Activation and regulation of store-operated calcium entry

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Abstract

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 5 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.

Keywords: store-operated Ca\(^{2+}\) entry • CRAC • calcium influx • calcium channel • STIM1 • STIM2 • Orai1 • Orai2 • Orai3 • TRPC

Introduction

The calcium ion is arguably the most ubiquitous, and certainly one of the most diverse signalling 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 non-excitable 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 ER via the IP\(_3\) receptor (IP\(_3\)R). In the second phase, the decrease in ER Ca\(^{2+}\) content that results from IP\(_3\)R 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\(_3\)R 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\(_{\text{CRAC}}\)) [6]. While I\(_{\text{CRAC}}\) is certainly the best-characterized SOCE current, it remains arguable whether I\(_{\text{CRAC}}\) is the only current that underlies SOCE. This review will focus primarily on the SOCE pathway that is mediated by I\(_{\text{CRAC}}\).

Since the discoveries of SOCE and I\(_{\text{CRAC}}\), intense research from numerous laboratories has focused on defining the molecular components of the pathway. The last 5 years have witnessed incredible breakthroughs in this endeavour. It is now evident that SOCE requires members of two families of proteins: the stromal interaction molecule (STIM) molecules (STIM1 and STIM2), which function as Ca\(^{2+}\) sensors in the ER, and the Orains (Orai1, Orai2 and

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Orai3), which function as pore-forming subunits of SOCE channels. These molecules cooperate in an elegant signalling 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.

**ICRAC**

Our understanding of SOCE has been greatly improved by the biophysical characterization of the underlying \(i_{\text{CRAC}}\) current [6, 7]. \(i_{\text{CRAC}}\) 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 \(i_{\text{CRAC}}\) 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 \(i_{\text{CRAC}}\), now known as the Orai family of proteins, of which there are three mammalian homologues (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 \(i_{\text{CRAC}}\).

**Orais**

Utilizing gene mapping of a family with a severe combined immunodeficiency (SCID) attributed to a loss of \(i_{\text{CRAC}}\) 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 ICRAC, 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 \(i_{\text{CRAC}}\) [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 \(i_{\text{CRAC}}\), 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 bio-physically from native \(i_{\text{CRAC}}\) [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 \(i_{\text{CRAC}}\) 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 \(i_{\text{CRAC}}\), 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 homologues contain putative coiled-coil domains. In Orai1 this region has been shown to be critical for the re-arrangement and colocalization 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 wild-type 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 (CRAC activation domain)) 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
Orai1-activating STIM1 fragment
CCb9

store-operated Ca\(^{2+}\) channels. Interestingly, Orai2 and Orai3 also form functional 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 cell 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

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**Table 1** Reported Orai-activating domains of STIM1

| References       | 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   | 233-450     |
| Kawasaki et al. [58] | Ccb9                      | 339-444     |

In vitro studies on overexpressed 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 overexpression 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\(^{2+}\) selective properties, 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 cell 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.
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 comprises several identifiable functional motifs, including an EF-hand Ca\(^{2+}\) binding domain and a sterile-\(\alpha\) 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] (Fig. 1). It is this recolocalization of STIM1 within the ER network towards 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 PIP2, may account for targeting of STIM1 oligomers to the plasma membrane [61]. However, in a separate report, depletion of plasma membrane PIP2 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 overexpressed Orai1, suggesting that interaction of STIM1 with Orai1 may contribute to STIM1 puncta formation [39]. However, the question of whether STIM1 directly interacts with 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

![Fig. 1 STIM1 and Orai1 colocalize in response to Ca\(^{2+}\) store depletion. (A) eYFP-STIM1 rearranges into near-plasma membrane puncta and colocalizes 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).](image)
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 iPLA2β may function as such a 
linker between STIM1 and Orai1 [69, 70] and although a role for 
iPLA2β 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, which 
then directly activates Orai1 channels [70]. Full analysis of the 
physiological role of Ca\(^{2+}\) influx factor 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 
recently 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 pro-
vided 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 direc-
tion 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 ICRAC [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 asso-
ciation 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 rever-
sal 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 inhibitor ML-9, which was previously shown to 
inhibit SOCE and \(\kappa_{CRAC}\) [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/ICRAC 
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 respon-
ble for SOCE inhibition appears not to be myosin light chain kinase 
and remains unknown [74]. A similar reversal of STIM1 puncta 
was also observed with 2-APB, one of the most commonly 
employed \(\kappa_{CRAC}\) 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 \(\kappa_{CRAC}\) [20]. 
Instead, 2-APB exerts a strong inhibitory effect on the Orai1 chan-
nel 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 interac-
tion between STIM1 and Orai1 that is accentuated when both mole-
ecules are co-expressed in excess.

It was first documented in 1988 [79], and subsequently con-
irmed [80–82], that SOCE is strongly suppressed during cell divi-
sion. This represents one of the only known physiological situa-
tions in which depletion of Ca\(^{2+}\) stores is dissociated from SOCE 
activation, and therefore represents an unparalleled system in 
which to study signalling 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\(^{2+}\) signalling during cell divi-
sion. A recent study has now demonstrated that during mitosis, 
STIM1 phosphorylation is up-regulated 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, res-
cued puncta formation and SOCE in mitotic cells (Fig. 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 nega-
atively 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 func-
tion 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 meiosis 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 signalling pathway has understandably generated a flurry of excitement in the Ca\(^{2+}\) signalling 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 stomatal adhesion of pre-B cells [86]. This function of STIM1 has not been explored further, but interestingly STIM1 was also attributed a tumour-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 (\(I_{\text{Ca,arachidonic acid}}\)), which is similarly activated downstream of PLC-coupled receptors but independently of Ca\(^{2+}\) store depletion. In contrast to \(I_{\text{Ca,arachidonic acid}}\), \(I_{\text{Ca}}\) 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, 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 human beings, 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 \(I_{\text{Ca,arachidonic acid}}\) 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\(^{2+}\) signalling 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 hrs) overexpressing STIM2 [96]. Our laboratory also observed a modest reduction in SOCE when STIM2 was overexpressed in HEK293 cells (24 to 48 hrs) [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 Orai1 [99]. These investigators showed that HEK293 cells expressing both STIM2 and Orai1 exhibit a large and transient \(I_{\text{Ca,arachidonic acid}}\)-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 co-expressing STIM1 and Orai1. Store depletion also generates detectable \(I_{\text{Ca,arachidonic acid}}\)-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
STIM1, STIM2 and Ca\textsuperscript{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\textsuperscript{2+} stores, for example by use of SERCA inhibitors. However, with physiological stimuli, Ca\textsuperscript{2+} stores seldom if ever undergo such massive loss of Ca\textsuperscript{2+}. Rather, the [Ca\textsuperscript{2+}]\textsubscript{i} signals generated with low, physiological levels of receptor agonists most commonly take the form of discrete repetitive discharges of Ca\textsuperscript{2+}, sometimes termed Ca\textsuperscript{2+} oscillations [103, 104]. These oscillations run down in the absence of extracellular Ca\textsuperscript{2+}, indicating a need for Ca\textsuperscript{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\textsuperscript{2+} oscillations appears to involve very small changes in intracellular Ca\textsuperscript{2+} store content [106], it would be expected that STIM2 would play the predominant role in signalling SOCE. However, in one study it was clearly shown that the Ca\textsuperscript{2+} entry supporting muscarinic receptor-driven Ca\textsuperscript{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\textsuperscript{2+} homeostasis by responding to small changes in store content around the resting level [96].

The thresholds for STIM1 is reached in oscillating cells because each oscillation produces a brief drop in ER Ca\textsuperscript{2+} that is sufficient to activate STIM1. In support of this conclusion, in many cells oscillatory movements of STIM1 towards the plasma membrane were observed slightly following, but in close synchrony with cytoplasmic Ca\textsuperscript{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\textsuperscript{2+} oscillations. It is clear that SOCE is necessary for maintaining adequate stores that drive the oscillatory Ca\textsuperscript{2+} release. But more importantly, it is also becoming increasingly clear that Ca\textsuperscript{2+} entry through plasma membrane channels is capable of providing localized Ca\textsuperscript{2+} signals that specifically couple to downstream effecter 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 signalling pathways will not be turned on except when bona fide Ca\textsuperscript{2+} signals are generated, either by high concentrations of agonists that induce substantial Ca\textsuperscript{2+} store depletion, or by low concentrations that induce spikes of Ca\textsuperscript{2+} release underlying Ca\textsuperscript{2+} oscillations. The recently published results of Di Capite et al. [116] demonstrated this concept very well. When mast cells were activated by leukotrienes, Ca\textsuperscript{2+} oscillations were produced, and this led to activation of the early gene, c-fos. However, by use of the technique of ‘Gd\textsuperscript{3+} insulation’ [106, 117–119] that blocks both Ca\textsuperscript{2+} influx and efflux, they showed that the global rise in cytoplasmic Ca\textsuperscript{2+} resulting from each spike or oscillation was not coupled to c-fos activation; rather, influx of Ca\textsuperscript{2+} through the SOCE (ICRAC) channels was absolutely required.

Previous studies have demonstrated the efficiency of Ca\textsuperscript{2+} oscillations as signals for downstream effectors [120–123]. However, the experiments have not generally distinguished the relative roles of Ca\textsuperscript{2+} release and Ca\textsuperscript{2+} entry through SOCE channels. The more recent findings suggest that in many cases it is the Ca\textsuperscript{2+} entering through the SOCE channels that is the primary source of signalling Ca\textsuperscript{2+}, and that in such cases the function of the oscillatory discharges of Ca\textsuperscript{2+} is to produce a sufficiently ample drop in ER Ca\textsuperscript{2+} levels to activate the signalling Ca\textsuperscript{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 (E$^{637}$D$^{640}$) and positively charged lysine residues in STIM1 (K$^{684}$K$^{685}$), 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 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 TRPC1 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*$_{SOCE}$ 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 TRPC1 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 MsCD 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 SOCE 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. 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 TG treatment [136]. These observations imply the possibility that TRPC1 functions as an intracellular Ca$^{2+}$ channel [134] and through this function, it might modulate 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$_3$R [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 pipette, 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 (Fig. 2). With hindsight this is perhaps not surprising given the rather unique nature of both the signalling mechanism, and channel properties associated with SOCE and $I_{\text{CRAC}}$. 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.

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**Conflict of interest**

The authors confirm that there are no conflicts of interest.

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