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STIM AND ORAI, THE LONG AWAITED CONSTITUENTS OF STORE-OPERATED CALCIUM ENTRY

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Abstract

Rapid changes in cytosolic Ca^{2+} concentrations $[\text{Ca}^{2+}]_i$ are the most commonly used signals in biology to regulate a whole host of cellular functions including contraction, secretion and gene activation. A widely utilized form of Ca^{2+} influx is termed store-operated Ca^{2+} entry (SOCE) due to its control by the Ca^{2+} content of the endoplasmic reticulum (ER). The underlying molecular mechanism of SOCE has eluded identification until recently when two groups of proteins, the ER Ca^{2+} sensors, STIM1 and -2, and the plasma membrane channels, Orai1, -2 and -3 have been identified. These landmark discoveries have allowed impressive progress in clarifying how these proteins work in concert and what developmental and cellular processes require their participation most. As we begin to better understand the biology of the STIM and Orai proteins, the attention to the pharmacological tools to influence their functions quickly follow suit. This review will briefly summarize recent developments in this exciting area of Ca^{2+} signaling.

I. Introduction

A select group of stimuli initiate cellular responses by acting on cell surface receptors to increase the cytosolic Ca^{2+} concentration. By the mid 1980s it was understood that stimulation of these receptors induces the hydrolysis of membrane phosphoinositides by phospholipase C (PLC) enzymes yielding the diffusible Ca^{2+} -mobilizing messenger, inositol 1,4,5-trisphosphate (InsP_3), to release Ca^{2+} from non-mitochondrial Ca^{2+} stores¹. However, Ca^{2+} release is also followed by a stimulated Ca^{2+} entry in such cells and in 1986 James Putney proposed that the ultimate reason for the Ca^{2+} influx is a decrease in the Ca^{2+} content of the endoplasmic reticulum (ER) introducing the concept of capacitative- or store-operated Ca^{2+} entry (SOCE)². The mechanism by which the ER Ca^{2+} stores communicate with the plasma membrane (PM) and the molecules participating in the Ca^{2+} influx process remained elusive until 3 years ago, when suddenly a series of key discoveries identified the STIM and Orai/CRACM proteins that serve as ER calcium sensors and calcium channels, respectively (see Box-1 for a brief historical overview). Several excellent reviews have summarized the exciting early developments on this research area in more detail^{3–6}. Here we describe the main features and the cell biology of these two classes of molecules as well as their biological significance. We also highlight some of the pharmacological means by which to manipulate the functions of these proteins.

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II. STIM1 is the ER Ca²⁺ sensor

STIM1 and -2 had been described and characterized before their roles in Ca²⁺ signaling were recognized. STIM (Stromal Interaction Molecule) was first identified as a cell surface molecule that mediates cell-stromal interactions acting as a recessive tumor suppressor gene⁷. STIM1 and its homologue, STIM2, are type-I membrane proteins with a luminal helix-turn-helix EF-hand Ca²⁺ sensing module followed by a sterile alpha motif (SAM) before the single transmembrane segment (see Figure 1 for the structural features of STIM proteins). STIM1 is localized to the tubular ER in quiescent cells^{8–10}, although it is also found in the PM^{7,11}. STIM1 is N-glycosylated within its luminal domain and indirect evidence suggest that N-glycosylation is required for its PM delivery^{12,13}. The most striking feature of STIM1 is its rapid clustering and relocation to PM-adjacent ER regions upon store depletion^{8,10,14} (Fig. 1). This “puncta formation” is initiated by decreased binding of Ca²⁺ to the EF-hand, causing the oligomerization of STIM1 followed by translocation of the multimers to membrane-adjacent ER areas where STIM1 can activate Ca²⁺ influx^{15,16}. Oligomerization of the isolated luminal domains of STIM1 is regulated by decreased luminal Ca²⁺ binding to the EF hand with a K_{1/2} of ~200–600 μM¹⁵. However, the whole process of Ca²⁺ unbinding, oligomerization and redistribution of STIM1 has a Ca²⁺ sensitivity of K_{1/2} of 180–210 μM^{17, 18} and a Hill-coefficient of ~4¹⁷ or 8¹⁸. Mutations within the EF hand that decrease Ca²⁺ binding localize STIM1 constitutively to the membrane-adjacent ER and activate SOCE^{8,9}. A recent elegant study used a drug-inducible heterodimerization approach to show that oligomerization of STIM1 is necessary and sufficient to activate Ca²⁺ influx¹⁷. Initially, it was suggested that STIM1 is also inserted in the PM upon store depletion⁹, but this was not confirmed in other studies^{8,10,19} and ER-localized STIM1 is sufficient for activation of SOCE^{13,14}. The STIM1 constructs luminally tagged with fluorescent proteins do not reach the PM^{8,10,19}. The PM pool of STIM1 was visualized with an N-terminally hexahistidine tagged STIM1 and a membrane impermeable small fluorescent chelator²⁰. The functional significance of the PM-localized STIM1 is controversial as antibodies targeting external epitopes of STIM1 were found to attenuate¹¹, or not to change¹³ SOCE upon store depletion.

Initial reports showed that STIM2 was also present in the ER but not the PM, and that it did not show puncta formation upon store depletion. In fact, STIM2 expression inhibited SOCE and only after co-expression with STIM1 was STIM2 able to cluster suggesting that it forms heteromeric complexes with STIM1²¹. In contrast, recent studies revealed a significantly reduced basal cytoplasmic Ca²⁺ concentration in STIM2-depleted cells and suggested that STIM2 controls basal cytoplasmic and ER Ca²⁺ levels¹⁸. This study showed that STIM2 does cluster and translocate to the ER-PM junctions and responds to significantly smaller ER-Ca²⁺ depletion (K_{1/2} ~ 400 μM) than STIM1¹⁸. The higher sensitivity of STIM2 to Ca²⁺ depletion could be partially related to the Ca²⁺ binding properties of its EF hand¹⁸. The EF-hands (with the SAM motifs) of STIM1 and -2 showed roughly comparable Ca²⁺ affinities, yet displayed different oligomerization properties²². According to recent studies, STIM2 has two modes of operation one dependent on store depletion, the other regulated by interaction with (and dissociation from) calmodulin²³.

III. The Orai/CRACM proteins form the channel pore

The predicted architecture of the integral membrane proteins, Orai1/CRACM1 (Fig. 2) already raised the possibility that they might be themselves the channel components of SOCE^{24–26}. This was unequivocally proven by mutational analysis that showed that the properties of the I_{CRAC} currents are determined by the Orai1 protein^{27–29}. The three Orai proteins display notable differences in their features despite a high degree of sequence similarity (Fig. 2). Orai1 is the most potent to reconstitute Ca²⁺ influx in most cells, and its

depletion has the highest impact on SOCE^{19,30}. Expression of Orai1 or Orai2 yields Ca²⁺ selective I_{CRAC} currents, while Orai3 results in small and slowly developing I_{CRAC}^{31,32}. Cross-linking studies suggest that Orai proteins form dimers in the resting state^{30,33}. However, the active pore consists of four Orai molecules^{34,35} that are induced by interaction with the cytoplasmic domain of STIM1³³. It is likely that the three Orai proteins form heteromeric channels with distinct properties.

Interestingly, in most studies expression of Orai1 alone significantly inhibits SOCE and only when expressed together with STIM1 does it induce a huge Ca²⁺ influx or manifest as an I_{CRAC} channel^{19,36,37}. Similar inhibitory effects were described also for the Orai2 but not the Orai3 protein¹⁹. This suggests a fine stoichiometry between Orai1, STIM1 and possibly other molecular components to determine the extent of Ca²⁺ entry. The most striking and distinctive feature of the Orai3 protein is its response to the drug 2-APB as it shows a store- and STIM-independent activation with a simultaneous change in its gating specificity (see details below).

IV. Interaction between STIM and Orai proteins

The Orai1 protein located in the PM shows a uniform PM distribution in resting cells but rapidly clusters after store depletion, and co-localizes with STIM1 puncta suggesting an interaction between the two proteins^{38–40}. The parsimonious assumption is that STIM1 proteins in the ER oligomerize once ER Ca²⁺ levels drop and they cluster the Orai1 proteins in the PM. This interaction then stabilizes the junctional sites between the ER and the PM (Fig. 3). Yet, direct interaction between Orai1 and STIM1 has been a controversial issue^{28–30}. Our recent studies in COS-7 cells also raised the possibility that Orai1 associates with a larger protein complex within the PM and this multiprotein platform could be the site where the two proteins interact (directly or indirectly)⁴⁰ (Fig. 3B). Physiological levels of agonist stimulation⁴⁰ or low concentrations of Tg⁴¹ affect the peripheral ER Ca²⁺ stores, which are also very efficiently refilled by the SERCA pump⁴², suggesting the existence of preferential ER sites where these interactions happen.

The C-terminal polybasic domains of clustered STIM could facilitate interaction with negatively charged phospholipids and hence facilitate the interaction with the PM¹⁶. Indeed, a polybasic-less STIM1 (Δ 672–685) was reported to cluster in the ER without translocation to the PM-adjacent regions¹⁶. However, deletion of the same polybasic domain (Δ 672–685) in other studies only slowed but did not eliminate I_{CRAC} activation⁴³. A larger deletion mutant (Δ M597–685) lacking the proline-rich region and putative phosphorylation sites still allowed I_{CRAC} activation although with altered inactivation properties¹¹. This could be consistent with recent findings that showed Ca²⁺-dependent calmodulin binding to peptides corresponding to the polybasic tails of both STIM1 and -2, suggesting Ca²⁺-feed-back regulation on STIM1⁴⁴. Deletions beyond the serine-proline rich region of STIM1 (Δ 391–685)¹⁴ and (Δ 425–685)⁴³ eliminated the clustering response and the ability of STIM1 to activate Ca²⁺ influx. Moreover, proline substitutions in the long coil-coil domain (L286P/L292P) also prevented STIM1 from activating I_{CRAC}, while still allowing puncta formation after store depletion⁴⁵. Stabilization of STIM1 PM interaction by PtdIns(4,5)P₂ (presumably via binding to the polybasic domain) was not confirmed as rapid PM PtdIns(4,5)P₂ removal neither prevented STIM1 movements toward the PM⁴⁰ nor did it affect Tg-induced Ca²⁺ elevations⁴⁶. The *Drosophila* STIM1 protein ends at what corresponds to position 499 of human STIM1 lacking a large part of the C-terminus including the polybasic domain (Fig. 1). This indicates that the core Ca²⁺ influx machinery probably works without large portions of the C-terminus but requires the 238–499 coiled-coil domain, which shows significant conservation among all STIM proteins. Soluble STIM1 constructs containing the entire cytoplasmic domain can activate Orai1-mediated Ca²⁺ influx or I_{CRAC}^{47–50}. Recent studies

using only the cytoplasmic segment of STIM1 suggested that electrostatic interactions mediated by the STIM1 polybasic tail are important for TRPC1 (see below) but not Orai1 activation⁵⁰.

In the case of Orai1, truncation of the first 73 residues ($\Delta 1-73$) decreased but did not eliminate store-operated Ca^{2+} entry⁴³. A larger deletion ($\Delta 1-88$), however, rendered Orai1 unable to support Ca^{2+} influx, yet it still showed clustering with the STIM1 protein after store depletion^{43,48}. In contrast, deletion of the C-terminal segment in the cytoplasmic tail (proposed but not yet proven to be a coiled-coiled domain) prevented Orai1 association with STIM1 and activation of the channel^{43,48}. A critical residue (L273) within this region was identified as essential for interaction and activation by STIM1⁴⁸. In the same studies a direct association was found between the soluble entire cytoplasmic C-terminus of STIM1 and the C-terminal (but not N-terminal) fragment of Orai1, also suggesting a direct association between these regions of the two proteins (Fig. 2).

Few studies are available on the distribution and movements of STIM1 and Orai1 proteins in natural lymphocytes or mast cells as these cells are either small or hard to obtain and transfect. Nevertheless, the formation of STIM1 puncta and Orai1 clustering have been reported in T-cells⁹, pre-B (DT40)¹⁴ and Jurkat T cells³⁹. In addition, recent studies showed the concentration of the activated STIM1/Orai1 complexes in the immunological synapse⁵¹, and curiously enough, these proteins also formed a prominent “distal cap” in the rear-end of the T-cells relative to the activation of their T-cell receptors⁵². These interesting observations suggest that the spatial organization of the Ca^{2+} signal mediated by the concentration of the STIM1/Orai1 machinery in specific membrane areas could have important consequences to Ca^{2+} signaling of polarized cells.

STIM and the cytoskeleton

Overexpressed STIM1 associates with microtubules in several cell types^{14,53} and STIM1 (and -2) have recently been shown to be binding-partners of the microtubule plus-end tracking protein, EB1⁵⁴. Therefore, STIM1 contributes to the shaping of the peripheral tubular ER along the growing microtubules, constantly dragging ER regions to the proximity of the PM (Fig. 3). Store depletion disrupts microtubular association and STIM1 switches to its PM binding partner (Orai1 or another, yet to be identified molecule) during puncta formation⁵⁴. Conversely, disruption of microtubules with nocodazole promotes STIM1-PM association⁵³. STIM1-microtubule association, however, is only a subtle regulatory mechanism, as the microtubule inhibitory agent, nocodazole, only partially interferes with endogenous SOCE and it is without effect in cells overexpressing STIM1^{14,39,53}.

A series of studies have suggested that the actin cytoskeleton had an important role in the control of SOCE and that the cortical actin had to be broken down in order to allow activation of SOCE⁵⁵, but a similar mechanism was not found in T cells⁵⁶ and RBL-1 cells⁵⁷. Also, the number of ER-PM junctions did not change after store depletion¹⁰ indicating that SOCE can be activated without major remodeling of the ER-PM junctions including the actin cytoskeleton. Moreover, STIM1 translocation from the tubular to the juxta-plasmamembrane ER and activation of Orai1/STIM1 mediated Ca^{2+} entry occurs even in the presence of blockers of actin polymerization³⁹, suggesting that actin is not required for the movements and clustering of these molecules but in some cell types it still could present a barrier interfering with the STIM1/Orai1 interactions.

Connection to TRP or other channels

Significant amount of data suggest that TRPC channels can mediate receptor-activated Ca^{2+} influx⁵⁸. Since some TRPC channels respond to depletion of intracellular Ca^{2+} stores⁵⁹, the

role of STIM1 in this process has been investigated. An interaction between STIM1 and the endogenously expressed TRPC1 was found in activated human platelets⁶⁰. In another study the isolated cytoplasmic domain of STIM1 could bind to some but not all TRPC channels⁴⁷. In these studies, soluble STIM1 was able to activate TRPC1 and TRPC4 channels, and knock-down of STIM1 directly inhibited TRPC1, TRPC4 and TRPC5 but not TRPC7⁴⁷. A functional interaction between endogenous TRPC1 and STIM1 was described in vascular smooth muscle cells, where knockdown of TRPC1 decreased SOCE activated by a constitutively active form of STIM1⁶¹. The physical and functional interaction between Orai and TRPC channels was recently shown by two independent laboratories^{62,63} raising the possibility that this association could add to the functional variety of SOCE. The role of lipid rafts in the formation of STIM1/TRPC1 complexes and regulation of SOCE has also been suggested recently⁶⁴.

Arachidonic acid-regulated Ca^{2+} channels (ARC) are a store-independent form of Ca^{2+} entry activated at low agonists stimulation⁶⁵. Although ARC regulation is distinct from that of I_{CRAC} channels, the biophysical properties of the two channels bear several similarities⁶⁵. Recent studies suggested that STIM1 also plays a role in the regulation of ARC. Depletion of endogenous STIM1 with simultaneous expression of a glycosylation-deficient mutant STIM1 (presumably eliminating the cell surface fraction of STIM1) greatly inhibited ARC but not I_{CRAC} , suggesting a role of PM STIM1 in the ARC activation process¹³ and pointing to its independence of the ER Ca^{2+} stores. In addition to STIM1, it was also suggested that ARC uses Orai1/Orai3 heteromers as its channel component⁶⁶. Whether ARC channels are a general feature of all cells and how arachidonic acid activates their opening are questions that need to be further explored.

V. Life without STIM and Orai proteins

Analysis of SCID patients carrying the R91W mutation has already indicated that Orai1 is not an essential protein, although critically important for proper immune cells functions⁶⁷. During T cell activation, the cytoplasmic Ca^{2+} increase triggers the dephosphorylation and nuclear translocation of the NFAT1 transcription factor (nuclear receptor for activated T-cells) by the Ca^{2+} dependent phosphatase, calcineurin⁶⁸. Apparently, this process relies upon Ca^{2+} influx supported by SOCE. The mild extraimmunological symptoms of SCID patients, such as non-progressive muscular dysplasia and a lack of sweat glands, suggested additional roles for Orai1 in skeletal muscle and ectodermal tissue physiology⁶⁹. Complete elimination of Orai1 causes perinatal lethality in C57BL/6, but not in outbred ICR mice⁷⁰. One study using a gene-trapping method reported severely impaired Ca^{2+} influx and degranulation responses in mast cells and a greatly suppressed anaphylactic reaction⁷¹. This study reported no T-cell developmental defects but an impaired cytokine secretory response. However, mice with targeted deletion of Orai1 are immunodeficient, showing a strong defect in SOCE in B cells and T cells but normal lymphocyte development⁷⁰. They also display mast cell defects and ectodermal symptoms such as hair loss and eyelid irritation⁷⁰. The differences between the two studies could be due to the possibility that the gene-trapped mice are hypomorphic and can express a low level of Orai1 protein in some tissues⁷⁰.

Elimination of STIM1 yields smaller mice with perinatal lethality of unknown reason. One gene-trapping study reported impaired skeletal muscle development and function, hypotonia and congenital myopathy. These defects correlated with the disappearance of I_{CRAC} from skeletal muscle membrane and with decreased expression of the SERCA1 ATPase⁷². The role of STIM1 in skeletal muscle development suggests that NFAT regulation in the developing myotube is also based on activation of SOCE⁷². In other studies, mast cells developed from fetal liver of STIM1^{-/-} mouse⁷³ or T-cells with conditionally deleted STIM1⁷⁴ showed greatly reduced SOCE after activation but their development was not

affected. STIM1-deleted T-cells showed similar defects to those observed in SCID patients: the lack of SOCE and I_{CRAC} , a small and transient NFAT1 nuclear translocation, impaired cytokine production and proliferation but normal differentiation from thymic precursors⁷⁴. In contrast, STIM2 deficient $CD4^+$ T-cells showed only slightly reduced Ca^{2+} influx and I_{CRAC} , but a relatively larger decrease in cytokine production in response to antigen stimulation⁷⁴ consistent with the more important role of STIM1 than STIM2 in T-cell activation. The combined elimination of STIM1 and -2 from $CD4^+/CD8^+$ thymic precursors has revealed an unexpected effect in T-cell development. These animals developed splenomegaly, lymphadenopathy and an autoreactive phenotype that was apparently due to a decreased number of autoregulatory T cells⁷⁴. This exciting novel finding exposed the potential link of SOCE with autoimmune diseases.

An activating mutation within the EF-hand of STIM1 in a mouse strain caused severe thrombopenia, and severe bleeding, apparently due to a reduced life span of platelets. However, T-cell development and functions were normal in the heterozygote animals⁷⁵. As expected, platelets from mutant animals showed elevated basal Ca^{2+} level and a larger but transient Tg-response. Surprisingly, these platelets showed a greatly reduced Ca^{2+} response to engagement of the collagen receptor (GPVI) but normal response to stimulation of G protein-coupled receptors. This complex Ca^{2+} signaling defect cannot be easily explained with the simple model of STIM1 regulation of SOCE and raises interesting questions on the more complex role of STIM1 in platelets (such as regulation of TRPC1 channels⁶⁰). Remarkably, complete elimination of STIM1 caused major defects in the Ca^{2+} responses of platelets to all activating agonists but this translated to an impaired aggregation response only in response to collagen but not thrombin or ATP. Deletion of STIM1 protected animals from arterial thrombosis with only minor effects on bleeding time offering a strategy for selective protection from diseases caused by increased thrombus formation⁷⁶.

VI. Inhibitors of the STIM1 and Orai1 protein-mediated Ca^{2+} influx pathways

Divalent cations and trivalent lanthanides have long been known to inhibit Ca^{2+} entry pathways. Among these, La^{3+} (IC_{50} : 10–100 μM) and Gd^{3+} (IC_{50} : < 1.0 μM) have been most widely used. However, these ions inhibit a wide range of Ca^{2+} entry channels and their apparent selectivity displayed in a narrow concentration range could depend not only on the channel itself but also on the activation mechanism of Ca^{2+} entry⁷⁷. Therefore, several chemical inhibitors of SOCE have been introduced over the years⁷⁸.

2-APB

2-APB (2-aminoethoxydiphenyl-borate) was initially introduced as an $InsP_3$ receptor-channel inhibitor⁷⁹. In the course of subsequent studies the actions of 2-APB on $InsP_3$ -mediated Ca^{2+} release are still debated but its inhibitory effects on SOCE have become established⁸⁰. 2-APB stimulates I_{CRAC} at low (less than 10 μM) and inhibits it at higher (up to 50 μM) concentrations⁸¹. This dual regulation is also observed with expressed STIM1/Orai1^{82,83}. Higher concentrations of 2-APB also reverse the store-depletion-induced clustering of STIM1 and the activation of Orai1 but this effect largely depends on the expression level of these proteins^{82,83}.

A remarkable “paradoxical” stimulatory effect of higher concentrations (50 μM) of 2-APB was reported on cytosolic Ca^{2+} levels³⁶ or I_{CRAC} ²³ in STIM2/Orai1 expressing cells. Curiously, G418, the selection antibiotic often used to maintain stable transfected cell lines, greatly suppressed the STIM2/Orai1 current²³. The activation kinetics of the 2-APB-activated I_{CRAC} current is much faster and only transient after drug application, and is independent of store depletion, although the latter also activates it with slower kinetics²³. This study suggested that 2-APB relieves STIM2 from inhibition by a cellular factor that

was identified as calmodulin. Furthermore, Orai3 shows a strong activation after high concentrations of 2-APB, apparently independent of STIM1 or store-depletion^{49,82–84}. Not only do Orai3 channels open after 2-APB treatment, but their ion selectivity changes as they show very high conductance at positive potentials³¹. These data suggest that 2-APB directly binds to the channel and dilates its permeability pore both increasing its conductance and limiting its selectivity⁸³. This does not happen to the Orai1 and -2 channels, but a mutation within the pore domain of Orai1 (E106D and E190A) enables 2-APB to activate it similarly to Orai3 supporting a direct binding of 2-APB to the channels. The same study also suggested that the inhibitory effects of 2-APB, in contrast, reflect an indirect action of the drug on the STIM1 protein⁸³.

In spite of the great value of 2-APB in deciphering the workings of the Orai1/STIM1 activation process, 2-APB should not be viewed as a specific inhibitor (activator) of SOCE as it was reported to inhibit (and even activate) several other calcium channels as recently reviewed in detail⁸⁵. In an effort to obtain more specific compounds, a series of 2-APB analogues have been tested for effects on SOCE and InsP₃-mediated Ca²⁺ increases⁸⁶. Some of these compounds showed significantly improved selectivity between these two processes, but more studies will be needed to test these compounds on the various forms of Ca²⁺ entry pathways.

SKF-96365

This imidazole compound (1-[2-(4-Methoxyphenyl)-2-[3-(4-methoxyphenyl)propoxy]ethyl-1H-imidazole hydrochloride) was introduced as an inhibitor of receptor-mediated Ca²⁺ influx⁸⁷. It was shown to inhibit SOCE and I_{CRAC} in the 20–30 μ M range in several cell lines^{88–90}. Other imidazole derivatives, such as econazole, miconazole and clotrimazole, also known for their inhibition of cytochrome P450, all inhibit SOCE (see⁸⁵ for more details). However, SKF-96365 has been shown to inhibit other channels, such as TRPs⁹¹, voltage gated Ca²⁺ channels⁸⁷, potassium channels⁹². Other derivatives, such as calmidazolium, elevate basal Ca²⁺, inhibit Ca²⁺ ATPases, and are considered as a calmodulin inhibitors⁸⁵. Most of these compounds have not been tested directly on pure Orai1/STIM1-mediated Ca²⁺ entry, but SKF-96365 inhibited STIM1-mediated Ca²⁺ influx⁸ and the nuclear translocation and activation of the NFAT transcription factor in cells expressing a constitutively active (EF-hand mutant) STIM1⁴⁷. This suggests that SKF-96365 does target the STIM1/Orai1 pathway. Because of their limited specificity among Ca²⁺ influx channels, these compounds are less likely to attract the same attention as 2-APB or ML-9 (below) and more studies are needed to evaluate their site of action in the STIM1/Orai1 activation process.

ML-9

ML-9 [1-(5-chloronaphthalene-1-sulfonyl)homopiperazine, HCl], a potent myosin light chain kinase (MLCK) inhibitor, inhibits SOCE in a variety of cell lines⁹³. Other MLCK inhibitors, such as ML-7 and wortmannin as well as knock down of MLCK was also shown to inhibit agonist or Tg-induced Ca²⁺ increases in smooth muscle and endothelial cells⁹⁴, leading to the conclusion that MLCK is important for SOCE. In a recent study, ML-9 reversed with remarkable speed the puncta formation and subplasmalemmal translocation of STIM1 that follows store depletion leading to the inhibition of Ca²⁺ influx⁵³. In ML-9 treated cells, STIM1 and its constitutively active EF mutant loses PM attachment and associates with the microtubules, therefore, ML-9 seems to inhibit STIM1 interaction with the PM. Notably, the potency of ML-9 to reverse Ca²⁺ elevations and STIM1 distribution is reduced with overexpression of STIM1⁵³. In the same study, inhibition of MLCK by other inhibitors (such as μ M concentrations of wortmannin) or RNAi mediated knock-down of the various MLCK isoforms failed to mimic the effect of ML-9 suggesting that MLCK is not the

relevant target of ML-9 in this case⁵³. Given the numerous phosphorylation sites present in STIM1 and -2, it is possible that constitutive phosphorylation by an ML-9-sensitive kinase is necessary for STIM1 function but a direct interaction of ML-9 with STIM1 or an interacting protein, such as calmodulin, cannot be ruled out. It is also important to note that the inhibition by ML-9 is not unique to the STIM1/Orai1 mechanism, as an MLCK-independent inhibition of TRPC6 channels⁹⁵ and MLCK-dependent trafficking of TRPC5 channels have also been described⁹⁶.

Bistrifluoromethyl-pyrazole derivative, BTP2

BTP2 has been identified as a strong immunosuppressant that inhibits IL-2 production, NFAT-dependent transcription and SOCE in lymphocytes⁹⁷. While this compound has been considered quite potent (IC_{50} ~10 nM) and specific, its effect on I_{CRAC} required longer time (~ 2h or more) to develop⁹⁸. In other studies BTP2 showed very potent acute stimulatory effect on the non-selective cation channel, TRPM4 and it was suggested that the drug affected SOCE and downstream events because of its depolarizing effects⁹⁹. BTP2 also blocked SOCE and TRPC3 and -5 but not TRPV6 channels with an (IC_{50} ~0.1–0.3 μ M)¹⁰⁰ questioning its specificity for I_{CRAC} channels. To improve the potency and selectivity, a series of BTP2 derivatives have been synthesized and compared in SOCE and voltage-gated channels¹⁰¹. These compounds will have to be tested on I_{CRAC} and other channels such as the TRP family to better understand their targets and mechanism of action.

In addition to these channel blockers, it has been found that inhibitors of phospholipase C and polyphosphoinositide synthesis potently inhibited SOCE and I_{CRAC} ¹⁰². Remarkably, the PLC inhibitor was only effective when added before but not after store depletion. These effects were found unrelated to $InsP_3$ formation or action, raising the possibility that phosphoinositides could directly affect the I_{CRAC} activation mechanism.

VII. Outstanding questions and future directions

A number of questions remain to be clarified, the most immediate ones related to the molecular determinants mediating the interactions between the STIM and Orai proteins. Additionally, soluble active STIM1 components capable of activating the Orai1 channels may allow a more direct manipulation and analysis of the electrical properties of these channels in excised membranes. More complex questions include whether specific areas of the ER are preferentially coupled to SOCE with special $InsP_3$ receptors regulating their Ca^{2+} content. These specialized areas of the ER could be analogous to the terminal cisternae of the sarcoplasmic reticulum of striated muscle. Keeping with this analogy, additional protein components may participate in the “conformational coupling” between the STIM1 and Orai1 proteins. The role of the PM localized STIM1 also needs further attention, even though it is dispensable for SOCE. However, STIM1 may have other functions in cell to cell or matrix to cell communication as suggested by the very early observations leading to STIM1 discovery¹⁰³. The comparison of the STIM1 and -2 proteins also holds additional mysteries. What is the advantage for a cell of having two sensors working in two ranges of ER Ca^{2+} concentration to set basal and stimulated Ca^{2+} entry separately? Why is then a T-cell lacking STIM2 able to respond only transiently with NFAT1 nuclear translocation in spite of a fairly preserved Ca^{2+} influx response? Why is STIM1 less important for lymphoid development than for mature T-cell activation? The Orai proteins will also have to be further explored to define roles of the Orai2 and -3 proteins in development and in differentiated tissues. What parts of the channels are responsible for the different electrophysiological or inhibitory (activation) behavior? Studies addressing this latter question with chimeric constructs have already started to surface⁴⁹. The relationship and importance of the TRPC channels in the functions of the Orai1 and STIM1 proteins are also an area of intense exploration as is the identity and source of the elusive calcium influx factor, CIF¹⁰⁴.

The practical benefits of STIM/Orai research are clear in light of the uniquely important roles of the STIM1/Orai proteins in lymphocyte, mast cell and platelet biology. Targeting these proteins with selective drugs is a sensible strategy in developing anti-inflammatory, immune suppressant or anti-allergic drugs. The benefits from new drugs treating these conditions could be enormous. It took 20 years to find the molecules responsible for SOCE and advances in many related fields helped it to happen. It is our hope that realizing the potentials of these great discoveries will take a significantly shorter time.

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GLOSSARY

ARC	arachidonic acid-regulated Ca^{2+} channel
2-APB	2-aminoethoxydiphenyl-borate
CIF	Ca^{2+} influx factor, a putative diffusible molecule that is liberated when the ER Ca^{2+} store becomes depleted and activates a Ca^{2+} influx pathway Cortical actin is the fraction of polymerized actin formed beneath the plasma membrane
CRAC	Ca^{2+} release activated channel, the electrophysiological correlate of STIM/Orai mediated Ca^{2+} entry
EB1	microtubule plus-end tracking protein. Microtubule plus-end tracking proteins accumulate at the distal end of growing microtubules
ER	endoplasmic reticulum
Gene-trapping	a method by which a cassette containing a selectable genetic marker is inserted in an intron of a gene such that the original transcript encoding the protein of interest is replaced by one coding for a truncated and dysfunctional fusion protein that can be easily detected
InsP₃	inositol 1,4,5-trisphosphate
ML-9	1-(5-chloronaphthalene-1-sulfonyl)homopiperazine, HCl
MLCK	myosin light chain kinase
NFAT	nuclear receptor for activated T-cells
PLC	phospholipase C
PM	plasma membrane
PtdIns(4,5)P₂	phosphatidylinositol 4,5 bisphosphate
SCID	severe combined immunodeficiency
SERCA	sarco- and endoplasmic reticulum Ca^{2+} pump
SKF-96365	1-[2-(4-Methoxyphenyl)-2-[3-(4-methoxyphenyl)propoxy]ethyl]-1H-imidazole hydrochloride)
SOCE	store-operated Ca^{2+} entry
STIM1	stromal interaction molecule 1

Tg	thapsigargin, a drug isolated from the roots of the mediterranean plant <i>Thapsia garganica</i> that selectively inhibits the ER Ca^{2+} pump leading to the depletion of the ER Ca^{2+} stores and increased Ca^{2+} influx without any receptor stimulation
Tg response	the cytoplasmic Ca^{2+} elevation induced by the addition of Tg
TRP	transient receptor potential

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Text BOX-1**A brief history of SOCE**

After the concept of SOCE had been introduced, much research was focused on the mechanism by which the ER Ca^{2+} status was communicated to the plasma membrane. The most attractive idea, known as the “conformational coupling” model, assumed that a physical contact exists between the plasma membrane and peripheral elements of the ER where proteins sensing the ER luminal Ca^{2+} concentration would transfer the information to a Ca^{2+} channel in the plasma membrane¹⁰⁵. Since InsP_3 receptors in the ER have a large flexible N-terminal domain that could, in theory, contact the plasma membrane, and because Ca^{2+} influx is tightly coupled to InsP_3 -induced Ca^{2+} release, the InsP_3 receptor seemed like a good candidate to serve as a coupling molecule. This was later discounted based on clear evidence that InsP_3 receptor-deficient cells still showed functional SOCE and I_{CRAC} ^{81,106,107}. Another coupling model was developed around a soluble diffusible factor that was isolated and named CIF (Ca^{2+} influx factor)¹⁰⁸, although its molecular nature and mechanism of action still wait identification¹⁰⁴. Concerning the nature of the Ca^{2+} channel, most of the last 20 years was dominated by studies on a group of non-selective cation channels, the TRP channels. The first TRP channel was identified in the *Drosophila* eye as a protein responsible for a characteristic light-induced membrane potential change (the acronym comes from: transient receptor potential)(see⁵⁹ for a comprehensive review on SOCE and TRP channels).

A major advance came with the description of a highly Ca^{2+} -selective current named I_{CRAC} in patch clamp recording from mast cells¹⁰⁹ and T-cells¹¹⁰ that was activated by store depletion and was designated as the electrophysiological correlate of SOCE. Another milestone was the discovery that a few patients presenting with an inherited form of severe combined immunodeficiency (SCID) had T cells that lacked SOCE and I_{CRAC} ^{67,111}. The real breakthrough, however, was brought about by the large-scale use of small interfering RNA (RNAi) for gene silencing, and its adaptation for functional screens targeting SOCE. Two such studies simultaneously identified STIM1 as a necessary component of SOCE and noted that this protein and its sister, STIM2, serve as sensors of the ER luminal Ca^{2+} concentration^{8,112}. Nonetheless, it was clear that the single membrane-spanning STIM proteins were not structurally suited as Ca^{2+} channels. Within a year, the protein Orai1 was identified as the mutated culprit causing the T-cell defect²⁴, and RNAi screens discovered the same protein (there named CRACM1) as a molecule required for I_{CRAC} ^{25,26}. STIM1 and Orai1/CRACM1 together reconstituted huge SOCE and I_{CRAC} ^{19,36,37,39} suggesting that the question concerning the nature of the elusive store-operated entry pathway has finally been settled.

Figure 1. Structural features and cellular localization of the STIM proteins

Comparison of Drosophila dSTIM (NM_078633) and the human hSTIM1 (NM_003156) and hSTIM2 (NM_020860) proteins show high degree of conservation (A). The luminal EF hand (EF) is responsible for Ca²⁺ binding while the sterile-alpha motifs (SAM) contributes to oligomerization¹¹³ and contains N-glycosylation sites (two in STIM1 and one in STIM2) (yellow circles). The single transmembrane domain (TM) separates the luminal and cytoplasmic domains. The cytoplasmic segment starts out with a longer and shorter stretch of coiled-coil domains (orange) and additional highly conserved regions (blue) that end with a highly acidic stretch (D). In addition the STIM2 protein contains another highly acidic proline-histidine-rich region (P/H/E) while STIM1 contains a serine-proline-rich domain (S/P). Both STIM1 and STIM2 have a polybasic sequence at their C-termini (K). The total amino acid number calculated from the ORF in hSTIM2 in the above GenBank entry is 833 but it was determined that the human STIM2 protein uses a non-standard non-AUG start site (corresponding to Leu88 downstream from the first methionine)⁷. Numbering here reflects the domain distribution of this 746 amino acid length protein. (B) Confocal images of COS-7 cells expressing low level of YFP-STIM1. Stimulation of the cell with a Ca²⁺ mobilizing agonist (ATP) induces puncta formation of STIM1 primarily at peripheral regions of the cell. More drastic and generalized depletion of ER Ca²⁺ pool by thapsigargin treatment (Tg) led to the complete translocation of all STIM1 proteins to peripheral puncta. (C) Higher expression of YFP-STIM1 resulted in the appearance of STIM patches developed without pool depletion. (D) Microtubular localization of YFP-STIM1, noticed in many studies, is due to the interaction between STIM1 and the microtubule positive end-tracking protein, EB1. (E) TIRF analysis of YFP-STIM1 showed the appearance of the protein complex in the close proximity of the plasma membrane upon store depletion (ATP/Tg). See text for original citations.

Figure 2. Structural features of the Orai/CRACM proteins

The Orai1/CRACM1 channels have four putative transmembrane domains both N- and C-termini facing the cytoplasm. It has been determined that a functional channel is built from 4 Orai1 molecules (not shown in the Figure). The channels are found evenly distributed in the plasma membrane in quiescent cells but show clustering (and co-localization with STIM1) after ER store depletion evoked by thapsigargin (Tg). Alignment of Orai1 (BC015369), Orai2 (BC069270) and Orai3 (BC022786) shows the high degree of conservation in the acidic residues (D110 and D112 and to a lesser degree D114 in human Orai1) located within the 9 amino acid stretch located in the extracellular loop between the 1st and 2nd transmembrane helices (blue), and the two Glu residues (E106 and E190) within the 1st and 3rd transmembrane helices that determine the cation selectivity of the pore^{27, 29} (red). Mutation of E106Q and E190Q in Orai1 result in dominant negative channels^{29, 30}. An additional recently reported mutant of the Orai1 channel (V102I) that changes the voltage dependence raised the possibility that Orai1 proteins have an intrinsic voltage sensing close to the selectivity filter, similar to KcsA K⁺ channels⁴⁵. The Orai1 protein (but not Orai2 and -3) is N-glycosylated, but a glycosylation defective mutant is still capable of restoring SOCE in T-cells³⁰. Transmembrane domains (TM1-4) are highlighted with grey boxes and the conserved stretch within the N-terminus just preceding the 1st TM domain and which is important for channel function is labeled with a green box. The region formed by the C-terminal cytoplasmic tail that is critical for activation from STIM1 is shown in red with the critical Leu/Ile in the middle (light blue).

Figure 3. Current model of the mechanism of store operated Ca^{2+} influx

(A) In quiescent cells Orai/CRACM proteins are found in the PM possibly as part of a larger molecular complex. STIM proteins are mostly located in the ER, although a fraction of STIM1 may be present in the PM (not shown for simplicity). Association of STIM proteins with the microtubule end-tracking proteins, EB1 helps pulling part of the peripheral ER to the proximity of the PM. During Ca^{2+} store depletion, oligomerization of STIM proteins is initiated followed by the engagement of STIM oligomers with the PM-ER contact sites. Orai1 is attracted to these contact sites and gets activated presumably by interaction with the STIM1 proteins. (B) An artificial tight contact forced between the PM and ER by a targeted molecular drug-induced heterodimerization (see⁴⁰ for technical details), allows formation of YFP-STIM1 containing clusters (green dots) only at the periphery of the contact zones (purple) after store depletion. This finding served the basis of the suggestion that Orai is part of a larger molecular complex as depicted by the cartoon on the left.