Store-Operated Calcium Channels: New Perspectives on Mechanism and Function

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Store-operated calcium channels (SOCs) are a nearly ubiquitous Ca^{2+} entry pathway stimulated by numerous cell surface receptors via the reduction of Ca^{2+} concentration in the ER. The discovery of STIM proteins as ER Ca^{2+} sensors and Orai proteins as structural components of the Ca^{2+} release-activated Ca^{2+} (CRAC) channel, a prototypic SOC, opened the floodgates for exploring the molecular mechanism of this pathway and its functions. This review focuses on recent advances made possible by the use of STIM and Orai as molecular tools. I will describe our current understanding of the store-operated Ca^{2+} entry mechanism and its emerging roles in physiology and disease, areas of uncertainty in which further progress is needed, and recent findings that are opening new directions for research in this rapidly growing field.

Calcium is a remarkably multifunctional signaling ion, at the heart of diverse biological processes that direct the birth, development, function, and eventual death of cells, tissues, and organisms. Cells use a diverse array of transporters and channels to regulate intracellular Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$). A major pathway present in nearly all metazoan cells is the store-operated Ca^{2+} channel (SOC). The defining feature of SOCs, the one that distinguishes them from all other classes of Ca^{2+} channels discussed in this volume, is their activation by the reduction of Ca^{2+} concentration in the lumen of the ER ($[\text{Ca}^{2+}]_{\text{ER}}$). Though they were originally described in nonexcitable cells (cells lacking the ability to fire action potentials), they are now known to be present in virtually all cells, including excitable cells like

skeletal muscle and neurons (Parekh and Putney 2005).

Physiologically, SOCs are most commonly activated by stimuli that release Ca^{2+} from the ER. This generally involves receptors that activate phospholipase C to produce inositol 1,4,5-trisphosphate (IP_3) and activate IP_3 receptors in the ER, but can also result from Ca^{2+} -induced Ca^{2+} release through ER/SR ryanodine receptors. The notion that ER Ca^{2+} depletion can control Ca^{2+} entry was first formulated by Jim Putney 25 years ago as the “capacitative calcium entry” hypothesis based on observations that Ca^{2+} entry triggered by muscarinic agonists was more closely linked to the emptiness of the ER store than to IP_3 elevation or occupation of the muscarinic receptor (Putney 1986). The introduction of thapsigargin

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(TG) (Thastrup et al. 1989), a sarcoendoplasmic reticulum Ca^{2+} -ATPase (SERCA) inhibitor that depletes ER Ca^{2+} independently of receptors and IP_3 , and methods for measuring $[\text{Ca}^{2+}]_i$ in single cells (fura-2, indo-1, etc.) (Gryniewicz et al. 1985) provided powerful tools that greatly accelerated progress in establishing store-operated Ca^{2+} entry (SOCE, as it was later renamed) as a ubiquitous Ca^{2+} entry pathway. TG-induced Ca^{2+} entry was soon shown to occur in dozens of cell types (Putney and Bird 1993; Parekh and Penner 1997), though nothing was known about the diversity of pathways that might be involved, let alone their molecular basis.

A major step forward was the identification of store-operated Ca^{2+} currents in mast cells and T cells. This achievement arose initially from attempts to identify Ca^{2+} conductances triggered by secretory agonists in mast cells and antigen receptors in T cells (Penner et al. 1988; Lewis and Cahalan 1989). In both cases, extremely small currents were detected and linked to large $[\text{Ca}^{2+}]_i$ rises, suggesting a high Ca^{2+} selectivity. In T cells, the current was shown to activate spontaneously during whole-cell recordings and in response to the T-cell mitogen phytohemagglutinin in perforated-patch recordings (Lewis and Cahalan 1989). Soon after, fluorescence-based studies showed that Ca^{2+} influx triggered through T cell mitogens shared several features with TG-induced influx, suggesting that T cell receptor agonists activated the store-dependent pathway (Mason et al. 1991; Sarkadi et al. 1991). These two paths of research converged when Hoth and Penner described a highly Ca^{2+} -selective current in mast cells that was activated in whole-cell recordings spontaneously (by Ca^{2+} chelators), by IP_3 , and by ionomycin, and called it the Ca^{2+} release-activated Ca^{2+} (CRAC) current (Hoth and Penner 1992). In Jurkat T cells, Zweifach and Lewis showed that TG activated a similar current, which appeared to be identical to the mitogen-stimulated current described earlier, and made the first estimate of its characteristically tiny conductance (~ 20 femtosiemens, far too small to resolve single-channel currents) (Zweifach and Lewis 1993). These

initial studies defined a membrane conductance that over the next decade would be described biophysically and pharmacologically in detail, providing a characteristic “fingerprint” culled from its ion selectivity, unitary conductance, and regulation by intra- and extracellular Ca^{2+} and pharmacological inhibitors (Parekh and Penner 1997; Prakriya et al. 2004).

Among several currents that were described as store-operated in different cells, the CRAC current emerged as the prototype because of its extensive characterization and the weight of evidence showing that it could be activated by ER Ca^{2+} depletion independently of surface receptors or changes in cytosolic $[\text{Ca}^{2+}]_i$. This included activation by intracellular Ca^{2+} chelators, SERCA inhibitors or ionomycin at constant intracellular $[\text{Ca}^{2+}]_i$, and by the Ca^{2+} chelator TPEN loaded into the ER (Prakriya et al. 2004; Parekh and Putney 2005). In fact, the CRAC channel is the only store-operated channel whose input–output relation is known. This relation, first examined by Hofer et al. (1998) and later quantified by Luik et al. (2008) using an ER-targetedameleon protein, shows that I_{CRAC} is a highly nonlinear function of $[\text{Ca}^{2+}]_{\text{ER}}$, with a Hill coefficient of ~ 4 and a $K_{1/2}$ of $170 \mu\text{M}$. Given a resting $[\text{Ca}^{2+}]_{\text{ER}}$ of $\sim 400 \mu\text{M}$, these results suggest that the ER must be depleted by $\sim 25\%$ before I_{CRAC} begins to activate significantly.

Over the two decades after Putney formalized the capacitative Ca^{2+} entry hypothesis, many mechanisms were proposed as the link between Ca^{2+} store depletion and SOCE. Among these, diffusible messengers released from the ER, insertion of CRAC channels into the plasma membrane, and conformational coupling of CRAC channels with IP_3 receptors in the ER were the most extensively studied, but in the absence of molecular substrates were difficult to establish (Prakriya et al. 2004; Parekh and Putney 2005). The CRAC channel fingerprint proved useful in ruling out a number of candidate genes for the CRAC channel itself, particularly members of the transient receptor potential (TRP) protein family, but its identity remained a mystery (Prakriya et al. 2004; Parekh and Putney 2005). The discoveries of the ER

Ca^{2+} sensor STIM1 in 2005 and the CRAC channel protein Orai1 a year later marked an unmistakable turning point in the field, as they provided the first and most essential molecular tools with which to dissect the SOCE mechanism. The history of these discoveries and the early revelations they afforded have been reviewed extensively (Cahalan et al. 2007; Wu et al. 2007; Fahrner et al. 2009; Putney 2009; Várnai et al. 2009; Hogan et al. 2010). In this review, I will summarize our current understanding of how Ca^{2+} store depletion leads to Ca^{2+} entry at a molecular level, and the role of STIM oligomerization and additional proteins in this process. I will also describe how these discoveries and the ensuing studies have increased awareness of SOCE roles in physiology and disease, and have created entirely new directions for research. Throughout I will emphasize work on STIM1 and Orai1 mainly because they have been the most extensively studied isoforms, but will discuss other STIM and Orai isoforms to highlight important functional differences. For more information on these other isoforms, the reader is referred to the reviews cited above.

STIM AND ORAI: A MOLECULAR BASIS FOR STORE-OPERATED CALCIUM ENTRY

RNAi screens for inhibitors of SOCE led to identification of the STIM protein family. Using small-scale RNAi screens Roos et al. (2005) identified STIM in *Drosophila* S2 cells and by homology the mammalian homologs STIM1 and STIM2, while Liou et al. (2005) identified STIM1 and STIM2 in a HeLa cell screen. Shortly thereafter, the Rao, Kinet, and Cahalan groups identified the Orai/CRACM protein family (Orai/CRACM1, 2, and 3) by genome-wide RNAi screens in S2 cells for inhibition of SOCE (Vig et al. 2006b; Zhang et al. 2006) or of NFAT translocation combined with linkage analysis of a family in which a loss of CRAC channel function led to severe combined immunodeficiency (SCID) (Feske et al. 2006).

STIM1 was quickly identified as a Ca^{2+} sensor for SOCE because it displayed an EF-hand-like sequence on the predicted luminal side, and neutralization of acidic residues to reduce Ca^{2+}

binding by the EF hand elicited constitutive Ca^{2+} entry independent of store depletion, in effect mimicking the store-depleted state (Liou et al. 2005; Zhang et al. 2005). Of the three Orai isoforms in mammals, Orai1 was most closely connected to the CRAC channel because a point mutation (R91W) in Orai1 led to a complete loss of I_{CRAC} in human T cells (Feske et al. 2005, 2006), Orai1 is the predominant isoform in these cells, and its properties (fast Ca^{2+} -dependent inactivation, sensitivity to 2-aminoethylidiphenyl borate (2-APB), and predominant expression in human T cells) most closely match those of CRAC (Lis et al. 2007). Orai1 was shown to be the pore-forming subunit of the CRAC channel by changes in I_{CRAC} calcium selectivity caused by mutating highly conserved glutamates (particularly E106D) in the *trans*-membrane domains (Prakriya et al. 2006; Vig et al. 2006a; Yeramini et al. 2006).

The Molecular Choreography of SOCE

Depletion of ER Ca^{2+} stores activates SOCE through a distinctive rearrangement of STIM1 and Orai1 in the cell. At resting levels of $[\text{Ca}^{2+}]_{\text{ER}}$ ($\sim 400\text{--}500\ \mu\text{M}$), STIM1 and Orai1 are both mobile proteins that are relatively diffusely localized throughout the ER and plasma membranes, respectively (Fig. 1A). Within seconds, store depletion causes STIM1 to accumulate at the cell periphery, forming “puncta” in confocal or total internal reflection fluorescence (TIRF) microscopic images of cells expressing fluorescently labeled STIM1 (Liou et al. 2005; Zhang et al. 2005). These peripheral puncta correspond at the ultrastructural level to specialized regions of smooth ER positioned within $\sim 10\text{--}20\ \text{nm}$ of the plasma membrane, known as ER-PM junctions (Fig. 1B) (Wu et al. 2006; Lur et al. 2009; Orci et al. 2009). In Jurkat T cells expressing ER-targeted horseradish peroxidase, electron microscopic studies show that native ER-PM junctions cover $< 5\%$ of the cell surface (Wu et al. 2006). Over roughly the same time period, Orai1 accumulates at PM sites directly opposite STIM1 (Luik et al. 2006; Xu et al. 2006), where STIM1 binds to Orai1 and opens the CRAC channel, generating highly localized

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Figure 1. The molecular choreography of store-operated calcium entry. (A) In this resting HEK 293 cell, mCherry-STIM1 is localized throughout the ER and eGFP-Orai1 is dispersed throughout the PM of the cell footprint. Following store depletion, both proteins redistribute into colocalized puncta. (Panel A is from Park et al. (2009) and reprinted with permission from Elsevier © 2009.) (B) Electron micrograph showing accumulation of HRP-STIM1 in the ER (arrows) at ER-PM junctions in a Jurkat T cell after store depletion. (Panel B is modified from Luik et al. (2006) and reprinted with permission from The Rockefeller University Press © 2006.) (C) A current model for SOCE, divided into four phases from left to right. For simplicity and to emphasize the interactions of STIM1 and Orai1, full stoichiometries are not shown (STIM1 is likely to be a dimer at rest and after store depletion at least a tetramer, and 8 STIM1s probably interact with each CRAC channel for full activation). At far left, STIM1 is pictured in its resting state when Ca^{2+} stores are replete. The Ca^{2+} -bound EF hand interacts with the SAM domain, and electrostatic interactions between acidic residues in CC1 (–) and basic residues in CAD (+) prevent CAD from interacting with Orai. On store depletion, Ca^{2+} is released from the EF hand, allowing STIM1 to oligomerize (shown here schematically as a dimer) and assume an extended conformation that exposes CAD and the polybasic domain. Oligomers move to ER-PM junctions by diffusion in the ER membrane, and accumulate there through interaction of the polybasic domain with phosphoinositides in the PM. At the junction, CRAC channels diffusing in the PM bind to the STIM1 CAD via electrostatic interaction of a coiled-coil region of the Orai1 carboxyl terminus (–) with basic CAD residues (+). This interaction traps CRAC channels and combined with CAD interactions with the Orai1 amino terminus (not shown) opens them at the ER-PM junction. EF, canonical EF hand; SAM, sterile α -motif; CC1, coiled-coil 1; CAD, CRAC activation domain; PBD, polybasic domain.

Ca^{2+} “hot spots” visible by TIRF microscopy (Luik et al. 2006). Puncta formation is reversible; store refilling causes both proteins to slowly disperse from the junctions and revert to their diffuse localization (Liou et al. 2005; Várnai et al. 2007; Malli et al. 2008; Smyth et al. 2008).

The assembly of these elementary units of SOCE in response to store depletion is in most cells quite slow, occurring over seconds to tens of seconds, which largely reflects the time required for STIM1 and Orai1 to migrate to ER-PM junctions. The timing of these events may have physiologic consequence; for example, in T cells, the slow speed of CRAC complex assembly and disassembly may generate Ca^{2+} oscillations by creating lags between changes in $[\text{Ca}^{2+}]_{\text{ER}}$ and CRAC channel activity (Dolmetsch and Lewis 1994). However, activation of SOCE is not always so slow. Recent studies indicate that STIM1 in skeletal muscle is prelocalized to the triads where the sarcoplasmic reticulum comes to within 10 nm of the plasma membrane transverse tubule (Stiber et al. 2008). In these cells, SOCE can occur in <1 sec of Ca^{2+} release from the SR, and is thought to help replenish SR Ca^{2+}

during high-frequency stimulation (Edwards et al. 2010). Thus, differential targeting of STIM1 and Orai1 in the resting state may adjust the activation kinetics of SOCE over orders of magnitude as needed in different cells.

Studies of the SOCE mechanism have reached a point where many of the underlying events can be understood in terms of the interactions of specific domains in STIM1 and Orai1 (Figs. 2 and 3). Below I discuss the functional roles of these domains in carrying out the major steps of the SOCE mechanism, diagrammed in Fig. 1C.

Oligomerization of STIM1 Is a Regulatory Switch for SOCE

The oligomerization of STIM1 is key to the regulation of its function. Early work on the isolated EF hand/sterile- α motif (EF-SAM) domain from STIM1 showed that removal of Ca^{2+} caused it to transition from a monomer to dimers and oligomers, prompting Ikura and colleagues to suggest that oligomerization of the EF-SAM domains may be the trigger for SOCE (Stathopulos et al. 2006). Supporting

Figure 2. Functional organization of STIM1. The major functional domains of STIM1 are indicated by residue numbers relative to the translation initiation site. SP, signal peptide; cEF1, canonical EF hand; hEF2, hidden (noncanonical) EF hand; SAM, sterile α -motif; TM, transmembrane domain; CC1–3, coiled-coil domains 1–3; CAD, CRAC activation domain; ID, inactivation domain; P/S, proline-serine-rich domain; K, polybasic domain. CAD is highly similar to SOAR (aa 344–442) and Ccb9 (aa 339–444), not shown. Sequences are displayed for regions with established functions: the Ca^{2+} sensing domain in cEF1, the basic region of CC2 involved in autoinhibitory binding to the acidic region of CC1 and stimulatory binding to Orai1, the acidic residues in the ID of STIM1 required for Ca^{2+} -dependent inactivation, the EB1 binding sequence (TRIP) that links STIM1 to the tips of growing microtubules, and the carboxy-terminal polybasic domain that targets STIM1 to the ER-PM junction. Phosphorylation sites S486 and S668 help suppress STIM1 activity and SOCE during mitosis. Acidic and basic residues are shown in red and blue, respectively.

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Figure 3. Functional organization of Orai1. This schematic layout of a single Orai1 subunit includes the four transmembrane domains (TM1–TM4), amino and carboxyl termini, and the connecting loops. Functionally significant residues and sequences are shown. The indicated residues in TM1 line the aqueous pore, E106 (red) forms the ion selectivity filter, and R91 inhibits channel function when substituted by bulky hydrophobic residues. D110/112/114 help determine sensitivity to block by lanthanides. In the amino terminus proximal to the PM, residues 68–91 form a Ca^{2+} /CaM binding domain, with residues essential for CaM binding and CDI indicated in bold green (A73, W76, Y80). This region also binds CAD and contains residues required for channel opening in bold blue (K85, R91). A domain in the intracellular loop is required for CDI, and a probable coiled-coil domain in the carboxyl terminus contains hydrophobic (bold green) and acidic (bold red) residues critical for binding STIM1 CAD.

this idea, its Ca^{2+} affinity of 0.2–0.6 mM (Stathopoulos et al. 2006) is similar to the $[\text{Ca}^{2+}]_{\text{ER}}$ dependence of STIM1 translocation into puncta ($K_{1/2} \sim 200 \mu\text{M}$) (Brandman et al. 2007; Luik et al. 2008). Importantly, increased Förster resonance energy transfer (FRET) between CFP- and YFP-labeled STIM1 after store depletion showed that STIM1 oligomerizes in cells after store depletion but prior to accumulation in puncta (Fig. 1C) (Liou et al. 2007; Malli et al. 2008; Muik et al. 2008). Subsequent NMR studies revealed a structural basis for this process. In the resting state, a single Ca^{2+} ion bound to the canonical EF hand exposes a hydrophobic cleft in STIM1 that interacts with

hydrophobic anchor side chains from the SAM domain (Stathopoulos et al. 2008). In this way, high $[\text{Ca}^{2+}]_{\text{ER}}$ prevents EF-SAM from engaging in intermolecular interactions, essentially providing a “brake” on spontaneous activation of SOCE. When Ca^{2+} is released from the EF hand (as during store depletion), hydrophobic regions are exposed, leading to oligomerization of the EF-SAM domains.

Although STIM1 and STIM2 are closely related in domain organization and show >65% sequence similarity, STIM2 appears to have a lower affinity for Ca^{2+} , such that it is partially Ca^{2+} -free and localized to ER-PM junctions in resting cells (Brandman et al. 2007).

The lower Ca^{2+} affinity of STIM2 may enable it to respond to smaller fluctuations of $[\text{Ca}^{2+}]_{\text{ER}}$, and by finely modulating SOCE act as a homeostatic regulator of cytosolic and ER $[\text{Ca}^{2+}]$ (Brandman et al. 2007). A reduced Ca^{2+} affinity may also enable STIM2 to play an active role in signaling, by sustaining a low level of Ca^{2+} entry through CRAC channels under conditions where stores have refilled enough to deactivate STIM1 (Oh-Hora et al. 2008). The full range of STIM2 functions is not yet understood.

The causal link between STIM1 oligomerization and SOCE was demonstrated by Luik et al. (2008), who replaced the EF-SAM domain of STIM1s with FRB or FKBP protein modules, and artificially cross-linked the modified STIM1 with a rapamycin analog. These conditions were sufficient to cause the modified STIM1s to accumulate in puncta and activate I_{CRAC} independently of store depletion. Thus, the reorganization of STIM1 and Orai1 into colocalized complexes appears to be an autonomous process that is held in check simply by the state of oligomerization of STIM1.

Interestingly, in addition to structural changes in the EF-SAM domain, interactions of the cytoplasmic domains are also critical for the oligomerization of full-length STIM1 following store depletion. Although a truncated STIM1 containing only the EF-SAM domain attached to the TM domain dimerizes in the ER on store depletion, these dimers appear to be unstable (Covington et al. 2010). Addition of the cytosolic coiled-coil 1 (CC1) and CRAC activation domain (CAD) (Fig. 2, and described below) is necessary for the protein to form stable higher-order oligomers after store depletion. Knowing at a structural level how these domains interact will be central to understanding how structural changes in the EF-SAM domains are transmitted across the ER membrane to trigger the conformational changes in the cytosolic region of STIM1 that lead to its activation.

The conformational changes in STIM1 that occur in response to oligomerization have recently begun to come to light. Several complementary studies suggest that oligomerization causes the STIM1 cytoplasmic domain to unfold

and unmask a hidden activation domain. Balla and colleagues first noted that an acidic sequence in the Orai1 carboxyl terminus, thought to interact with a basic region in coiled-coil 2 (CC2) of STIM1 (K384–386) (Calloway et al. 2009, 2010), also resembles a region in CC1 of STIM1 (Korzeniowski et al. 2010). They hypothesized that the CC2 basic residues may engage this CC1 acidic domain in the resting state, creating an autoinhibitory clamp when stores are full (Figs. 1C and 2). Neutralizing the CC1 acidic domain led to spontaneous activation, whereas neutralizing the CC2 basic domain prevented interaction with Orai1, supporting a model in which oligomerization destabilizes the intramolecular interaction, exposing CAD so that it can interact instead with the Orai1 carboxyl terminus. Intramolecular binding of the acidic and basic domains in STIM1 remains to be shown directly; however, concurrent studies of soluble STIM1 cytoplasmic fragments support the model. From a combination of FRET measurements and biophysical analysis, Muik et al. (2011) suggest that the cytoplasmic domain of STIM1 adopts a folded conformation at rest but elongates on binding to Orai1. Mutations of hydrophobic residues in CC1, CC2, and coiled-coil 3 (CC3) appeared to elongate the STIM1 fragment in the absence of Orai1, suggesting that coiled-coil formation in all three regions is involved in maintaining the resting folded conformation.

Accumulation of STIM at ER-PM Junctions

Two main mechanisms have been proposed to explain how STIM1 moves to ER-PM junctions. FRAP studies have suggested that STIM1 moves passively by diffusion with an estimated diffusion coefficient (D) of $0.1\text{--}0.15\ \mu\text{m}^2/\text{s}$ in the ER membrane (Liou et al. 2007; Covington et al. 2010). Thus, a simple but attractive model is that STIM1 oligomers diffuse passively to junctions where they are trapped by interaction with the PM or Orai (Fig. 1C and see below). However, STIM1 may also be transported within cells by an active microtubule-dependent mechanism. When growing microtubules cross the

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ER, the microtubule tip-binding protein EB1 binds to STIM1 and pulls out a new ER tubule through a “tip attachment complex” mechanism (Grigoriev et al. 2008; Honnappa et al. 2009). Although this interaction is weakened by store depletion and does not appear necessary for puncta formation (Grigoriev et al. 2008), one could imagine that STIM1-laden ER tubules are propelled in this way toward the cell periphery by microtubules, and when they encounter local sites of elevated IP_3 , local ER depletion releases the tubule near the PM with STIM1 primed to interact with the PM and establish or stabilize a junction. Interestingly, in two EM studies, store depletion caused the number of ER-PM junctions to increase, and overexpression of STIM1 further amplified this effect (Wu et al. 2006; Orci et al. 2009). Microtubules have also been observed aligned with cortical ER, perhaps as a memory trace of the ER’s journey to the PM (Orci et al. 2009).

STIM1 movement to the cell periphery after store depletion may be regulated by the energy state of the cell. Mitochondrial depolarization impedes the movement of STIM1 oligomers to ER-PM junctions and correspondingly inhibits SOCE (Singaravelu et al. 2011). This effect appears to depend on mitofusin-2, a mitochondrial membrane protein that helps form short tethers between mitochondria and ER. One idea is that this interference with SOCE may provide protection from Ca^{2+} overload in damaged cells with de-energized mitochondria (Singaravelu et al. 2011).

The formation of STIM1 puncta itself is a highly nonlinear function of $[Ca^{2+}]_{ER}$ ($K_{1/2} \sim 200 \mu M$, Hill coefficient of 4–8), consistent with the idea that multiple STIM1s, each bearing a single Ca^{2+} -binding EF hand, must oligomerize to be able to accumulate at ER-PM junctions (Brandman et al. 2007; Luik et al. 2008). To form puncta in the nominal absence of Orai, STIM1 requires an intact carboxy-terminal polybasic domain, which has been proposed to form an amphipathic α -helix that interacts with phosphoinositides in the plasma membrane, in effect creating a diffusion trap (Huang et al. 2006; Liou et al. 2007; Park et al.

2009). In line with this hypothesis, conditions that deplete phosphatidylinositol 4,5-bisphosphate (PIP_2) and phosphatidylinositol 3,4,5-trisphosphate (PIP_3) together strongly inhibit STIM1 puncta formation, and STIM1/2 polybasic domains bind to liposomes containing these lipids in vitro (Ercan et al. 2009; Walsh et al. 2010a). Depletion of PIP_2 alone by recruitment of an active 5-phosphatase to the PM only partially inhibits STIM1 puncta formation (Korzeniowski et al. 2009; Walsh et al. 2010a). However, the phosphatidylinositol 3-kinase and phosphatidylinositol 4-kinase inhibitor LY294002 largely eliminates SOCE without affecting STIM1 puncta, revealing effects of phosphatidylinositol 4-phosphate ($PI\ 4-P$) more directly on Orai1 (Korzeniowski et al. 2009). Together, these studies reveal potentially complex functions of lipids in SOCE, which may involve effects of PIP_2 and PIP_3 on STIM1 localization and of $PI\ 4-P$ on Orai1 opening, and further work will be needed to sort out these various roles.

Deletion of the C-terminal polybasic domain reveals a second mechanism for STIM1 accumulation at junctions, mediated by binding to Orai1 through the CAD domain. While STIM1 lacking the polybasic domain forms puncta if coexpressed with Orai1 (Park et al. 2009), I_{CRAC} activation is delayed under these conditions (Li et al. 2007). These results suggest that the polybasic domain-PM interaction may act to accelerate I_{CRAC} activation by creating clusters of STIM1 that more efficiently trap CRAC channels as they diffuse through ER-PM junctions.

Accumulation and Activation of CRAC Channels at ER-PM Junctions

Orai1 is highly mobile in the plasma membrane with a diffusion coefficient of $\sim 0.1 \mu m^2/s$ (Park et al. 2009; Madl et al. 2010), and most evidence supports a diffusion-trap mechanism in which Orai1 randomly encounters STIM1 at the ER-PM junctions and is trapped there by binding directly to STIM1 (Fig. 1C and see below). A recent study suggests that binding of only 1–2 STIM1s is sufficient for trapping a

CRAC channel (Hoover and Lewis 2011). Lateral movement of Orai1 into the junctions is consistent with a visible loss of Orai1 in the spaces between STIM1 puncta after store depletion. However, store depletion may also contribute to SOCE by regulating Orai1 levels in the PM. In resting oocytes Orai1 appears to recycle constantly between the PM and an endosomal compartment, with ~80% of the total Orai1 pool residing in the PM (Yu et al. 2010). Interestingly, the endosomal pool of Orai1 disappears on store depletion, prompting the suggestion that Orai1 binding to STIM1 at ER-PM junctions prevents its reuptake into endosomes. In platelets there is evidence for Orai1 insertion into the PM, but in this case in response to elevated $[Ca^{2+}]_i$ following activation of SOCE (Woodard et al. 2008). A powerful example of how regulation of Orai1 trafficking can modulate SOCE occurs during meiosis, when internalization of Orai1 helps inactivate SOCE (Yu et al. 2009).

Several groups have identified a minimal domain of STIM1 that binds to and powerfully activates CRAC channels, called CAD [CRAC Activation Domain; aa 342–448 (Park et al. 2009)], SOAR [STIM1-Orai Activating Region; aa 344–442 (Yuan et al. 2009)], or Ccb9 [aa 339–444 (Fig. 2) (Kawasaki et al. 2009)]. Park et al. showed that the binding is direct, based on GST pulldowns and copurification of Orai1 and CAD. Constitutive activation of I_{CRAC} by CAD binding to Orai1 offers the strongest evidence to date in favor of a conformational coupling mechanism involving direct protein–protein interactions, and argues against a diffusible messenger model (though it does not rule out the possibility that a diffusible messenger could modulate CRAC or provide a parallel activation pathway).

CAD and longer fragments of the STIM1 cytosolic domain appear to interact more strongly with the carboxyl terminus of Orai1 than to the amino terminus in vitro (Park et al. 2009; Zhou et al. 2010a). Although the carboxyl terminus is necessary and sufficient for Orai1 to colocalize with STIM1 in puncta, the amino terminus is neither necessary nor sufficient (Li et al. 2007; Muik et al. 2008).

The carboxyl terminus is weakly predicted to form a coiled-coil, and interactions with STIM1 and colocalization of Orai1 with STIM1 are inhibited by mutations predicted to destabilize the coiled-coil, such as L273S/D and L276D (Fig. 3) (Navarro-Borelly et al. 2008; Muik et al. 2008; Frischauf et al. 2009). The structural basis of the STIM-Orai carboxyl terminal interaction is not known, but may involve the formation of heteromeric STIM-Orai coiled-coils (Frischauf et al. 2009) or more restricted electrostatic interactions between acidic residues in the Orai1 carboxyl terminus (aa 272–291) and a basic region in the CC2 domain within CAD (K384–386) (Calloway et al. 2009, 2010; Korzeniowski et al. 2010).

Binding of STIM1 to the Orai1 amino terminus, though weaker than to the carboxyl terminus, does appear to be critical for channel activation. Because channels with a deletion of residues 1–73 from Orai1 still open (though at a reduced level), most attention has been focused on the highly conserved aa 73–91 (the approximate beginning of TM1) (Li et al. 2007). Deletion of aa 73–84 prevents CAD binding to the amino terminus and channel opening (Park et al. 2009). Mutations such as K85E (Lis et al. 2010) and R91W, the mutation found originally in T cells from SCID patients (Feske et al. 2006), completely prevent channel opening, apparently without preventing STIM1-Orai1 binding (Navarro-Borelly et al. 2008; Derler et al. 2009a), although R91W may interact with STIM1 somewhat less strongly than wild-type Orai1 (Fig. 3) (Muik et al. 2011). Interestingly, K85E does not prevent CAD binding to the amino terminus, suggesting that this residue is required for allosteric changes leading to gate opening. R91 may serve a similar function (Derler et al. 2009a). Interestingly, this membrane-proximal amino-terminal region overlaps with a calmodulin (CaM) binding site (aa 68–91), and several residues in this region (A73, Y80, W76) appear to be essential for CaM binding and Ca^{2+} -dependent inactivation (Fig. 3) (Mullins et al. 2009). Thus, the membrane-proximal region of the amino terminus is intimately tied to both activation and inactivation of CRAC channels and may provide important

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clues about the coupling of STIM1 binding to channel gating.

Questions of Stoichiometry: STIM1, Orai1, and the CRAC Channel Complex

A number of persistent questions apply to the native stoichiometries of STIM1 and Orai1 that are central to understanding the functional interactions of these proteins. In resting cells inactive STIM1 is often referred to as a dimer, based on coimmunoprecipitation of orthogonally labeled STIM1s and the high resting FRET between STIM1s labeled with CFP and YFP (Baba et al. 2006; Covington et al. 2010). However, the possibility that protein overexpression in these studies may have favored formation of dimers from endogenous monomers through mass action cannot be excluded. It is important to note that even if resting dimers are an artifact of overexpression, they do not seem to perturb either the inactivity of STIM1 in resting cells or the activation of SOCE following depletion. Unfortunately, measuring the resting STIM1 stoichiometry in vivo is not a simple task. Dimeric interactions may be too labile to survive solubilization, and measuring single-molecule photobleaching of GFP-tagged STIM1 is problematic because of its mobility and by the fact that fixation depletes Ca^{2+} stores, possibly promoting higher-order oligomerization (E. Covington and R. Lewis, unpubl.).

More importantly, the stoichiometry of “active” STIM1 following store depletion is unknown. The CAD fragment forms a tetramer in solution (Park et al. 2009), whereas the longer STIM1 cytosolic fragments generally appear as dimers (Ji et al. 2008; Penna et al. 2008; Muik et al. 2009; Yuan et al. 2009; Zhou et al. 2010a) that may need to unfold to expose the CAD and activate Orai1 (Korzeniowski et al. 2010; Muik et al. 2011). These findings raise the intriguing possibility that on store depletion, STIM1 undergoes a conformational change that results in formation of a tetramer through interactions among multiple CADs (Covington et al. 2010), an idea that remains to be tested.

The subunit stoichiometry of the CRAC channel has also been the subject of debate. A tetramer of Orai1 subunits was first proposed by Mignen et al. based mainly on the ability of a dominant-negative pore mutant (E106Q) to inhibit currents produced by dimeric or trimeric but not tetrameric Orai1 concatemers (Mignen et al. 2008). In cells transfected with monomeric Orai1-GFP and subsequently fixed, quantitation of photobleaching steps of single Orai1-GFP particles also supported a tetrameric stoichiometry (Ji et al. 2008), consistent with population brightness analysis of mobile Orai1-GFP after photobleaching in unfixed cells (Madl et al. 2010). On the other hand, biochemical analysis and single-molecule photobleaching of *Drosophila* Orai-GFP (dOrai-GFP) in the presence or absence of dSTIM cytosolic fragments suggested instead that Orai in resting cells is dimeric, and that STIM binding brings Orai dimers together to form the tetrameric functional channel (Penna et al. 2008). These studies raise fundamental questions about how the subunit stoichiometry of the CRAC channel is regulated, the role of STIM1 in this process, and whether changes in stoichiometry are reversible. New approaches, including structural studies will likely be necessary to resolve these questions.

Finally, recent studies have begun to show how the STIM1:Orai1 binding stoichiometry determines the extent of CRAC channel activation. Li et al. (2011) knocked variable numbers of STIM1 binding sites out of tetrameric Orai1 concatemers, revealing a graded relationship between the number of STIM1s bound and activity. Occupation of four sites was needed to evoke maximal activity, with each site thought to bind two STIM1s, leading to the conclusion that eight STIM1s must bind to fully activate the channel. Hoover and Lewis (2011) came to similar conclusions using native STIM1 and Orai1 coupled to mCherry and GFP. In cells with the same STIM1 expression, current magnitude increased with increasing amounts of Orai1, but dropped abruptly to near zero when the STIM1:Orai1 ratio in puncta fell below ~ 2 (or 8/channel). Both studies may explain the enigma that overexpression of Orai1 alone often

suppresses endogenous I_{CRAC} (Mercer et al. 2006; Peinelt et al. 2006; Soboloff et al. 2006; Li et al. 2007); by depleting a limited supply of STIM1, excess Orai1 may reduce the STIM1:Orai1 binding stoichiometry to a level that cannot open the channel. The two studies differ in the nonlinearity of CRAC channel activation, which appeared to be a much steeper function of the STIM1:Orai1 ratio with monomeric Orai1 than with tetrameric concatemers, raising the question of whether activation of SOCE at the single-channel level is “all-or-none” or graded with the number of STIM1s bound. A previous study using noise analysis concluded that store depletion, Ca^{2+} -dependent potentiation, and low doses of 2-APB through the stepwise recruitment of silent channels to a highly active state ($P_o = 0.8$) rather than a smooth increase in open probability of all the channels (Prakriya and Lewis 2006). Thus, even if channel activation is a graded function of occupancy by STIM1, when a diffusing CRAC channel encounters a cluster of STIM1 at the ER-PM junction, it may quickly become fully occupied and activate in an “all-or-none” manner.

Are STIM and Orai the Only Players in SOCE?

Overexpression of STIM1 and Orai1 in HEK cells generates massive CRAC-like currents after store depletion, up to 100 times the size of endogenous currents (Mercer et al. 2006; Peinelt et al. 2006; Zhang et al. 2006). These findings initially suggested that these two proteins may be sufficient on their own to account for SOCE. The ability of cytosolic STIM1 fragments to activate CRAC channels outside of ER-PM junctions and independently of store depletion lends further support to this view. Zhou et al. tested the idea more rigorously using a mutant yeast system in which Orai1 was expressed and trapped in secretory vesicles (Zhou et al. 2010a). Because yeast does not express any homologs of Orai or STIM, it was considered unlikely they would express any possible cofactors of SOCE. Purified cytosolic fragments of STIM1 were able to bind to the vesicles and elicit Ca^{2+} flux, strongly

suggesting that the two proteins can in fact function autonomously.

Although these findings support the idea that interactions of STIM1 and Orai1 alone are sufficient to elicit SOCE, they do not rule out important modulatory roles for additional proteins in vivo. An apt analogy can be made to the mechanism of exocytosis, in which purified SNARE proteins are capable of reconstituting membrane fusion to some degree by themselves in vitro but auxiliary proteins are needed to attain the high efficiency, speed, and control of secretion observed in living cells (Südhof and Rothman 2009). Orai1 in resting cells was first proposed to associate with another protein based on its exclusion from ER-PM junctions engineered by chemical crossbridging to have a reduced gap of ~ 9 nm (Várnai et al. 2007). One candidate for such a partner could be CRACR2A, a widely expressed cytosolic protein that copurifies with Orai1 in HeLa cells (Srikanth et al. 2010). CRACR2A binds to both Orai1 and STIM1 to form a ternary complex, and contains two EF hands that trigger its dissociation at elevated Ca^{2+} . Knockdown of CRACR2A in Jurkat cells reduced SOCE by about half, consistent with a role of enhancing the coupling between STIM1 and Orai1. Interestingly, CRACR2A's binding site on the Orai1 amino terminus overlaps with that of Ca^{2+} -CaM, whose binding is linked to Ca^{2+} -dependent inactivation (CDI) of I_{CRAC} (Mullins et al. 2009). Increased local $[Ca^{2+}]_i$ would be expected to cause CRACR2A to unbind, but it is not yet known whether this is necessary to allow Ca^{2+} -CaM to bind and trigger CDI.

STIM1 may also associate with proteins other than Orai. Although a truncated STIM1 containing only the luminal and transmembrane domains diffuses at the expected rate for a single transmembrane ER protein, addition of the cytosolic CC1 domain slows it by about twofold (Covington et al. 2010), and oligomerization in response to store depletion slows it by another factor of two (Liou et al. 2007; Covington et al. 2010). These changes in diffusion rate are too great to be easily explained by multimerization of the transmembrane

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domains, suggesting instead that STIM1 interacts with cytoskeletal, ER, or other cytoplasmic proteins through its CC1 domain (Covington et al. 2010). Slowing could reflect the steric hindrance of a long coiled-coil structure protruding up to ~15 nm from the ER membrane, weak binding of STIM1 along the length of microtubules (Smyth et al. 2007; Honnappa et al. 2009), or interactions with proteins like CRACR2A (Srikanth et al. 2010) or protein complexes associated with mitochondria-ER tethers (Singaravelu et al. 2011). Golli, a widely expressed, alternatively spliced form of myelin basic protein, is another recent candidate for a STIM1-binding protein. Knockdown and over-expression experiments suggest that it inhibits SOCE in T cells when localized to the PM via a myristoylation site (Feng et al. 2006). Golli appears to interact with the isolated carboxyl terminus of STIM1 in a Ca^{2+} -dependent manner, but thus far evidence for interaction with full-length STIM1 in cells is somewhat weak (Walsh et al. 2010b). Given the large number of hits in the original RNAi screens for SOCE suppression, continuing efforts to pull out STIM1- and Orai-interacting proteins are likely to reveal additional mechanisms that fine-tune SOCE under physiological conditions.

Toward a Molecular Basis for CRAC Channel Properties

Ion Selectivity and Permeation

The CRAC channel is unusual among Ca^{2+} channels in its pore properties. It has among the highest selectivity for Ca^{2+} over monovalent cations, comparable to that of Ca_v channels (Hoth 1995), but has a narrow pore diameter (3.9 Å) and a conductance that is among the smallest of any ion channel, being estimated from noise analysis at ~20 fS for Ca^{2+} (Zweifach and Lewis 1993; Prakriya and Lewis 2006). Initial studies identified mutations in several highly conserved residues that reduced the divalent ion selectivity or the sensitivity to block by trivalent cations. E106 and E190 (and their equivalent positions in dOrai) emerged as being critical for divalent selectivity, with

D110/112/114 participating in block by trivalent cations (Fig. 3) (Prakriya et al. 2006; Vig et al. 2006; Yeromin et al. 2006). To attain a more precise picture of the pore, subsequent studies applied scanning cysteine mutagenesis and probed for disulfide crosslinking (Zhou et al. 2010b) or accessibility to thiol-reactive reagents (McNally et al. 2009) as indicators of pore-lining positions. The shared conclusion was that TM1 residues line the ion-conductive pore, but those in TM3 do not, indicating that the effects of E190 mutations on ion selectivity were most likely the results of allosteric changes in protein structure (Fig. 3). The ability of Cd^{2+} to block the cysteine-substituted pore are consistent with a narrow pore diameter along its length (McNally et al. 2009). The emerging model of the CRAC channel pore is that of a rather flexible vestibule at the extracellular surface, a selectivity filter formed by a quartet of glutamates near the extracellular end of the pore, followed by a long narrow channel that may explain the low overall conductance. By comparing cysteine accessibility in closed vs. open channels, these methods should also be applicable to identifying the location of channel gates.

Ca^{2+} -dependent Inactivation (CDI)

Like many Ca^{2+} -permeable channels, CRAC channels are regulated by negative feedback from the Ca^{2+} that enters the cell. Rapid Ca^{2+} -dependent inactivation (CDI) occurs over tens of ms and is thought to result from binding of Ca^{2+} to sites several nm from the mouth of the channel (Hoth and Penner 1992, 1993; Zweifach and Lewis 1995). A role for STIM1 in this process was first suggested by the observation that CAD peptide activates Orai1 without allowing CDI (Park et al. 2009). Studies of truncated STIM1 subsequently led to the identification of a region carboxy-terminal to CAD that fully restored CDI (Derler et al. 2009b; Lee et al. 2009; Mullins et al. 2009). The inactivation domain (ID_{STIM}; aa 470–491) contains a stretch of seven acidic residues that exert a powerful influence over the extent of CDI (Fig. 2). Neutralization of pairs of residues either reduced or enhanced CDI (Mullins et al. 2009),

whereas neutralization of 5–7 residues completely eliminated CDI (Derler et al. 2009b; Lee et al. 2009; Mullins et al. 2009). Preliminary evidence from ^{45}Ca overlays suggested that this domain may bind Ca^{2+} weakly, but the effects of neutralizing mutations on Ca^{2+} binding were not in every case consistent with their effects on CDI (Mullins et al. 2009). Thus, its potential role as a Ca^{2+} binding site for CDI is at present undetermined.

Evidence is stronger for CaM as the Ca^{2+} sensor for CDI. Early work by Rychkov et al. showed evidence for a role of CaM, based on partial suppression of CDI by overexpressing a mutant Ca^{2+} -nonbinding CaM or a CaM inhibitory peptide (Litjens et al. 2004). Subsequent work revealed that CaM binds in a Ca^{2+} -dependent manner to the Orai1 amino terminus in a region adjacent to the start of TM1 (aa 68–90; Fig. 3) (Mullins et al. 2009). Mutations that prevent CaM binding (A73E, W76E/A/S, and Y80E) completely eliminate CDI, whereas mutations that preserve binding (Y80A/S) display CDI with accelerated kinetics. Much remains to be understood about how CaM operates to control CDI, such as the location of the apo-CaM binding site, and the role of the CaM binding domain in controlling CDI kinetics.

In addition to the amino terminus, other regions of Orai1 also participate in CDI. Alanine substitutions in the intracellular II-III loop at aa 151–154 eliminate CDI, whereas application of a soluble wild-type peptide containing this region reduces current unless it bears the alanine mutations (Fig. 3) (Srikanth et al. 2010). Srikanth et al. suggest that the II-III loop of Orai1 may function as a blocking particle in CDI to inhibit conduction. Another study has shown that mutations that increase Orai1 pore size (E106D, E190Q) greatly reduce or eliminate CDI (Yamashita et al. 2007). These findings raise the possibility that the inactivation gate may involve elements of the selectivity filter, as has been proposed for Ca_v and K_v channels.

One complication in designing and interpreting CDI experiments is that the STIM1:Orai1 binding stoichiometry has a powerful effect on the extent of CDI. Scrimgeour et al. (2009)

initially reported that CDI was normal in cells expressing a high ratio of STIM1:Orai1 but was lost in cells with low expression ratios. In an extension of this work, we found that CDI fell in parallel with channel activation as the STIM1:Orai1 ratio fell below ~ 2 , suggesting that full inactivation requires binding of eight STIM1s to the channel (Hoover and Lewis 2011). Thus, the number of STIM1s bound to a CRAC channel determines not only its ability to open, but its propensity to inactivate in response to Ca^{2+} entry.

One important concept that has emerged from the work on CDI described above is that the CRAC channel itself is not merely a multimer of Orai1, but is more accurately considered as a multiprotein complex. It binds CaM, which acts as a subunit to promote CDI. In addition, the requirement for STIM1 in enabling the channel to gate in response to an extracellular ligand (Ca^{2+}) implies that STIM1 also acts as a channel subunit and not merely as an activating ligand.

FUNCTIONAL ROLES OF STIM AND ORAI: INSIGHTS FROM GENETICS

Determining the precise functions of STIM and Orai in intact organisms has long been frustrated by a lack of highly specific pharmacological inhibitors. However, new insights and unexpected roles have been revealed by the phenotypes of genetically engineered STIM1/2 and Orai1 mutant mice as well as human patients with naturally occurring loss-of-function mutations in STIM1 or Orai1. Thus far, these studies have established important functions for STIM and Orai in the immune system, skeletal muscle, and platelets, among other cells and tissues.

Three distinct mutations causing loss of Orai1 function have been described in unrelated human families, as well as one frameshift mutation in STIM1 leading to a loss of protein expression; in mice, Orai1⁻, STIM1⁻, and STIM2-deficient mice have been engineered by homologous recombination or insertional mutagenesis (for reviews see Feske 2010). It is noteworthy that the clinical pathophysiologies caused by a loss of STIM1 or Orai1 function

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mostly overlap, suggesting that STIM1's critical functions in humans are performed primarily through Orai1 rather than other targets (Feske 2010). There are differences in phenotypes between humans and mice that may be caused by environment (the real world vs. animal care facilities or pathogen-free conditions), genetic background (e.g., highly inbred mouse strains), or the expression patterns of different Orai isoforms. Here I will attempt to identify the generalities that apply to both humans and mice and describe some of the unexpected results; a fuller discussion of these genetic studies is available in several excellent reviews (Baba and Kurosaki 2009; Feske 2009, 2010; Vig and Kinet 2009; Hogan et al. 2010).

Immune Cells

Loss of STIM1 or Orai1 function in humans causes severe combined immunodeficiency (SCID), a lethal condition marked by recurrent opportunistic infections, the only therapy being a functional hematopoietic stem cell transplant. Although normal numbers of T cells are present in these patients, they appear largely nonfunctional because of an inability of antigen receptor engagement to generate Ca^{2+} signals that regulate gene expression during T cell activation (Feske et al. 2001). These findings have triggered intense interest in developing specific pharmacologic inhibitors of the CRAC channel to treat a variety of autoimmune disorders. In mice, the absence of Orai1 or STIM1 function causes multiple defects of cytokine expression in T cells (interleukin (IL)-2, interferon- γ , IL-4, IL-10) (Gwack et al. 2008; Oh-Hora et al. 2008), and in mast cells, defective synthesis or release of inflammatory mediators (tumor necrosis factor- α , IL-6, serotonin, and leukotriene C_4) leads to a reduced anaphylaxis response in vivo (Baba et al. 2008; Vig et al. 2008). B-cell targeted deletion of STIM1 and STIM2 revealed a novel role for SOCE in enabling B cells to limit autoimmunity; in these animals reduced secretion of the anti-inflammatory cytokine IL-10 was linked to increased severity of experimental autoimmune encephalomyelitis, a model for multiple sclerosis (Matsumoto et al. 2011).

Interestingly, loss of STIM or Orai1 function does not eliminate all immune activity. T cell-dependent antibody responses and graft vs. host disease response appear relatively unaffected in $\text{STIM1}^{-/-}$ mice (Beyersdorf et al. 2009). Partial function of B cells is also evident in STIM1- or Orai1-deficient patients who develop lymphoproliferative disease and an autoimmune attack on their own neutrophils and thrombocytes (McCarl et al. 2009; Picard et al. 2009). A related autoimmune phenotype was observed in STIM1/STIM2 deficient mice (Oh-Hora et al. 2008; Oh-hora 2009). The autoimmunity is likely caused by defective generation of regulatory T (T_{reg}) cells that normally act to suppress T and B cell function. Finally, while there is much evidence supporting a role of sustained TCR-mediated Ca^{2+} signals in positive and negative selection of T cells during development in the thymus, development appears to proceed normally in the absence of STIM1 or Orai1; the same is true for development of mast cells and platelets. These findings raise a number of intriguing questions. Can other Ca^{2+} channels substitute for STIM1 and Orai1 (e.g., Orai2 or 3, Ca_v , or TRPC channels, P2X receptors, or something else?), or are the Ca^{2+} requirements for some cell functions low enough that ER Ca^{2+} release alone is sufficient? Finally, what makes the development of T_{reg} cells relatively sensitive to the loss of STIM or Orai1, while that of the other T cell subsets is not affected?

Platelets

Ca^{2+} is an essential signal for platelet activation events leading to clotting, hemostasis and thrombosis. Platelets express several parallel Ca^{2+} entry pathways, which made it difficult to define the role of SOCE in specific platelet functions until the generation of genetically engineered mice. In mice that are STIM1- or Orai1-deficient or homozygous for the non-functional Orai1 R93W gene, platelets show deficient Ca^{2+} responses to collagen and thrombin with defects in activation and thrombus formation in vitro (Varga-Szabo et al. 2008; Bergmeier et al. 2009; Braun et al. 2009).

Interestingly, these mice are protected in experimental models of arterial thrombosis and ischemic brain infarction, while displaying only a mild prolongation of bleeding time. Thus, STIM1 and Orai1 may not be absolutely essential for most of the rapid responses of platelets to activating factors (e.g., shape change, integrin activation, and secretion), but are perhaps more important for late responses such as stabilization of the thrombus, clot retraction, coagulant activity, and recruitment of cells to sites of injury (Varga-Szabo et al. 2009). These findings suggest the potential therapeutic use of CRAC channel blockers to treat ischemic cardiovascular or cerebrovascular disease.

Sweat Glands and Teeth

STIM1 and Orai1 appear to be required for the normal development and function of some ectodermally-derived tissues, such as sweat glands and teeth. In humans lacking Orai1 function, eccrine sweat glands do not function properly (whether this is a developmental or a purely functional defect is unclear), and this leads to dry skin and defects in thermoregulation manifest as recurrent fever (McCarl et al. 2009). The dental enamel in patients lacking STIM1 or Orai1 expression also fails to develop properly, implicating SOCE in the transepithelial transport of Ca^{2+} during formation of enamel (Picard et al. 2009; McCarl et al. 2009). STIM1- and Orai1-knockout mice may provide a useful experimental model for this poorly understood process.

Skeletal Muscle

More unexpected roles of STIM1 and Orai1 have emerged from genetic studies of skeletal muscle. The first hints for a function came from observations that CRAC-deficient SCID patients display a mild muscle myopathy with reduced strength and endurance (Partiseti et al. 1994; McCarl et al. 2009). STIM1 and Orai1 were later shown to be highly expressed in both human and mouse skeletal muscle (Williams et al. 2001; Gwack et al. 2008; Stiber et al. 2008; Vig et al. 2008; McCarl et al.

2009), and STIM1-deficient mice exhibit reduced muscle mass and susceptibility to fatigue (Stiber et al. 2008). The reduced mass is presumably caused by a defect in muscle development, as knockdown of STIM1, STIM2, or Orai1 or 3 inhibits the differentiation of myoblasts into myotubes and underlying gene activation events (Darbellay et al. 2008, 2010). Early fatigue is consistent with a role for STIM1 and Orai1 in maintaining SR Ca^{2+} content during periods of high frequency muscle activity, when slow depletion of Ca^{2+} from the SR would normally activate SOCE to replenish the SR Ca^{2+} deficit and thus maintain Ca^{2+} release transients and the force of contraction (Pan et al. 2002; Stiber et al. 2008). An intriguing feature of SOCE in muscle is that it is orders of magnitude faster than in nonexcitable cells (Launikonis and Ríos 2007), commencing in some cases within milliseconds of Ca^{2+} release from the SR (Edwards et al. 2010). Several models have been proposed to explain how prelocalization of STIM1 and Orai1 at the triadic junction may confer these extraordinarily rapid activation kinetics (Dirksen 2009; Launikonis et al. 2010).

FUTURE DIRECTIONS

Formation and Function of the ER-PM Junctions

Because SOCE depends absolutely on the direct contact of STIM and Orai at ER-PM junctions, the number, size, and intermembrane dimensions of these structures will exert profound effects on SOCE. However, little is known about how ER-PM junctions form and disassemble. Depletion of stores and STIM1 overexpression increases the number and extent of ER-PM junctions (Wu et al. 2006; Orci et al. 2009), suggesting a role for STIM1 in their formation or stabilization. Similarly, overexpression or knockdown of the widely expressed SR/ER protein junctate increases or decreases the abundance of junctions, respectively, although this was store-independent (Treves et al. 2004, 2010). Overexpression of TRPC3 also increased junction formation, suggesting that multiple

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proteins may participate in bridging the two membranes. Periodic electron densities have been reported between the ER and PM at junction-induced junctions, but thus far their molecular basis is unknown, and the relationship to junctions in which STIM and Orai accumulate is unclear (Treves et al. 2010).

The highly restricted region of Ca^{2+} entry at ER-PM junctions offers attractive opportunities for local signaling within Ca^{2+} microdomains to effector proteins (Luik et al. 2006). SOCE has been shown to activate or repress particular enzymes with high specificity, including adenylate cyclases (Willoughby and Cooper 2007), the plasma membrane Ca^{2+} pump (Bautista and Lewis 2004), and phospholipase A_2 (Chang et al. 2008). The evidence for local signaling is mostly based on two approaches. One is to show that BAPTA, a fast Ca^{2+} chelator, can interrupt the activation of the target by SOCE, whereas the slower buffer EGTA cannot, suggesting that the target is located somewhere between the calculated capture distances of the two chelators (tens of nanometers from the channels). The other approach is to show that when SOCE and non-SOCE pathways are engaged to produce the same global $[\text{Ca}^{2+}]_i$ increase, only SOCE activates the target. Using these approaches, recent studies suggest that transcriptional pathways may be activated preferentially near CRAC channels (di Capite et al. 2009; Ng et al. 2009). Ca^{2+} entry through CRAC channels has long been known to drive gene expression through NFAT and other factors, and the efficiency and specificity of gene activation depends on the amplitude and duration of sustained Ca^{2+} signals and the frequency of Ca^{2+} oscillations triggered through CRAC channels (Dolmetsch et al. 1997, 1998). Recently, Parekh and colleagues presented evidence that NFAT activation, assayed by nuclear accumulation, is triggered preferentially in the close vicinity of CRAC channels rather than in the cytosol (Kar et al. 2011). This finding is remarkable, given that the NFAT-activating phosphatase calcineurin (CN) is present in the cytoplasm and its Ca^{2+} sensitivity is sufficiently high to dephosphorylate NFAT at sub-micromolar levels of global cytosolic Ca^{2+}

(Stemmer and Klee 1994). One possible explanation is that cytoplasmic CN activity is weak enough to be subverted by cytoplasmic NFAT kinases, whereas junctional CN activity more completely dephosphorylates NFAT, enabling it to run the cytoplasmic kinase gauntlet and reach the nucleus. Further studies may shed light on the details of this mechanism.

Regulation of SOCE in a Physiological Context

One expansive goal for further research is to determine the many levels at which SOCE can be regulated under physiological conditions. The great majority of studies to date have been conducted using TG, high intracellular IP_3 , ionomycin, or chelators, all of which deplete the ER completely throughout the cell. Physiological responses are likely to be much more localized, particularly when stimuli are spatially restricted. A particularly interesting example occurs during immune synapse formation between T cells and antigen-presenting cells (APCs), in which the T cell receptors engage peptide-MHC complexes on the APC. Shortly after cell–cell contact, STIM1 and Orai1 change their localization, accumulating initially at the synapse and then appearing to move in tandem to the distal end of the cell (Barr et al. 2008; Lioudyno et al. 2008). Ca^{2+} imaging shows a domain of elevated $[\text{Ca}^{2+}]_i$ at the synapse, where it could potentially affect synapse stability, cytoskeletal remodeling, secretion, and other Ca^{2+} -dependent events (Lioudyno et al. 2008). These studies raise many interesting questions about the mechanisms involved in polarizing the SOCE machinery and the potentially different consequences of localizing STIM1-Orai1 complexes to the two poles of the cell.

STIM1 is known to be phosphorylated in situ (Manji et al. 2000), but functional effects are only beginning to be explored. Among multiple sites of phosphorylation, several (S575, S608, S621) are phosphorylated by ERK1/2 and this increases after store depletion (Pozo-Guisado et al. 2010). Alanine mutations at these sites significantly repressed SOCE, suggesting that phosphorylation may serve as a powerful

positive modulator. Conversely, Smyth et al. linked phosphorylation in the cytoplasmic region to the suppression of SOCE during mitosis (Smyth et al. 2009). Among multiple sites, phosphorylation at S486 and S668 were found to be most potent in preventing STIM1 puncta formation and suppressing SOCE (Fig. 2). The physiological role of SOCE suppression is not yet clear, although a slight slowing of cell proliferation was seen in cells overexpressing a truncated STIM1 that functions during mitosis. It will be interesting to see if phosphorylation affects cell function independently of suppressing Ca^{2+} entry. From these early studies it is already clear that phosphorylation has the potential to modulate CRAC channel activity in response to multiple environmental cues.

Oxidation may also affect STIM1 and Orai1 function independently of ER Ca^{2+} , possibly modulating SOCE under conditions of cellular stress or inflammation. Oxidation by H_2O_2 or buthionine sulfoximine can cause STIM1 to form puncta and open Orai1 channels (Hawkins et al. 2010). One proposed mechanism involves S-glutathionylation of C56, which then reduces Ca^{2+} binding by the adjacent EF hand. In this way STIM1 may act as a cellular sensor for oxidant stress, although an indirect effect of oxidants on SOCE through depletion of Ca^{2+} stores has not been excluded. On the other hand, oxidation also inhibits SOCE by reducing Orai1 activity (Bogeski et al. 2010). This effect has been traced primarily to C195, and occurs with Orai1 but not Orai3. Interestingly, SOCE in effector T cells is more resistant to inhibition by ROS than in naïve T cells, which may result from increased expression of Orai3 relative to Orai1 in effector cells. Bogeski et al. propose that the increased Orai3:Orai1 ratio is an adaptation that allows effector T cells to proliferate and optimize cytokine production in the oxidizing environment of injured or inflamed tissue. Understanding how the stimulatory and inhibitory effects of oxidation are integrated during responses to physiological stimuli is an important goal.

Temperature is another factor that has been largely overlooked in studies of STIM1-Orai1 function; even though the majority of studies

use mammalian proteins, room temperature has been the norm. Interestingly, Xiao et al. (2011) showed that STIM1 is in fact highly temperature-dependent, and it begins to form puncta without store depletion at temperatures $>35^\circ\text{C}$. Interestingly (and fortunately for us), this does not by itself activate Ca^{2+} entry, but Orai1 channels open during subsequent cooling. It is not known whether binding to Orai1 is completely inhibited at the high temperature, or some more subtle change is involved. One proposal is that this thermosensitivity may prime immune cells for greater activity during fever as they circulate between the body core and the cooler periphery, or play a role in skeletal muscle physiology during intense exercise (Xiao et al. 2011). These findings also raise the possibility that at mammalian physiological temperatures the $[\text{Ca}^{2+}]_{\text{ER}}$ threshold for STIM1 oligomerization may be shifted to higher $[\text{Ca}^{2+}]_{\text{ER}}$ than has been measured at room temperature (Brandman et al. 2007; Luik et al. 2008), making STIM1 more responsive to small changes in $[\text{Ca}^{2+}]_{\text{ER}}$ than currently thought.

Multiple Roles for STIM1 in Ca^{2+} Channel Regulation

TRPC Channels

Mammalian TRPC channels are weakly Ca^{2+} permeable channels with a long history of association with SOCE, but whether they are truly store-dependent is still being debated (DeHaven et al. 2009; Wang et al. 2010). The strongest evidence of store-dependent activation comes from charge-swapping experiments suggesting that a di-lysine motif in STIM1 interacts with a conserved di-aspartate domain in TRPC1 and other TRPCs to activate these channels following store depletion (Zeng et al. 2008). One complication in assessing whether TRPCs are store-operated has been that PLC-coupled agonists have been used in most cases to activate the channels, stimulating second-messenger (diacylglycerol, PIP_2 , IP_3) and store-depletion pathways in parallel. The definitive tests for store-operated function (i.e., channel activation by store depletion independent of receptor

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activation or changes in $[Ca^{2+}]_i$) have in most cases not been performed, or have given ambiguous results. For example, TRPC7, once thought to be store-operated because it is activated by TG, was later found not to be activated by IP_3 or ionomycin even though they also empty the ER (DeHaven et al. 2009). One step toward settling this debate would be to test whether a variety of receptor-independent store-depletion stimuli (SERCA blockers, cytosolic and ER Ca^{2+} chelators, lipophilic Ca^{2+} ionophores, TPEN) all activate TRPC channels under conditions of heavy cytosolic buffering at resting $[Ca^{2+}]_i$ levels. Unfortunately, these tests also complicated by potential crosstalk between Orai1 channels and TRPC channels mediated through local increases in $[Ca^{2+}]_i$ entering via Orai1. In fact, the relation between STIM1, Orai1, and TRPCs could be quite complex, given evidence that TRPC channel activation or insertion into the PM may be affected by local changes in $[Ca^{2+}]_i$ through other channels (Gross et al. 2009; Cheng et al. 2011). A critical challenge for future studies is to understand this relation and how TRPC channels act in concert with STIM1 and Orai1 to shape store-dependent Ca^{2+} signals.

ARC Channels

Arachidonate-regulated Ca^{2+} (ARC) channels are similar in terms of Ca^{2+} selectivity and conductance to the CRAC channel, but differ in lacking CDI and sensitivity to 2-APB, and most importantly are activated by arachidonic acid rather than store depletion (Shuttleworth 2009). STIM1 is necessary for their activation, but only the fraction of STIM1 that is constitutively present in the PM (comprising about 10%–20% of the total STIM1 pool in most cells) (Mignen et al. 2007). Concatemer studies suggest that ARC channels form as a pentamer of Orai subunits ($3 \times \text{Orai1} + 2 \times \text{Orai3}$), and glutamine substitutions for glutamates at E106 in Orai1 or the corresponding location in Orai3 (E81) both inhibit conductance, suggesting the formation of a pentameric glutamatergic selectivity filter (Mignen et al. 2009). Ion selectivity studies with less extreme aspartate

substitutions and sizing the wt pentameric pore under divalent-free conditions will help validate this model and help relate its ion selectivity mechanism to that of the tetrameric CRAC channel. Tail swapping experiments have indicated that arachidonic acid selectivity requires two amino-terminal Orai3 regions in the pentamer (Thompson et al. 2010). Much remains to be learned about how STIM1 regulates the ARC channels, and how ARC and CRAC channel synthesis are controlled in cells that express both Orai1 and Orai3.

Ca_v Channels

STIM1 was recently and unexpectedly discovered to exert reciprocal actions on store-operated and voltage-gated Ca^{2+} ($Ca_v1.2$) channels (Park et al. 2010; Wang et al. 2010). STIM1 inhibits $Ca_v1.2$ channel function in two ways. First, store-depletion acts through STIM1 to acutely inhibit voltage-gated opening of $Ca_v1.2$; this effect ranges from a modest 15% (Park et al. 2010) to nearly total inhibition (Wang et al. 2010). In addition, STIM1 expression can nearly completely suppress $Ca_v1.2$ by stimulating its internalization (Park et al. 2010). These effects appear to be caused by binding of the CAD domain of STIM1 to the carboxyl terminus of $Ca_v1.2$. Many of the details of the mechanisms remain to be worked out, and further studies will be needed to understand how general these phenomena are and whether the crosstalk extends to other members of the Ca_v family. The effects of STIM1 on $Ca_v1.2$ channel function may help explain how PLC-coupled receptors inhibit voltage-gated Ca^{2+} signaling, and in cells that express both $Ca_v1.2$ and Orai1, the reciprocal regulation of these channels provides a new mechanism to enable the selective activation of channel-specific signaling pathways.

CONCLUSION

The identification of the STIM and Orai genes has led to a remarkable wave of progress in understanding the mechanism of SOCE, to the point where the major steps are now known

and have been described in at least a qualitative way. There clearly remains much that is not understood at a mechanistic level, and application of increasingly sophisticated physiological, cell biological, biochemical and structural approaches will be needed to fill in these gaps. It is hoped that with a more detailed mechanistic picture of CRAC channel function and regulation, we may better understand the full diversity of their functions *in vivo*, and ultimately develop new strategies to control them in the treatment of human disease.

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