery [92]. Thus, STIM1 is involved in a diverse array of cellular functions, and it will be interesting to determine the influence and interdependence of each of these functions on one another.

STIM2

STIM2 is the second member of the vertebrate STIM protein family [93]. In humans, STIM2 is widely expressed in various tissues [93]. However, unlike STIM1, which is localized in both the plasma membrane and the ER membrane, STIM2 has been shown to be exclusively present in the ER membrane [94].

STIM2 has a high amino acid sequence homology and similar domain architecture to STIM1. Like STIM1, STIM2 is a single-pass transmembrane protein with an unpaired N-terminal EF-hand and a SAM domain located in the ER lumen and two C-terminal coiled coil domains, a Pro/Ser-rich region, and a Lys-rich region located in the cytoplasm [56;93]. Nevertheless, the contribution of STIM2 to native SOCE is less obvious than that of STIM1. Liou *et al.* showed that knockdown of STIM2 partially attenuates SOCE [50], whereas Roos *et al.* failed to see such an effect [49]. Further, conditional ablation of STIM2 in mice resulted in a small reduction of SOCE in fibroblasts but no effect on CD4+ T cells, whereas STIM1 deficiency led to a complete loss of SOCE in both cell types [95]. In whole-cell patch-clamp recordings, STIM1-deficient T cells also had almost complete loss of I_{CRAC} whereas STIM2 deficient T cells showed no such effect [95]. However, in helper T cells, STIM2 deletion substantially impaired the nuclear transport of NFAT similar to STIM1 deficiency, suggesting an important role of STIM2 in maintaining Ca^{2+} signaling of T cells.

When STIM1 is overexpressed, SOCE is either modestly increased or unaffected [19;22;49]. However, overexpression studies with STIM2 have been less clear. SOCE was dramatically inhibited in HEK293 cells stably overexpressing STIM2 [94] but was slightly increased in cells transiently (9 hr) overexpressing STIM2 [96]. Our laboratory also observed a modest reduction in SOCE when STIM2 was overexpressed in HEK293 cells (24 to 48 hr) [97]. Interestingly, unlike STIM1, transient overexpression of STIM2 causes constitutive Ca^{2+} entry, suggesting a distinct role for STIM2 in resting cells with full Ca^{2+} stores [22;96;98].

Ratiometric Ca^{2+} imaging and electrophysiological current measurements showed that co-expression of STIM1 and Orai1 recapitulate huge SOCE [19;21;22]; however, overexpression of Orai1 in stable STIM2 cells only modestly enhanced SOCE [22]. Furthermore, Parvez *et al.* suggested that STIM2 plays a dual role in both store-dependent and -independent activation of Orai [99]. These investigators showed that HEK293 cells expressing both STIM2 and Orai1 exhibit a large and transient I_{CRAC}-like current in response to 2-APB even when stores are full. This observation was not seen in cells overexpressing either STIM2 or Orai1 alone, or coexpressing STIM1 and Orai1. Store depletion also generates detectable I_{CRAC}-like currents from cells expressing both STIM2 and Orai1 but only in the absence of the aminoglycoside antibiotic G418, which the authors suggest is a specific inhibitor of STIM2-mediated Orai activation [99].

In an effort to understand the functional roles in SOCE of STIM1 and STIM2, Zheng *et al.* examined the N terminal Ca^{2+} binding regions of STIM1 and STIM2 (an EF-hand motif and a SAM domain) by using far UV circular dichroism spectroscopy [100]. These investigators found that both STIM1 and STIM2 EF-SAM exist as monomers in the presence of Ca^{2+}, but that STIM2 EF-SAM forms more stable structures than STIM1 EF-SAM and does not readily aggregate in the absence of Ca^{2+}. Furthermore, unfolding and oligomerization of STIM2 EF-SAM are slower than STIM1 [101]. These differences might play important roles

in kinetic differences in redistribution of STIMs into puncta and activation of Ca^{2+} permeable channels. Indeed, a recent investigation showed that STIM2-mediated Orai1 activation is much slower than that mediated by STIM1 [99]. To address whether STIM2, like STIM1, also redistributes into near-plasma membrane puncta, Soboloff *et al.* demonstrated that, when overexpressed, STIM2 puncta formation by store depletion is dependent on STIM1 overexpression, suggesting that STIM2 functions as an accessory to STIM1 [94]. However, recent studies utilizing TIRF microscopy demonstrated that overexpressed STIM2 is capable of translocation to ERPM junctions with endogenous levels of STIM1 following store depletion [98].

Brandman *et al*. [96] demonstrated that STIM2 is also an ER Ca^{2+} sensor by showing that the STIM2 EF-hand mutant constitutively forms punctate structures at ERPM junctions in the absence of store depletion. Interestingly, the same group showed in biochemical assays that STIM2 is partially active even at the resting state and initiates puncta formation rapidly in response to a small degree of Ca^{2+} depletion [96], consistent with the observation that STIM2 has a lower Ca^{2+} binding affinity than STIM1 [100]. When the onset of translocation of STIM1 and STIM2 was compared, it was clear that STIM2 began to move almost immediately, while STIM1 began to move after some delay, indicating that because of its higher Ca^{2+} affinity, a significant threshold of Ca^{2+} release was needed to reach the concentration range in which STIM1 is activated [96;98]. However, the lower Ca^{2+} affinity of STIM2 probably does not explain the slow kinetics of STIM2-mediated Orai1 activation and lower efficiency in activation of Orai1 than STIM1. In an attempt to address this discrepancy, Zhou *et al.*, showed that the short N-terminal domains of STIM proteins localized in the lumen of the ER are responsible for differences in the activation kinetics of Orai1 [102]. These investigators showed by using chimeric replacement that STIM1 with the N-terminal domains of STIM2 slows the kinetics of Icrac activation and suppresses SOCE, whereas STIM2 with the N-terminal domain of STIM1 results in faster Icrac development and increase in SOCE. Taken together, these observations suggest that STIM2 plays a major role in Ca^{2+} homeostasis by maintaining basal cytosolic and ER Ca^{2+} levels, rather than being a strong activator of SOCE.

STIM1, STIM2 and Ca^{2+} Oscillations

Much of the work discussed to this point has involved investigation of SOCE by use of experimental conditions that induce maximal or near maximal depletion of Ca^{2+} stores, for example by use of SERCA inhibitors. However, with physiological stimuli, Ca^{2+} stores seldom if ever undergo such massive loss of Ca^{2+}. Rather, the $[Ca^{2+}]_i$ signals generated with low, physiological levels of receptor agonists most commonly take the form of discrete repetitive discharges of Ca^{2+}, sometimes termed Ca^{2+} oscillations [103;104]. These oscillations run down in the absence of extracellular Ca^{2+}, indicating a need for Ca^{2+} influx to maintain the intracellular stores. Despite some initial debate on the nature of this influx mechanism [105], it now seems clear that in many cell types, it is SOCE that provides this necessary influx [104;106;107]. Since the influx associated with Ca^{2+} oscillations appears to involve very small changes in intracellular Ca^{2+} store content [106], it would be expected that STIM2 would play the predominant role in signaling SOCE. However, in one study it was clearly shown that the Ca^{2+} entry supporting muscarinic receptor-driven Ca^{2+} oscillations depended entirely on STIM1 and not STIM2 [98]. Apparently, STIM2 does not contribute significantly because of its very low efficacy in activating Orai channels. This is consistent with its suggested role in maintaining cellular Ca^{2+} homeostasis by responding to small changes in store content around the resting level [96]. The authors suggested that STIM1 therefore is specialized for responding to significant changes in ER Ca^{2+} that are associated with the generation of Ca^{2+} signals [98]. The threshold for STIM1 is reached in oscillating cells because each oscillation produces a brief drop in ER Ca^{2+} that is sufficient

to activate STIM1. In support of this conclusion, in many cells oscillatory movements of STIM1 toward the plasma membrane were observed slightly following, but in close synchrony with cytoplasmic Ca^{2+} oscillations [98].

The reason the cell has developed this high threshold mechanism for activation of SOCE becomes clearer when one appreciates the cellular function of the SOCE underlying Ca^{2+} oscillations. It is clear that the oscillations are necessary for maintaining adequate stores that drive the oscillatory Ca^{2+} release. But more importantly, it is also becoming increasingly clear that Ca^{2+} entry through plasma membrane channels is capable of providing localized Ca^{2+} signals that specifically couple to downstream effector pathways [108], and this concept has been shown repeatedly to apply to SOCE [109–115]. Thus, the threshold required for activating STIM1 and SOCE assures that downstream signaling pathways will not be turned on except when bona fide Ca^{2+} signals are generated, either by high concentrations of agonists that induce substantial Ca^{2+} store depletion, or by low concentrations that induce spikes of Ca^{2+} release underlying Ca^{2+} oscillations. The recently published results of Di Capite *et al.* [116] nicely demonstrated this concept. When mast cells were activated by leukotrienes, Ca^{2+} oscillations were produced, and this led to activation of the early gene, *c-fos*. However, by use of the technique of "Gd^{3+} insulation" [106;117–119] that blocks both Ca^{2+} influx and efflux, they showed that the global rise in cytoplasmic Ca^{2+} resulting from each spike or oscillation was not coupled to *c-fos* activation; rather, influx of Ca^{2+} through the SOCE (I_{crac}) channels was absolutely required.

Previous studies have demonstrated the efficiency of Ca^{2+} oscillations as signals for downstream effectors [120–123]. However, the experiments have not generally distinguished the relative roles of Ca^{2+} release and Ca^{2+} entry through SOCE channels. The more recent findings suggest that in many cases it is the Ca^{2+} entering through the SOCE channels that is the primary source of signaling Ca^{2+}, and that in such cases the function of the oscillatory discharges of Ca^{2+} is to produce a sufficiently ample drop in ER Ca^{2+} levels to activate the signaling Ca^{2+} sensor, STIM1.

TRPCs and SOCE

Besides the Orai proteins, canonical transient receptor potential (TRPC) proteins have also been suggested to be channels that mediate calcium influx upon store depletion. TRP channels were first identified as cation channels activated in response to photoreceptor stimulation in *Drosophila*. TRPC channels are the closest mammalian homologues of *Drosophila* TRP and are activated downstream of phospholipase C activation. Among the seven isoforms of TRPC channels, TRPC1,3,4,5 and 7 have been reported to contribute to SOCE [2]; however, their channel properties are different from those of CRAC channels. While I_{CRAC} is highly selective for Ca^{2+} ions, endogenously and exogenously expressed TRPC channels are non-selective cation channels. Therefore, it is generally thought that in cases in which TRPCs are activated by store depletion, they form Ca^{2+}-permeable non-selective cation channels distinct from CRAC channels. Following the discovery of STIM and Orai proteins, several groups have begun to address whether TRPCs function in coordination with STIM1 and/or Orai1.

Studies from the Muallem laboratory provided evidence that STIM1 interacts with TRPC1, 2, 4 and 5 but not with TRPC3, 6 and 7 [124]. Subsequently, the same group reported that STIM1 can also regulate TRPC3 and TRPC6 through TRPC1 and TRPC4 respectively [125]. Activation of TRPC1 by STIM1 required the STIM1 C-terminus, which includes the ERM, S/P and polybasic domains [124]. Further studies suggested that gating of TRPC1 is mediated by electrostatic interactions between negatively charged aspartate residues in TRPC1($^{639}DD^{640}$) and positively charged lysine residues in STIM1 ($^{684}KK^{685}$), at

molecular sites distinct from the gating of Orai1 by STIM1 [126]. These results suggested that SOC channels formed by TRPCs, and CRAC channels formed by Orai1, are expressed and function independently on the plasma membrane. However, in our laboratory, we could not find any evidence of TRPC regulation by STIM1 in a variety of cell types [97].

Several studies have also suggested that STIM1, Orai1, and TRPC1 form ternary complexes and that Orai1 knockdown causes suppression of TRPC1-mediated SOCE activity [127;128]. Further, STIM1 and TRPC1 co-expression resulted in an increase of SOCE compared to TRPC1 expression alone, and this TRPC1-mediated SOCE was sensitive to Gd^{3+} and 2-APB at the same concentrations as CRAC channels. It should be noted, however, that the current-voltage relationship of the TRPC1-mediated SOCE current was different from that of I_{CRAC}. Therefore, endogenously expressed Orai1 may be involved not only in CRAC channels but also in TRPC1 channel complexes [127]. More recently, Kim *et al.* showed that simultaneous knockdown of Orai1 and TRPC1 abolished 90% of native SOCE in HEK293 cells, and SOCE was not restored by expression of Orai1 or TRPC1 alone [129]. This result supports the hypothesis that the formation of functional SOCE channels requires both TRPC1 and Orai1. Interestingly, this mutual requirement of Orai1 and TRPC1 is restricted to the TRPC1-mediated SOCE current in HEK293 cells, because endogenous I_{CRAC} was not affected by TRPC1 knockdown in Jurkat T cells. These authors suggest that I_{CRAC} is completely mediated by Orai channels while I_{SOC} is mediated by a TRPC1-Orai1 complex [129]. A problem with these conclusions is that other investigators have investigated the store-operated currents in HEK293 cells, and concluded that the current was identical to I_{crac} and essentially completely eliminated by knockdown of Orai1 alone [16;24].

Birnbaumer's group has also produced results that support functional and physical coupling between Orais and TRPCs, by demonstrating that expression of exogenous Orai1 increased SOCE and I_{CRAC} in a TRPC-dependent manner [130–132]. Furthermore, Orai1-overexpression in TRPC stable HEK293 cells resulted in an increase of receptor-operated Ca^{2+} entry which is thought to be predominantly mediated by TRPCs. Interestingly, direct activation of TRPC3 by 1-oleoyl-2-acetyl-*sn*-glycerol (OAG) was also inhibited by the expression of the SCID mutant form of Orai1 (R180W).

Although several groups have suggested functional interactions between TRPCs, STIM1, and/or Orai1, we have recently reported that TRPC channels function independently of STIM1 and Orai1 [97]. First, co-expression of STIM1 did not induce a significant increase of expressed TRPC activity upon agonist stimulation in HEK293 cells [97], a direct contradiction of the earlier reports from the Muallem lab [124;125]. Further, in a smooth muscle cell line, endogenous TRPC6 activity was not affected by knockdown of endogenous STIM1. And lastly, although disruption of lipid microdomains by MβCD attenuated OAG-activated TRPC3 activity, it had no effect on TG-induced STIM1 puncta formation or I_{CRAC} development. Based on these results, we concluded that TRPC channels are activated downstream of PLC but not regulated by STIM1.

Numerous studies have reached different and often conflicting conclusions as to the role of TRPCs in SOCE, generally based on somewhat alternative strategies and often different cell backgrounds. Because it is known that TG- or IP$_3$-induced Ca^{2+} release causes the Ca^{2+}-dependent activation of PLC, careful experimental design is required to discriminate store-operated Ca^{2+} entry from Ca^{2+}-activated or PLC-dependent second messenger-activated Ca^{2+} entry. Furthermore, there are several intriguing observations regarding TRPC1. TRPC1 is known to be expressed on intracellular membrane compartments in addition to the plasma membrane [133;134]. Knockout of TRPC1 in chicken DT40 cells caused diminished Ca^{2+} release from internal Ca^{2+} stores [135]. It has also been reported that TRPC1 knockdown

reduced the rate of passive release from stores upon thapsigargin (TG) treatment [136]. These observations imply the possibility that TRPC1 functions as an intracellular Ca^{2+} channel [134] and through this function, it might modify SOCE. In fact, the reported STIM1-TRPC1 interaction can be observed even in resting cells and does not change, or increases only modestly, with store depletion or receptor activation [124;128;137]. Thus, it will be important to exclude the possibility that TRPC1 and STIM1 form complexes on the ER membrane that regulate luminal ER Ca^{2+} content, as opposed to a direct role in mediating SOCE.

An additional possibility is that TRPCs might contribute to SOCE in a less direct way, for example if TRPCs were activated in parallel to, or downstream of I_{crac}. Despite their clear abilities to be activated by phospholipase C products, TRPCs can also, in some instances, be regulated or activated directly by Ca^{2+} [138–142], or by IP_3 receptors [143–146]. An excellent example is the situation in RBL-2H3 cells wherein the standard method for activating I_{crac}, whole cell current measurement with IP_3 in the pipet, appears to activate both Orai channels as well as TRPC1 [146]. However, it appears that the TRPC1 activation depends on IP_3, but not store depletion.

Conclusions

The major goal of this review has been to summarize and highlight recent advances in our knowledge of the molecular mechanisms underlying store-operated or capacitative calcium entry. The phenomenology has been appreciated for well over 20 years, but it was only in the past few years with the development of newer genome-focused strategies that the key molecules and their mechanisms of action and interaction were revealed (Figure 2). With hindsight this is perhaps not surprising given the rather unique nature of both the signaling mechanism, and channel properties associated with SOCE and I_{crac}. Progress is rapidly continuing, even as this review is being written, and we can look forward to soon learning the structural and cell biological characteristics of Orai and STIM molecules, eventually we hope leading to useful information for the treatment of specific diseases.

Acknowledgments

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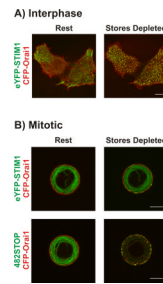
Figure 1.
STIM1 and Orai1 co-localize in response to Ca^{2+} store depletion. A) eYFP-STIM1 rearranges into near-plasma membrane puncta and co-localizes with CFP-Orai1 in response to Ca^{2+} store depletion in interphase HEK293 cells. B) eYFP-STIM1 fails to rearrange into near-plasma membrane puncta in response to Ca^{2+} store depletion in mitotic HEK293 cells (upper panel). Rearrangement in mitosis is rescued with a STIM1 truncation mutant (482STOP) that lacks mitosis-specific phosphorylation sites (lower panel).
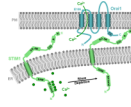
Figure 2.
Predicted topologies and functional domains of STIM1 and Orai1 proteins. Shown for STIM1 are the Ca^{2+}-sensing EF-hand domain and the sterile-alpha motif (SAM) in the ER lumen, and the STIM-Orai activation region (SOAR), the Ca^{2+}-dependent inactivation (CDI) domain, the regulatory domain that is modified by phosphorylation (R), and the poly-lysine (K) domain in the cytoplasm. As illustrated, STIM1 rearranges within the ER membrane in response to Ca^{2+} store depletion, allowing it to interact with and activate Orai1 channels in the plasma membrane. Orai1 is predicted to have four transmembrane regions, such that its N- and C-termini are cytoplasmic. Shown are E106, an amino acid within the channel pore that confers Ca^{2+} selectivity, and a putative coiled-coil in the C-terminus that is required for interaction with and activation by STIM1.

Table 1

Reported Orai-activating domains of STIM1

Reference	Nomenclature	Amino Acids
Yuan *et al.* [38]	STIM1 Orai-activating region (SOAR)	344–442
Park *et al.* [39]	CRAC activation domain (CAD)	342–448
Muik *et al.* [57]	Orai1-activating STIM1 fragment (OASF)	233–450
Kawasaki *et al.* [58]	CCb9	339–444