I. INTRODUCTION

Calcium as an intracellular second messenger serves a remarkable diversity of roles that span a range of biological processes from birth through the development, function, and death of cells, tissues, and organisms (23, 42, 59). In metazoans, one of the primary sources of Ca²⁺ signals in both excitatory and particularly in nonexcitable cells is the family of store-operated calcium channels (SOCs). These channels are typically activated by the engagement of cell surface receptors that through G proteins or a tyrosine kinase cascade activate phospholipase C to cleave phosphatidylinositol 4,5-bisphosphate (PIP₂) and produce inositol 1,4,5-trisphosphate (IP₃). SOCs are so named because they respond to the reduction of ER intraluminal Ca²⁺ ([Ca²⁺]ER), a consequence of IP₃-induced Ca²⁺ release through IP₃ receptors in the ER membrane (283) (FIGURE 1A).

Store-operated channels are unique among ion channels, from their molecular basis to their biophysical properties and mode of regulation. Because of their intimate physical and functional connections to the ER, they play a homeostatic role in providing Ca²⁺ to refill the ER after Ca²⁺ has been released and pumped out across the plasma membrane (PM) (95, 163, 261, 283). Importantly, however, Ca²⁺ entry through SOCs also serves a much wider set of signaling functions by elevating the cytosolic Ca²⁺ concentration ([Ca²⁺]),. Because of the finite Ca²⁺ capacity of the ER, Ca²⁺ release can only generate transient signals; however, prolonged store depletion can evoke Ca²⁺ entry through SOCs that is sustained for minutes to hours, driving a wide assortment of basic biological processes such as secretion, motility, and gene transcription, and modulation of enzymatic activity and motility. Because they are not voltage dependent, SOCs can conduct Ca²⁺ at negative membrane potentials at which depolarization-sensitive channels [e.g., voltage-gated Ca²⁺ (Caᵥ) or NMDA-R channels] are inactive, thus enabling complementary roles. In addition, their small but selective conductance for Ca²⁺, coupled with their tight localization at ER-PM junctions gives SOCs preferential access to Ca²⁺ response pathways within microdomains (23, 279). Together, these features enable SOCs to fulfill unique functional roles within the panoply of cellular Ca²⁺-dependent pathways.

For two decades following the first proposal of the store-operated Ca²⁺ entry (SOCE) pathway (307), its molecular...
basis remained a complete mystery (282, 283, 298). The identification of STIM proteins as ER Ca\(^{2+}\)/H\(_{11001}\) sensors in 2005 (199, 321) and Orai proteins as SOC subunits a year later (103, 398, 446) were major breakthroughs that spurred a wave of advances in elucidating the molecular mechanisms and functions of SOCs in many cells and tissues (192). Since then, the combined efforts of many groups have revealed the key cellular events involved in SOC activation by store depletion as well as the molecular determinants of SOC biophysical properties. Most recently, structural models for STIM1 (368, 370, 424) and Orai (153) are beginning to illuminate the molecular mechanisms that control activation and ion permeation of store-operated Ca\(^{2+}\) channels.

This review begins with a brief historical overview of some of the milestones in the study of store-operated Ca\(^{2+}\) entry that provide context for more recent mechanistic studies. Thereafter, the focus is on our current understanding of the molecular and cellular mechanisms underlying SOCE; how specific molecular determinants of STIM and Orai proteins control SOC channel gating, permeation, modulation, and pharmacology; and how STIM and Orai as molecular probes have been used to reveal many previously unknown functions of SOCE in health and disease. We have included tables compiling the many mutations that have been found to affect STIM and Orai function, to provide both an overall view of the functionally important domains and to help guide future studies. While SOCE operates in many species, we focus here on mammalian systems where the most work has been done, with appropriate references to differences in other species. Our overall goal is to describe key findings that have helped establish our current level of understanding, while highlighting significant controversies or uncertainties that remain to be resolved.

II. STORE-OPERATED CALCIUM ENTRY: AN HISTORICAL OVERVIEW

A. The Capacitative Ca\(^{2+}\) Entry Hypothesis

Soon after the discovery of IP\(_3\) and its role in triggering Ca\(^{2+}\) release from the ER (24, 374), it was well recognized that an almost universal consequence of IP\(_3\) production was a transient increase in \([\text{Ca}^{2+}]_{\text{ER}}\) resulting from the release of Ca\(^{2+}\) from intracellular stores, followed by a sustained \([\text{Ca}^{2+}]_{\text{ER}}\) plateau generated by influx across the plasma membrane (21, 22, 57, 307). While the molecular mechanism of Ca\(^{2+}\) release was understood to result from IP\(_3\) acting on the IP\(_3\) receptor/Ca\(^{2+}\) channel in the ER (21, 374), the mechanism underlying Ca\(^{2+}\) entry was considerably more mysterious. Several studies suggested that the Ca\(^{2+}\) permeability of the plasma membrane was more closely related to the degree of filling of internal stores than to either agonist receptor occupancy or IP\(_3\) levels. Thus, after transient activation of phospholipase C (PLC)-coupled receptors, a PM pathway that allowed extracellular Ca\(^{2+}\) to reload the
stores remained active long after IP$_3$ or its metabolites returned to baseline levels (8, 308). Based on these and related results from Casteels and Droogmans (44), Putney (307) proposed that Ca$^{2+}$ store depletion rather than IP$_3$ was responsible for triggering influx. This process was initially called “capacitative Ca$^{2+}$ entry” (CCE) to reflect the idea that Ca$^{2+}$ entered the cytoplasm from outside by traversing the ER (the “capacitor”), which was assumed at that time to be directly connected to the external milieu. Subsequent experiments showed that Ca$^{2+}$ actually enters the cytoplasm before it is taken up into the stores, leading to a revision of the original hypothesis (308). However, the nature of the coupling between ER depletion and PM Ca$^{2+}$ entry, as well as the permeation pathway itself, was unknown (FIGURE 1A). In 1995, CCE was renamed “store-operated Ca$^{2+}$ entry” (SOCE) to refer more explicitly to its mode of activation and to distinguish it from receptor-, ligand-, and voltage-operated Ca$^{2+}$ channels.

The first direct identification of the SOCE permeation pathway came from electrophysiological studies, conducted shortly after the original CCE proposal, aimed at identifying Ca$^{2+}$ channels underlying secretion in mast cells and mitogenic activation of T cells. Using a combination of patch-clamp and Ca$^{2+}$ imaging techniques, Penner, Matthews, and Neher (230, 293) described a small (1–2 pA), low-noise current that was activated by agonists such as substance P and intracellular dialysis with IP$_3$ in rat mast cells. This small current developed in parallel with a rise in [Ca$^{2+}$]$_i$, leading to the suggestion that it was responsible for the [Ca$^{2+}$]$_i$ rise caused by agonists and IP$_3$. Lewis and Cahalan (193) described a similarly small, highly selective Ca$^{2+}$ current that developed slowly during whole cell recordings in Jurkat human leukemic T cells with buffered low-Ca$^{2+}$ internal pipette solutions. Interestingly, in perforated-patch recordings, the same current was activated by phytohemagglutinin (PHA), a T cell mitogen that crosslinks the T cell receptor (TCR) and generates IP$_3$. A number of unique features of the Ca$^{2+}$ current were described, including a lack of voltage-dependent gating, high Ca$^{2+}$ selectivity with an inwardly rectifying current-voltage relationship, low conductance based on the absence of obvious current noise, feedback inhibition by Ca$^{2+}$, and blockade by Ni$^{2+}$ and Cd$^{2+}$. At the time, the mechanism for its activation was not understood, although the possibility that the current flowed across the PM and directly through the ER on its way to the cytosol as depicted by the original CCE model was considered (193).

The link between these Ca$^{2+}$ currents and SOCE became clearer following the introduction of SERCA inhibitors as experimental tools. The most widely used of these was thapsigargin (TG), a plant-derived lactone that inhibits SERCA family ATPases in the ER and SR with high selectivity and potency (384). The main advantage of TG and other SERCA inhibitors (cyclopiazonic acid, BHQ) is that they directly deplete Ca$^{2+}$ from the ER while bypassing receptors and associated biochemical signals. By inhibiting ongoing uptake, SERCA inhibitors unmask an ongoing Ca$^{2+}$ “leak” from the ER, leading to luminal Ca$^{2+}$ depletion without concomitantly generating IP$_3$ (FIGURE 1A). The sources of the leak are not yet well defined but may include the protein translocon that spans the ER membrane (271, 392). The introduction of furan 2 and related Ca$^{2+}$-sensitive fluorescent dyes by Tsien and colleagues (129) made it possible to detect Ca$^{2+}$ release in response to TG added in the absence of extracellular Ca$^{2+}$ followed by Ca$^{2+}$ entry through SOCs after subsequent re-addition of Ca$^{2+}$. These kinds of experiments led to an avalanche of reports of SOCE in diverse cell types, primarily nonexcitable cells (oocytes, hepatocytes, lymphocytes, epithelial acinar cells, etc.), but in some excitable cells as well (282, 310). With these tools, Putney and colleagues (381) made the critical observation that Ca$^{2+}$ influx across the plasma membrane was triggered by the depletion of an intracellular Ca$^{2+}$ pool and importantly that IP$_3$ and TG had nonadditive effects, suggesting that they activate the same Ca$^{2+}$ influx pathway.

It soon became evident that the Ca$^{2+}$ entry induced by TCR agonists and SERCA inhibitors shared key properties, suggesting that the currents in T cells and most likely in mast cells were store operated (122, 228, 333). Hoth and Penner (151) demonstrated this first in mast cells by identifying a Ca$^{2+}$-selective current activated in whole cell recordings by intracellular EGTA, IP$_3$, or ionomycin, and named it the Ca$^{2+}$ release-activated Ca$^{2+}$ (CRAC) channel (151). In Jurkat T cells, Zweifach and Lewis (458) used perforated-patch recording and TG to identify a similar Ca$^{2+}$-selective current that appeared identical to the PHA-activated current in terms of ion selectivity, sensitivity to Ni$^{2+}$, and an extremely small unitary conductance (10–25 fS as estimated from noise analysis). The similarity between store depletion-mediated and TCR-stimulated currents (302, 458), as well as the parallel loss of CRAC current and TCR-mediated Ca$^{2+}$ influx in T cells from human SCID patients (286) and in mutant Jurkat T cells (95, 342), argued strongly that the TCR is coupled to the CRAC channel. Similarly, in mast cells, $I_{CRAC}$ was shown to be activated by stimulation through Fc receptors (442). Together, these early studies established physiological links between $I_{CRAC}$ and receptor stimulation in mast cells and T cells and laid the basis for more than a decade of detailed biophysical studies of CRAC channel properties and behavior. By enabling the direct measurement of channel activity, patch-clamp current recordings made it possible to unravel intrinsic channel properties and understand their modulation, which was simply not possible based on [Ca$^{2+}$]$_i$ measurements alone because of the many additional parameters that regulate [Ca$^{2+}$]$_i$, such as membrane potential, buffering, and ATP-driven pumps.
B. The Ca\(^{2+}\) Release-Activated Ca\(^{2+}\) (CRAC) Channel Is a Prototypic SOC

A variety of ionic currents have been described that satisfy a limited set of criteria for being store operated in various cells (e.g., activation by TG or intracellular Ca\(^{2+}\) chelators), but have not been extensively characterized and their molecular basis is still not understood (reviewed in Ref. 283). One complication in testing whether a channel is store-operated is that agents that release Ca\(^{2+}\) from the ER (e.g., TG or IP\(_3\)) will elevate [Ca\(^{2+}\)]\(_i\), which in some cases can by itself activate channels directly or through Ca\(^{2+}\)-induced changes in membrane potential. Likewise, intracellular dialysis with Ca\(^{2+}\) chelators can in principle activate channels by removing tonic inhibition by resting cytosolic [Ca\(^{2+}\)]\(_i\).

CRAC channels are considered to be SOCs because they are activated by agents that deplete Ca\(^{2+}\) from the ER lumen without a significant change in cytosolic [Ca\(^{2+}\)]\(_i\). By this criterion the most direct evidence for the store dependence of CRAC channels is their activation by TPEN, a membrane-permeant Ca\(^{2+}\) chelator that reduces luminal Ca\(^{2+}\) (144), as well as by TG or ionomycin (a membrane-permeant ionophore that transports Ca\(^{2+}\) out of the ER) when applied in the presence of high concentrations of intracellular Ca\(^{2+}\) buffers to clamp cytosolic Ca\(^{2+}\) at its resting level (96, 458) (FIGURE 1A). The essential operating characteristic of the CRAC channel is its dependence on [Ca\(^{2+}\)]\(_{ER}\). This input-output relation has been examined directly by measuring [Ca\(^{2+}\)]\(_{ER}\) with Ca\(^{2+}\)-sensitive dyes loaded into the ER (144) or ER-targeted cameleons (209) while recording \(I_{CRAC}\) in whole cell or perforated-patch mode. A quantitative analysis by Luik et al. (209) showed that \(I_{CRAC}\) increases steeply (Hill coefficient of \(\sim 4\)) as [Ca\(^{2+}\)]\(_{ER}\) is reduced from its resting value of \(\sim 400 \mu M\), with half-maximal activation at \(\sim 200 \mu M\) luminal Ca\(^{2+}\) (FIGURE 1B, and discussed below). Together, these results demonstrate that CRAC channels are activated by reduction of free [Ca\(^{2+}\)]\(_{ER}\) rather than by changes in total ER Ca\(^{2+}\) or [Ca\(^{2+}\)]\(_i\); this, together with extensive characterization of their biophysical and pharmacological properties, led to the widespread acceptance of the CRAC channel as the prototypic SOC and the primary target in the search for a molecular mechanism for SOCE.

C. The Fingerprint of the CRAC Channel

Electrophysiological studies from 1989–2006 revealed essentially all that is known about the biophysical and pharmacological properties of the CRAC channel (283, 298). There are several notable characteristics in addition to its activation by Ca\(^{2+}\) store depletion that distinguish the CRAC channel among the large number of known Ca\(^{2+}\)-permeable channels. It is extremely Ca\(^{2+}\)-selective with a Ca\(^{2+}\):Na\(^{+}\) permeability ratio of \(>1,000\), comparable to the most selective Ca\(^{2+}\) channels known (e.g., the voltage-gated L-type Ca\(^{2+}\) channel) (148). However, its unitary conductance is \(>100\)-fold smaller than that of Ca\(_V\) channels, making single-channel currents too small to measure directly; based on noise analysis, the unitary Ca\(^{2+}\) conductance is only \(\sim 10–35\) fs (299, 458). In the absence of extracellular divalent ions, CRAC channels conduct Na\(^+\) with a unitary conductance of \(\sim 1\) pS and low permeability to Cs\(^+\) (299). Ca\(^{2+}\) modulates CRAC channel activity in several distinctive ways. On a millisecond time scale, the channels undergo fast Ca\(^{2+}\)-dependent inactivation (CDI) due to binding of Ca\(^{2+}\) close to the intracellular face of the pore (105, 152, 459). Over seconds to tens of seconds, intracellular Ca\(^{2+}\) accumulation causes slow inactivation (276, 460), while extracellular Ca\(^{2+}\) potentiates channel activity by severalfold (54, 461). CRAC channels are insensitive to most of the common Ca\(_V\) channel inhibitors, but are inhibited by a number of weakly selective compounds (reviewed below) as well as by submicromolar concentrations of lanthanides (9, 324, 430). Overall, the combination of store-dependent activation, lack of V-dependent activation, high Ca\(^{2+}\) selectivity and low conductance, low Cs\(^+\) permeability, Ca\(^{2+}\)-dependent inactivation and potentiation, and high sensitivity to lanthanide block emerged as a stringent set of criteria by which to screen candidates for the CRAC channel gene.

D. The Search for the CRAC Channel and Its Activation Mechanism

From 1992–2005, numerous candidate genes were proposed to encode the CRAC channel, and many mechanisms were hypothesized to explain its coupling to ER Ca\(^{2+}\) content (283). Based on early speculation that "Drosophila" TRP channels were store operated (419), the most prominent CRAC channel candidates were mammalian TRP homologs (including TRPC1, TRPC3, and TRPV6) (67, 251, 295, 435). However, when expressed heterologously their Ca\(^{2+}\) selectivity and conductance failed to match those of \(I_{CRAC}\) (399), and it was not clear in many cases whether the channels were truly store operated (74). In the meantime, ironically the founding member of the family, dTrp, was definitively shown to be store independent (313). Additional confusion arose when the use of Mg\(^{2+}\)-free intracellular solutions inadvertently resulted in the slow induction of Mg\(^{2+}\)-inhibited cation (MIC/TRPM7 channel) currents (140, 177, 301) that were initially mistaken for CRAC channel currents (169, 170).

The mechanism coupling ER Ca\(^{2+}\) depletion to CRAC channel activation was also obscure. Among over a dozen proposed mechanisms (282, 283), the three most extensively studied were a diffusible activating factor released from the ER, called calcium influx factor (CIF) (316), insertion of active CRAC channels in the PM by membrane fusion (426), and conformational coupling between the CRAC channel and a Ca\(^{2+}\) sensor protein in the ER (22,
Most proposals were supported at best by indirect evidence, and were best by conflicting data from followup studies so that none achieved wide acceptance (283). In retrospect, the conformational coupling model (158) came closest to the actual mechanism as we now know it, although the molecular components as originally proposed were incorrect (IP₃ receptors in the ER serving as Ca²⁺ sensors that interact with and open IP₄ receptor channels in the PM; Ref. 158).

A number of factors compounded the difficulty of identifying molecular constituents in the SOCE pathway. No biological tissue was known to express a sufficient abundance of SOCE components to support an approach based on biochemical purification. The absence of specific, high-affinity inhibitors for SOCE precluded approaches based on ligand binding. Cells commonly used as heterologous gene expression systems display endogenous SOCE so that expression cloning was undermined by high background activity, and attempts to use CRAC-deficient mutants (95, 342) for expression cloning were unsuccessful. Finally, the properties of the CRAC channel are unique compared with those of all other known channels, making a homology-based search risky at best (which was later borne out by the unique sequences of the Orai gene family members). Fortunately, the introduction of RNAi screening and advances in human linkage analysis methods ultimately made possible the identification of the STIM and Orai protein families in 2005–2006.

E. Identification of STIM Proteins as Ca²⁺ Sensors for SOCE

STIM (stromal interaction molecule) proteins were initially discovered in a screen for stromal cell transmembrane and secreted proteins that bind to pre-B lymphocytes (273). Mammals express two homologs, STIM1 and STIM2. Both are type I single-pass ER membrane proteins with a luminal NH₂ terminus and a cytoplasmic COOH terminus with >74% sequence similarity (54% sequence identity) and molecular weights of 77 kDa (STIM1) or 84 kDa (STIM2) (for reviews, see Refs. 43, 61, 357, 371). While early studies linked STIM proteins to suppression of tumor growth (285, 327), a role in Ca²⁺ signaling was unsuspected until two groups rediscovered them in focused RNAi screens for inhibitors of TG-evoked SOCE. Following the recognition that Drosophila S2 cells express a CRAC-like current (430), Stauderman, Cahalan, and colleagues screened these cells for suppression of SOCE using 170 dsRNA probes that included channel-like domains, transmembrane regions, Ca²⁺-sensing domains and TRP and other putative SOC genes. They identified STIM dsRNA as the sole potent inhibitor of SOCE and the CRAC current in their library (321). While Drosophila expresses only one STIM isoform (dSTIM), vertebrates express two homologs, STIM1 and STIM2. Importantly, they showed that knockdown of STIM1 suppressed SOCE and IₐCRAC in Jurkat T cells and SOCE in HEK 293 cells, firmly linking the protein to CRAC channel function. During the same period, Liou, Meyer, and colleagues (199) performed a similar siRNA screen for suppression of SOCE in HeLa cells based on 2,304 proteins with known signaling domains and independently identified STIM1 and STIM2 as the strongest hits.

The first clue that STIM proteins function as the long-sought ER Ca²⁺ sensors came from their intracellular location and the organization of their functional domains. STIM1 is primarily localized throughout the ER in resting (i.e., Ca²⁺-replete) cells (223, 244), but fluorescently labeled STIM1 was seen to redistribute into clusters (“puncta”) near the PM upon store depletion (199, 443). STIM1 also was known to be a single-pass transmembrane (TM) protein with a Ca²⁺-binding EF hand domain predicted to lie within the ER lumen (FIGURE 2). The notion that STIM proteins act as ER Ca²⁺ sensors for SOCE was quickly accepted, largely because SOCE was not merely reduced by STIM knockdown but was also activated by mutagenesis. Overexpression of STIM1 proteins with EF hand mutations designed [and later confirmed (155)] to reduce Ca²⁺ affinity (D76A, D76A/D78A, E87A) triggered constitutive formation of puncta as well as activation of SOCE and IₐCRAC in unstimulated cells (199, 239, 362, 443), essentially mimicking the effects of ER Ca²⁺ depletion (TABLE 1).

In the years since these pioneering studies, quantitative in vivo and in vitro measurements have added further support for the role of STIM proteins as ER Ca²⁺ sensors for SOCE. The Ca²⁺ affinity of the isolated EF-SAM domains of STIM1 (~200 μM) (367) and STIM2 (~500 μM) (449) in vitro are comparable to the Kᵦ₅ᵢ values for the redistribution of STIM1 (187–210 μM) and STIM2 (406 μM) into puncta in intact cells (32, 209). Furthermore, the [Ca²⁺]ER dependence of STIM1 redistribution into puncta and IₐCRAC activation are indistinguishable (209). These complementary studies demonstrated that STIM1 is the Ca²⁺ sensor for SOCE and that the store-dependent activation of the CRAC channel can be entirely accounted for by the release of Ca²⁺ from STIM1.

STIM1 and STIM2 are glycosylated and are constructed from a number of structural modules, including several protein interaction motifs (FIGURE 2). Among the most critical for SOCE are the EF hand and sterile alpha motif (SAM) domains on the luminal side, and on the cytoplasmic side several coiled-coil domains, including the CRAC activation domain (CAD; also known as STIM-Orai activation region, or SOAR) which binds to Orai, and a polybasic domain at the extreme COOH terminus which interacts with the PM. The functions of these and other protein interaction domains are described in greater detail below. For simplicity
the CAD/SOAR domain will be referred to as the CAD domain throughout this review.

F. Identification of Orai Proteins as Store-Operated Ca$^{2+}$ Channels

Soon after the emergence of STIM proteins as SOCE Ca$^{2+}$ sensors, several groups independently converged on the CRAC channel gene using RNAi approaches and human genetic linkage analysis. Beginning in the 1990s, several human patients were identified with severe combined immunodeficiency (SCID) that was attributable to the absence of CRAC channel function (102, 104, 286). Rao, Feske and colleagues (103) used modified linkage analysis and positional cloning to localize the mutated gene to a region of chromosome 12 covering ~74 genes (103). In the same lab, a genome-wide RNAi screen in S2 cells for inhibition of nuclear translocation of NFAT (a marker for sustained SOCE) yielded a number of hits, one of which (olf186-F) had a homolog in the region of human chromosome 12 identified by the linkage analysis. This molecule, a widely expressed and previously uncharacterized 33-kDa cell surface protein with four predicted transmembrane domains and intracellular NH$_2$ and COOH termini, was named Orai1. At the same time, the Kinet and Cahalan groups (398, 446), conducting their own screens of the S2 RNAi library for suppression of SOCE, identified the same olf186-F gene in S2 cells, and Vig et al. (398) named the closest mammalian homolog CRACM1 (CRAC modifier 1). In all, three Orai homologs (Orai1-3, or CRACM1-3) were identified in the human genome. Although all three proteins are highly homologous to each other (~62% overall identity, increasing to ~92% in the transmembrane domains), they exhibit negligible sequence similarity to other ion channels, which likely explains the failure of earlier homology-based searches to identify them. An overview of the transmembrane topol-
ogy and functional domains of the Orai family members is depicted in FIGURE 3.

Initial studies quickly linked Orai1 and Drosophila Orai (dOrai) to CRAC channel function. First, the SCID patients were found to be homozygous for a single missense mutation in Orai1 (R91W) that abrogated CRAC channel activity, and transformation of the patient’s T cells with the wild-type Orai1 gene rescued SOCE and $I_{\text{CRAC}}$ (103). Knockdown of dOrai suppressed the endogenous CRAC current in S2 cells (446). Overexpression of Orai1 with STIM1 in HEK293 cells generated store-operated currents up to 100 times larger than endogenous $I_{\text{CRAC}}$ that displayed the ion selectivity, pore dimensions, and pharmacological sensitivity of native CRAC channels (239, 291, 358, 420, 446). These results suggested that STIM and Orai alone may be sufficient to reconstitute the basic SOCE mechanism, an idea that was later supported by in vitro reconstitution studies (453). However, one should note that these studies were conducted with massively overexpressed STIM and Orai; at the much lower expression levels typical of native cells, STIM and Orai function may be regulated in important ways by other proteins (242, 363, 364) (discussed below). An interesting footnote is that overexpression of Orai1 alone actually suppresses endogenous SOCE in many cells (147, 206, 239, 291, 358) by reducing the STIM1:Orai1 binding stoichiometry (147). Thus, in retrospect, it was fortuitous that STIM1 was discovered before Orai1; otherwise, Orai1 might have been identified as a CRAC channel suppressor based on effects of overexpression alone.

While the overexpression and knockdown studies as well as the inhibitory effect of the R91W mutation suggested that Orai1 could be the CRAC channel structural gene, they did not exclude the possibility that it merely encoded an accessory protein required for channel opening. The known role of glutamate side chains in tuning the selectivity of $Ca^{2+}$-selective channels (334) suggested a way to distinguish these possibilities. Several groups soon reported that mutagenesis of highly conserved acidic residues in the transmembrane domains of hOrai1 and dOrai significantly reduced the
Ca\(^{2+}\) selectivity and lanthanide sensitivity of CRAC channels or blocked conduction (297, 396, 429) (FIGURE 3; discussed in sec. V). Just as mutagenesis of the EF hand helped establish STIM1 as a Ca\(^{2+}\) sensor for SOCE, the effects of altering acidic residues on selectivity and block quickly led to the acceptance of Orai1 as a key component of the CRAC channel pore.

While Orai1 is most closely associated with the endogenous CRAC channel of mast cells and T cells, all three Orai homologs produce Ca\(^{2+}\)-selective store-operated channels when coexpressed with STIM1 in HEK293 cells (72, 126, 133, 200, 239), although differences in ion selectivity, activation, depotentiation, pharmacology, affinity for STIM1, redox sensitivity, and activation by STIM2 have been reported (29, 72, 109, 190, 200) (discussed below). Overall, Orai1 is the best characterized of these proteins and has proven the most popular for structure-function studies of SOCs due to its close similarity to the single Orai proteins found in Drosophila and C. elegans, well-established functional links with endogenous CRAC channels and effector functions in many immune cells including human T cells, as well as its relatively high expression level in heterologous systems. Despite the ability of homomeric Orai proteins to form SOCs, it is noteworthy that Orai1 and Orai3 proteins may also assemble as heteromultimers to form STIM1-dependent but store-independent Ca\(^{2+}\) channels that are regulated by arachidonic acid (ARC channels) (245, 247, 386) or leukotriene C\(_4\) (LRC channels) (120). In this way, heteromultimeric assembly may serve to increase the functional diversity of the Orai channel family.

It is important to note that identification of the CRAC channels and Orai proteins as store-operated does not preclude other channels from functioning in a similar way. For example, several TRPC channels have been proposed to act as SOCs (3, 203, 413), although this assignment continues to be debated (53, 58, 74). The most compelling argument in favor of TRPCs as SOCs comes from charge-swapping experiments by Muallem and colleagues in which charge reversal of either the last two lysines in STIM1 (K684/K685) or two key aspartate residues in TRPC1 (D639/D640) abrogated TRPC1 activity, while the two mutants expressed together restored activity (189, 441). On this basis, Zeng et al. (441) proposed an electrostatic model in which positive and negative charges on either protein must interact for STIM1 to activate TRPC1. Despite this evidence, it has been difficult to build a widely accepted case for TRPCs as SOCs, largely due to a number of confounding factors (53, 125). TRPC channels respond to diverse stimuli, including DAG, G proteins, Ca\(^{2+}\), and redox compounds, and several of the TRPC proteins form heteromultimers with other TRPC members which can alter their preferred mode of activation (434a). The complexities introduced by heteromultimer formation have been put forward to explain the inconsistent conclusions among laboratories regarding TRPC store dependence (53). Added to this is the complication that many studies use agonists of PLC-linked receptors to activate TRPC channels, making it difficult to separate the stimulatory effects of biochemical pathways from those of [Ca\(^{2+}\)]\(_{ER}\). As a result, there is as yet no definitive measurement of the dependence of TRPC activity on [Ca\(^{2+}\)]\(_{ER}\), or on store depletion alone without parallel activation of receptors and associated biochemical pathways. A final complication is that TRPC channel activity in some contexts also depends on Orai1 activity, which has been attributed by Ambudkar and colleagues to the insertion of TRPC channels into the PM in response to Ca\(^{2+}\) influx through Orai1 (48) but may also involve activation by Ca\(^{2+}\) entering through Orai1 (125). A complex picture is emerging in which the behavior of TRPC channels in a particular cell may depend on the relative amounts and accessibility of STIM1, Orai1, and the various TRPC homologs. The difficulty of measuring and controlling these parameters poses persistent challenges to achieving wider acceptance of the function of TRPCs as SOCs in a physiological as opposed to an experimental setting (53, 58, 74).

G. Evolution, Tissue Distribution, and Diversity of STIM Isoforms

STIM proteins are highly conserved in metazoans from C. elegans and Drosophila to Homo sapiens. STIM homologs are not found in lower organisms including yeast and Dictyostelium, and first appeared during evolution with the choanoflagellates (Monosiga brevicollis) (61). While the Ca\(^{2+}\)-sensing NH\(_2\)-terminal regions of STIM proteins are fairly well conserved from worms and flies to humans, there is wide divergence in the cytosolic domains. Invertebrate STIM lacks several domains COOH-terminal to the coiled-coil domains, including the proline-rich and polybasic domains. A phylogenetic analysis revealed that STIM may have evolved in two stages from invertebrate to vertebrate forms (37). The missing COOH-terminal domains first appeared in the primordial vertebrate form of STIM expressed in urochordates (sea squirts). Subsequently, a gene duplication event that is thought to have occurred at the start of the vertebrate Euteleostomi lineage created the STIM1 and STIM2 isoforms. Interestingly, a second duplication in bony fishes generated four divergent STIM homologs that may have evolved to carry out additional functions (37). The presence of specific regulatory domains in STIM proteins varies according to species and protein isoform; although in most cases the consequences of this variation are unknown, the differences between STIM1 and STIM2 are believed to create distinct but overlapping functions as described below.

In mammals, STIM proteins are broadly expressed in many organs and tissues. Northern blot analysis in human tissue samples reveals robust expression of STIM1 and STIM2
RNA in pancreas, skeletal muscle, brain, and heart as well as modest levels in liver and lung (407). Expression analysis with affinity-purified antibodies confirms the presence of both STIM1 and STIM2 in a wide variety of human cells and cell lines (223, 407). Additionally, a STIM1-LacZ fusion protein is strongly expressed in all types of muscle as well as cerebellum, spleen, and thymus (373). There is also evidence that the expression may be developmentally regulated, as STIM1-LacZ expression increases significantly during myotube differentiation and correlates with increased SOC activity (373). Coexpression of STIM1 and STIM2 in the same tissues and cells raises the possibility of heteromeric association. Indeed, immunoprecipitation analyses of endogenous proteins in cell lines expressing both isoforms as well as FRET between labeled STIM1 and STIM2 indicates that they can form heterooligomers in situ (70, 315, 407). Given differences in the response of the two isoforms to Ca\(^{2+}\) store depletion and their ability to activate CRAC channels (32, 402), heteromerization of these proteins may have important implications for the amplitude and dynamics of SOCE-mediated Ca\(^{2+}\) signals (270).

The diversity of STIM protein function is further increased by alternative splicing. An alternatively spliced long variant of STIM1 (STIM1L) has been described in which an extra 106 residues are inserted in the STIM1 cytosolic domain (FIGURE 2) (69). STIM1L is expressed in several tissues but most prominently in muscle, and its abundance increases during myotube differentiation. The insert in STIM1L is an actin-binding domain and leads to altered localization and activation kinetics (69). Two recent reports describe a STIM2 splice variant (STIM2\(\beta\), or STIM2.1) in which a highly conserved set of eight residues is inserted in the CAD domain (FIGURE 2), converting it into the first known inhibitory STIM protein (243, 315). STIM2\(\beta\) cannot bind appreciably to Orai1 by itself, but is recruited to Orai1 by forming heterodimers with STIM1 or STIM2, and inhibits SOCE in part through sequence-specific, direct inhibition of Orai channel function (315). Considering >95% of all multiexon loci in vertebrates are alternatively spliced to greatly expand the functional diversity of a limited number of genes, more STIM splice variants are to be expected.

## H. Evolution, Tissue Distribution, and Diversity of Orai Isoforms

Orai proteins are expressed in metazoans from nematodes to primates (38). Orai homologs first appeared in evolution with nematodes and insects, but proteins with distant homology to Orai are found even in organisms such as green algae and moss that do not express recognizable STIM proteins, suggesting activation through other mechanisms (61). Invertebrates express a single Orai isoform (38, 61). The first appearance of two Orai homologs is associated with vertebrates, and Orai3 appears even later, only in mammals, and appears to have evolved from Orai1 from a gene duplication event. Thus, in evolutionary terms, Orai1 is the oldest family member, and Orai3 the newest (347). Orai3 has a significantly longer III–IV loop than Orai1 and little sequence similarity in this region, as well as a shorter COOH terminus that exhibits a greater coiled-coil probability than the COOH terminus of Orai1 (109).

Evidence from a variety of techniques (Northern blot, Western blot, rtPCR, immunohistochemistry) indicates broad tissue expression of Orai transcripts or proteins in mammals (126, 132, 133, 411). A monoclonal antibody that specifically targets the second extracellular loop of Orai1 has provided evidence that rodents and primates have similar expression patterns of Orai1 except in male gonad tissue (132). In addition to immune cells such as macrophages and lymphocytes, significant expression of Orai1 was also reported in the brain, pancreas, kidney, and skin. Genetic studies in humans and knockout studies in mice complement these expression profiles and confirm an important role for Orai1 in a variety of immune cells (T cells, mast cells, B cells, NK cells) as well as nonimmune cells (fibroblasts, skeletal muscle, platelets, microglia, endothelial cells, smooth muscle, hepatocytes, and neural stem cells) (12, 34, 103, 133, 233, 269, 360, 373, 393). The expression of Orai1 in the nervous system, especially in the cortex and hippocampus is noteworthy and raises the prospect of important functions in the brain (see sect. VIII). Comparable expression studies of Orai2 and Orai3 are lacking, due to the scarcity of high quality antibodies that can distinguish among the Orai isoforms. However, from analysis of RNA levels and ability of the small molecule 2-aminodiphenylborate (2-ABP) to stimulate Orai3 channel activity (see sect. VII), some reports have indicated that Orai3 expression is enhanced in cancerous cells and promotes tumorigenesis (252, 253). These differences in expression may have important implications for cell function as well as development of isoform-specific drugs for therapeutic applications.

Expression studies have revealed that the three mammalian Orai homologs are often present in overlapping patterns in different tissues, including but not limited to the brain, the lung, and immune cells (126, 133, 411). This begets the question of whether there exist heteromultimeric channels, and if so, what are their unique functional features? Recombinant overexpressed Orai homologs clearly interact with each other, as shown by coimmunoprecipitation experiments and the ability of a nonconductive mutant of Orai1 to act as a dominant negative for all three Orais (200). The functional diversity of multimeric Orai channels, however, remains largely unknown. In one study, coexpression of Orai1 and Orai3 channels yielded store-operated currents with reduced Ca\(^{2+}\) selectivity compared with homomeric Orai1 or Orai3 channels (336; but see Refs. 245, 247). The reduction in Ca\(^{2+}\) selectivity was attributed to differences in acidic residues in the first extracellular loop of Orai1 and Orai3 (E85 in Orai3 vs. D110 in Orai1). However, Orai1 and Orai3 channels also differ in

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their COOH termini, which can affect the strength of STIM binding (109), potentially generating secondary effects on ion selectivity (237).

While alternatively spliced isoforms of Orai proteins have not yet been described, a short form of Orai1 is generated by translation initiation at a second site (M64/71) (113). Both forms are expressed in most human cell lines, and with heterologous expression the relative amount of each is determined by the strength of the Kozak sequence in the expression vector. The shorter form lacks a polybasic sequence that may interact with negatively charged phospholipids and appears to diffuse faster than its “full-length” counterpart, although the functional consequences of this are not known (113).

III. MOLECULAR CHOREOGRAPHY OF STORE-OPERATED CALCIUM CHANNELS

A. Overview

The activation process of CRAC channels is highly unusual in that it involves contact between membrane proteins located in two different cell compartments, the ER and the PM. In most cells, 75–85% of endogenously expressed STIM1 resides in the ER membrane, with the remainder in the PM (223, 244). Early studies were divided on the issue of whether STIM1 must translocate to the PM to evoke SOCE (142, 208, 362, 443) or whether it functions within the ER membrane (199, 219, 256, 332, 354). Interestingly, one study suggests that ER refilling by itself may not be sufficient for reverting the SOCE process and may require Ca\(^{2+}\) entry through Orai1 into the cytosol in addition to the rebinding of luminal Ca\(^{2+}\) to the STIM1 EF hand (344) (see also Ref. 219). The Ca\(^{2+}\) sensor for this process is not known, but SARAF (274), CRACR2A (364), and CaM bound to the PBD (17) or other parts of STIM1 (111) are possible candidates.

It is interesting to note that while the choreographic process underlying SOCE is unique, functional coupling between Ca\(^{2+}\) signaling proteins in the PM and internal organelles is not without precedent. In the triadic junctions of skeletal muscle, depolarization-activated Ca\(_{\text{c}}\) channels (DHPR) in the PM bind directly to ryanodine receptors (RyR) in the sarcoplasmic reticulum (SR) to trigger Ca\(^{2+}\) release, a process called excitation-contraction (e-c) coupling. While SOCE and e-c coupling are similar in terms of signal transmission by protein contacts across a narrow 10- to 20-nm gap, they differ in that signal propagation during e-c coupling is outside-in rather than inside-out, and RyR-DHPR complexes in muscle are preassembled while the SOCE complexes form only “on demand,” i.e., after ER Ca\(^{2+}\) depletion. Accordingly, SOCE in most cells develops over seconds to tens of seconds, being limited by the time it takes STIM1 and Orai1 to accumulate by passive diffusion, as well as possible delays in binding and activation (43, 414). In contrast, SR Ca\(^{2+}\) release occurs within milliseconds of PM depolarization, being limited only by the kinetics of protein conformational changes and thereby ensuring the precision, reliability, and speed of e-c coupling (83, 323). Interestingly, STIM1 and Orai1 in skeletal muscle appear to be prelocalized at the triad (373, 406), and SOCE is reported to activate within milliseconds of SR Ca\(^{2+}\) depletion (91, 187), most likely because of the removal of protein diffusional delays. The underlying mechanisms that prelocalize STIM1 and Orai1 to these sites are not well understood.

B. The ER-PM Junction: the Nexus of Store-Operated Calcium Entry

Initial light microscopy studies showed that upon store depletion YFP-STIM1 puncta formed near the PM (within ~200 nm, the length constant of the TIRF evanescent field) (199). Subsequent electron microscopic localization of a
STIM1-horseradish peroxidase chimera (HRP-STIM1) indicated that in store-depleted cells STIM1 accumulates at ER-PM junctions that correspond to the puncta visible at the light microscopic level (FIGURE 4) (414). Together with observations that STIM1 and Orai1 are colocalized in puncta and that Ca\(^{2+}\) entry is precisely restricted to these sites, these studies defined the elementary unit of SOCE to be clusters of closely apposed STIM1 and Orai1 at ER-PM junctions (210). EM studies in different cells are in general agreement that STIM1 accumulates at sites where the average ER-PM gap ranges from 10 to 17 nm (212, 272, 414). A detailed immunoelectron microscopic analysis by Orci et al. (272) delineated different types of junctional ER, including “cortical” (cER) and “thin cortical” structures where the tubules narrow from a diameter of ~70 to ~25 nm and exclude KDEL-containing ER proteins. In this study, store depletion caused STIM1 accumulation only in thin cER, whereas in the initial work by Wu et al. (414), cER containing HRP-STIM1 appeared similar to cER visualized with an ER-targeted, KDEL-containing HRP. The basis for this difference and possible functional distinctions between cER and thin cER are not yet understood.

The ability of STIM1 to return to the same sites after multiple rounds of depletion and refilling suggests that ER-PM junctions are stable over minutes, at least at room temperature (199, 354). In general agreement, EM studies have reported that the majority of cortical ER-PM junctions preexist in resting cells, but that their number increases to varying extents after store depletion (272, 414). This increase is not due to a bulk translocation of ER towards the PM (414) but is instead likely to involve movements of individual tubules. Junction stability and the store dependence of junction abundance may vary among cells, as polymerization of cortical actin, which is thought to interfere with junction formation, inhibits SOCE in some cells but not others (210, 289).

The proteins involved in creating and maintaining ER-PM junctions are a subject of intense interest (306). STIM1 itself
could be involved through multiple mechanisms. STIM1 is known to bind to the microtubule (MT) tip attachment protein EB1 through its TRIP sequence (Figure 2), and STIM1 knockdown reduces the rate of ER tubule formation, suggesting that endogenous STIM1 regulates the formation and extension of new ER tubules (123). In this way, STIM1 could promote junction formation by enabling MTs to drag nascent ER tubules towards the PM. Such a role would be consistent with observations of microtubules closely associated with precortical and cortical ER (272, 345). STIM proteins could also promote junction formation through interactions of their COOH-terminal polybasic domains with the PM. Overexpression of STIM1 is well known to increase the spatial extent of junctions; with high enough expression, this extent can increase from a baseline level of a few percent of the cell surface to cover the entire footprint of the cell (272, 394, 414). STIM2 may also play a role (94) as it has a higher affinity for PIP2 (26) and is prelocalized at junctions in resting cells (32). Thus STIM proteins could potentially function both in the extension of ER towards the PM and in promoting adhesion forces to help stabilize ER-PM junctions.

Another promising candidate for enabling ER-PM junction formation is the extended synaptotagmin family (45, 119). These ER membrane proteins localize to ER-PM junctions and interact via C2 domains with PIP2 in the PM (119). Interestingly, the E-Syts may link Ca2+ signaling and PIP2 homeostasis, as a local rise in [Ca2+]i, appears to recruit E-Syt1 to junctions (119) where it pulls the ER closer to the PM and recruits Nir2, a phosphatidylinositol transfer protein which helps replenish PIP2 in the PM (45). Surprisingly, while knockdown of all three E-Syts greatly reduced the number of junctions in HeLa cells, it had little effect on SOCE, raising the possibility that E-Syts may be more directly involved in controlling lipid transfer than Ca2+ entry (119). The ER membrane protein jucnate is a third candidate for promoting ER-PM contacts, as it localizes to junctions, and jucnate overexpression increases the number and sizes of junctions (388, 389) while knockdown reduces SOCE (363, 388). Jucnate is also reported to have a luminal EF hand which senses [Ca2+]ER and may enhance the accumulation of STIM1 at junctions after store depletion by binding to the luminal STIM1 domain (363).

Further studies and approaches will be needed to positively identify the mechanisms involved in ER-PM junction formation and turnover. Unfortunately, existing tools for studying junctional dynamics in living cells are quite limited; all of the current marking methods involve probes that interact in some way with both the ER and PM and therefore perturb junctional stability. This caveat applies to STIM1 as well as a rapamycin-inducible heterodimerizer system that irreversibly links the ER and PM (394). A recent study described an ER membrane protein engineered to constitutively localize to junctions through binding of a COOH-terminal polybasic domain to the PM, much like STIM1/2, but without interacting with STIMs (45). The general challenge in applying such tools to study junction dynamics will be to reduce expression to a low enough level that they do not affect junctional stability or dynamics yet generate enough signal to illuminate junctional contacts with high sensitivity.

C. STIM Activation and Accumulation With Orai at ER-PM Junctions

The maintenance of ER Ca2+ homeostasis and generation of receptor-activated SOCE signals depends critically on the precise control of STIM protein activity. In cells with replete Ca2+ stores, STIM1 must be kept in an inactive state and diffusely localized throughout the ER, yet after ER Ca2+ depletion it must effectively adopt an active conformation and become highly concentrated at ER-PM junctions where it can interact with Orai1 to generate SOCE. Many of the critical events in this process are becoming understood and support a diffusion trap mechanism that commences with Ca2+ release from STIM1, followed by oligomerization and conformational changes that trap STIM1 at junctions and enable it to bind and trap Orai1. This section reviews the current understanding of how these events are controlled at a molecular level.

1. The resting (inactive) state of STIM: structural basis and mobility

Multiple lines of evidence suggest that STIM1 is a dimer in resting cells with full Ca2+ stores. STIM1 coimmunoprecipitates with itself through interactions involving the cytoplasmic region (11, 408), and isolated fragments of the cytosolic domain generally form dimers in solution (154, 254, 453, 455). Although the CC1 domain by itself can support some dimerization, these oligomers are unstable (64) as CC1-CC1 interactions are inherently weak (see also Ref. 455). The CAD domain appears to play the dominant role in determining the dimeric stoichiometry of STIM1, as STIM1-CAD (residues 1–448) is a stable dimer in solution (64) as CC1-CC1 interactions are inherently weak (see also Ref. 455). The CAD domain appears to play the dominant role in determining the dimeric stoichiometry of STIM1, as STIM1-CAD (residues 1–448) is a stable dimer in solution (64), and cytosolic fragments containing CC1+CAD are dimeric in solution (254, 455).

The physical basis for the dimerizing ability of CAD is not yet clear. A crystal structure of human CAD (residues 345–444) reveals several reciprocal hydrophobic and hydrogen bond interactions between the two CAD monomers which have been proposed to hold the dimer together (Figure 5, D AND E) (424). Alanine substitutions at these locations prevented STIM1 accumulation in puncta and interactions with Orai1 (424), but the effects on STIM1 dimerization need to be tested directly. The structure also revealed extensive interactions between the CC2 and CC3 domains within each monomer that are expected to stabilize the closed structure of the protein (Figure 5D).
The structure of dimeric full-length STIM1 in its resting inactive state is not known. However, the conformation of the luminal EF hand and SAM domains and how it regulates the stability of the resting state is relatively well understood. In the presence of Ca$^{2+}$, the isolated EF-SAM domain in solution is a relatively compact and stable monomer (367). Based on NMR solution structures of Ca$^{2+}$-bound EF-SAM fragments from STIM1 and STIM2, the EF-SAM
domain is predominantly α-helical and consists of a canonical EF hand (cEF) with a typical helix-loop-helix structure that binds one Ca$^{2+}$ ion between loops 1 and 2 (amino acids 73–88 in STIM1, amino acids 87–103 in STIM2); a noncanonical EF hand (nEF) that does not bind Ca$^{2+}$ (but which helps stabilize the canonical EF hand through hydrogen bonding); and the SAM domain (Figure 5C) (370, 450). With Ca$^{2+}$ bound in the resting state, the two EF hands of each monomer are in the “open” conformation, creating a hydrophobic cleft which binds hydrophobic residues extending from the SAM domain to stabilize the compact monomeric structure (Figure 5C).

STIM1 and STIM2 differ in their functional properties, and these differences may be traced in large part to differential stability of their resting states. Unlike STIM1, STIM2 is partially active in resting cells, attributable to its lower overall affinity for Ca$^{2+}$ (32). Its lower Ca$^{2+}$ affinity allows STIM2 to respond to changes of [Ca$^{2+}$]$_{ER}$ around the resting level and engage in homeostatic functions, whereas STIM1 responds only to the larger extent of store depletion generally resulting from stimulation of PM receptors (32, 209). A series of cEF and SAM chimeras (450) showed that for STIM1 a higher affinity cEF hand is paired with a relatively less stable SAM domain (producing weaker cEF-SAM binding). In contrast, the cEF hand of STIM2 has a lower affinity for Ca$^{2+}$, but increased hydrophobic and electrostatic interactions make its EF-SAM domain more stable. Interestingly, these properties create metastable states, such that STIM1 responds only to larger degrees of store depletion but does so rapidly, while STIM2 responds to smaller changes in [Ca$^{2+}$]$_{ER}$ but in a slower, more damped fashion. These characteristics appear to be well matched to the agonist response versus homeostatic roles of the two proteins (369, 450). The poorly conserved random coil STIM sequences NH$_2$-terminal to the EF-SAM domain also enhance the stability of STIM1 and STIM2 resting conformations (369). Chimeric swap experiments show that the NH$_2$-terminal region of STIM2 reduces the speed and extent of CRAC channel activation relative to that of STIM1 (452). Thus, together with the EF-SAM domain, the NH$_2$ terminus acts to dampen activation of SOCE by STIM2. These results raise the possibility that large variations observed in the sequence and length of the NH$_2$-terminal regions of STIM from different species may generate species-specific differences in the behavior and functions of STIM proteins.

Mobility of STIM1 in cells has been studied using fluorescence recovery after photobleaching (FRAP) as well as single-particle tracking. FRAP studies suggest that STIM1 in cells with full Ca$^{2+}$ stores diffuses passively within the ER membrane at a slow rate (~0.1 μm$^2$/s) compared with many single-pass ER membrane proteins (64, 198). Its speed is greatly affected by the cytosolic domain; while STIM1-ΔC (STIM1 amino acids 1–237) diffuses at a similar speed as many other ER membrane proteins, the addition of CC1 (amino acids 1–344) or the entire cytosolic domain (amino acids 1–685) slows it down by approximately twofold (64). These results suggest that STIM proteins are slowed significantly by interactions of their cytosolic domains with elements of the cytosolic environment. These ensemble measurements have been recently extended by single-particle tracking methods, which confirm that STIM1 moves by Brownian diffusion in the resting state (416).

A second mode of STIM1 transport arises from association with MTs. This mode is particularly evident when fluorescently tagged STIM1 is overexpressed, generating a striking pattern of fluorescent “comets” that radiate in relatively linear paths from a central site, presumably the microtubule organizing center (11, 198, 415). This behavior has been attributed to STIM1 binding via its TRIP sequence to end-binding (EB) proteins that associate with the plus ends of microtubules (123) (Figure 2A). A combination of immunohistochemistry, coimmunoprecipitation, biochemistry, and in vitro studies of purified proteins indicates that STIM1 associates with EB1 and EB3 at MT tips (123, 146). While STIM1 moves both along existing microtubules and with the growing plus-end of microtubules, only the association with MT ends is enhanced by STIM overexpression. Although this mode of MT-associated transport was initially thought to be involved in the delivery of STIM1 to ER-PM junctions (11), later work deemed this unlikely, as knockdown of EB1 or stabilization of microtubules with taxol failed to affect puncta formation or SOCE (123). In addition, single-particle tracking of GFP-STIM1 failed to find evidence for active transport, suggesting that MTs make a negligible contribution to STIM1 transport at endogenous levels of expression (416).

2. Sensing of ER Ca$^{2+}$ depletion by STIM: conformational changes and oligomerization

Activation of STIM proteins is initiated by the release of Ca$^{2+}$ from the luminal EF hand, which triggers unfolding of the EF-SAM domain and conformational changes in both luminal and cytosolic domains. The $K_D$ for Ca$^{2+}$ binding of the isolated EF-SAM domains of STIM1 and STIM2 in solution are ~200 and ~500 μM, respectively (450). Structural and biochemical studies of the isolated STIM1 EF-SAM domain show that unfolding of the EF-SAM domain exposes hydrophobic surfaces that promote the formation of dimers and higher order aggregates in solution (367). These findings led Ikura, Stathopulos, and colleagues (367) to make the initial proposal that oligomerization of the EF-SAM domains initiates the STIM activation process, an idea supported later by observations that mutations designed to destabilize interactions of the EF hands with the SAM were shown to create dimers or aggregates of the peptide in solution and promote puncta formation and SOCE when introduced into full-length STIM1 (370) (Table 1). The monomeric Ca$^{2+}$-bound EF-SAM domain may
act as a "brake" on STIM activation, as its removal and replacement with a fluorescent protein promotes spontaneous puncta formation and SOCE (206).

The role of STIM1 oligomerization as an early event in SOCE also received support from FRET studies of full-length STIM1 in cells. FRET between YFP-STIM1 and CFP-STIM1 increases upon store depletion and is reversed when stores refill (64, 198, 256). The increased FRET occurs at locations within the cell that are distinct from ER-PM junctions and prior to accumulation of STIM1 at the cell periphery and FRET between STIM1 and Orai1, indicating that STIM1 oligomerization precedes binding and activation of Orai1. Likewise, refilling of stores caused STIM1-STIM1 FRET to decrease prior to STIM1-Orai1 FRET, again supporting a link between oligomerization and Orai1 binding (256).

The nature of active STIM oligomers is at this point an open question. Are they formed from the association of multiple STIM1 dimers (“interdimeric oligomers”) or do they represent dimers in which the EF-SAM domains of a single dimer are bound to each other (“intradimeric oligomers”)? An early interpretation of FRET studies was that the moderate degree of resting FRET comes from dimers containing a donor and an acceptor fluorophore, and the FRET increase after store depletion reflects the formation of interdimeric, higher order oligomers that bring more donors and acceptors together. This interpretation is supported to some degree by an approximately twofold reduction of the STIM1 diffusion rate after store depletion (64, 198), although this effect may be more directly related to increased interactions with the local environment as the cytosolic domain extends than to increased stoichiometry per se (64). Formation of higher order oligomers is also supported by the ability of STIM1-ΔC (STIM1 1–237) proteins lacking the cytosolic domain to be recruited to ER-PM junctions by wt-STIM1 after store depletion (64), presumably through interactions with the transmembrane/luminal regions of full-length STIM1.

On the other hand, the observed increases in STIM-STIM FRET could simply result from a conformational change that brings the fluorophores in a single dimer closer together (intradimeric oligomerization; for an example see Figure 5A). Based on the crystal structure of the CAD domain, Yang et al. (424) suggested that the CC1 helices in the resting state may be splayed apart. In support of this idea, FRET studies of the CT-STIM1 fragment in solution suggest that the ER-proximal ends of the dimer are well separated (455). Interestingly, bringing the two NH2 termini together via crosslinking of introduced cysteines creates a COOH-terminal conformational change that may allow the CAD domain to bind to Orai (455). A recent study by Zhou and colleagues (214) provides a different view as to how this conformational change may occur. They found

<table>
<thead>
<tr>
<th>Domain</th>
<th>Mutation</th>
<th>Reference Nos.</th>
</tr>
</thead>
<tbody>
<tr>
<td>STIM1 cEF hand</td>
<td>D76A/N, D78A/N, N80A, D84G, E87A/Q</td>
<td>11, 124, 199, 239, 260, 359, 362, 370, 443</td>
</tr>
<tr>
<td>STIM1 nEF hand</td>
<td>F108D/G110D</td>
<td>370</td>
</tr>
<tr>
<td>STIM1 SAM</td>
<td>L167R, T172R, L195R</td>
<td>370</td>
</tr>
<tr>
<td>STIM1 TM</td>
<td>I220W, C227W</td>
<td>214</td>
</tr>
</tbody>
</table>

> Naturally occurring human mutations are indicated in bold.
that the TM domains associate with each other in the membrane and that a C227W mutation in the STIM1 TM domain reorients the TM helices and causes elongation of the cytoplasmic domain and constitutive STIM1 activity (214). Thus, with the assumption that the C227W mutant mimics the structure of depletion-activated WT STIM1, Ca\(^{2+}\) depletion may activate STIM by driving the intradimeric binding of 2 EF-SAM domains, which then triggers conformational changes in the TM domain that propagate further downstream to expose the PBD and allow CAD to bind and activate Orai (FIGURE 5A). It should be noted that intradimeric binding does not preclude interdimeric associations that could create larger clusters of STIM proteins, and it is not yet clear which of these events is responsible for the FRET changes that have been reported. Clearly more work will be needed to resolve these issues, in particular to establish the conformation and stoichiometry of full-length STIM1 proteins in intact membranes. For purposes of this discussion, we will hereafter use the term oligomerization to refer to the EF-SAM-mediated process that causes the FRET increase and leads to STIM activation, without implying any particular type of oligomer.

In addition to the EF hand and SAM domains, the CAD region of STIM1 is also critical for STIM1 oligomerization in response to store depletion. Based on FRET measurements of STIM1 proteins truncated at various cytosolic locations, it appears that CC1 alone (STIM1 1–344) is not sufficient to support oligomerization, but addition of CAD (STIM1 1–448) restores the normal response (64). Within CAD, both CC2 and CC3 domains are essential for stabilizing the oligomerized state following store depletion. It is not clear whether this requirement reflects the role of CAD in stabilizing the resting dimeric state which is likely to be necessary for further conformational changes during activation, or rather a specific role of CAD in stabilizing the conformation of the activated cytosolic structure. With regard to the latter possibility, a recent FRET study has demonstrated homomultimerization to explain the role of CC3 in higher order oligomerization of STIM1 (94a).

A fundamental question is how the conformational changes associated with luminal sensing of Ca\(^{2+}\) depletion by EF-SAM drive changes in the cytosolic regions of STIM that enable targeting to ER-PM junctions and binding and activation of Orai. A concept that is emerging involves the operation of two “brakes” on STIM activation. The first is the EF-SAM domain itself, which is a compact monomer in the Ca\(^{2+}\)-bound resting state (see above), and the second is an interaction between CC1 and CAD. With store depletion, Ca\(^{2+}\) release causes EF-SAM to separate, and hydrophobic interactions could bring together the two EF-SAM domains of the dimer. This propagates a conformational change through the STIM TM domains to the cytoplasmic domain to override a CC1 structural clamp that keeps CAD in an inactive conformation, allowing it to adopt the active conformation.

The notion that CC1 interacts with CAD to keep it in an inactive state was first elaborated by Balla and colleagues (175) in the form of an electrostatic clamp between a highly conserved acidic region in CC1 (318EEELE322) and basic region in CC2 (382KIKKK386). This was based largely on the inhibitory effects of neutralizing the quartet of glutamates in CC1. Later studies showed that this CC1 acidic region is unlikely to be in proximity to the basic region in CC2 (424, 455) and that the amphipathic properties of the helix determine its inhibitory capacity rather than charge (438). However, the concept of CC1 as an inhibitory clamp on CAD activity has continued to garner support. Romanin, Hogan and colleagues have convincingly shown that the isolated STIM1 cytosolic domain assumes a compact form in the resting state, based on high FRET or LRET values observed with STIM1 fragments labeled at the ER membrane insertion site and the COOH terminus (255, 455). FRET values decline upon binding the wild-type fragments to Orai1 or introducing mutations in CC1 that activate STIM1 (4EA, L248S, L251S, L258S, or the deletion of CC1 a1 helix) leading to the idea that the cytosolic domain elongates as it transitions to the active state (94a, 255, 455). In addition, the L251S mutation increases the binding of the STIM1 fragment to PIP3, suggesting that the polybasic domain also becomes exposed as a result of elongation (455).

The structural details of the interaction between CC1 and CAD that controls activation are not yet fully understood. Yang et al. (424) initially proposed that amino acids 308–337 (helix a3) constituted an “inhibitory helix” that suppresses CAD activity, based on the 4EA results described above, a crystal structure of C. elegans CAD in which a short segment of CC1 interacts with CAD, and the activating effects of deleting residues 310–337. Mutation of Y316 in CC1 a3 also causes partial activation of STIM1 (439). However, other regions are also clearly involved in stabilizing the inactive structure. Mutations of hydrophobic residues in helix a1 of CC1 (L248, L251, L258) activate (255, 455), as do mutations of residues predicted to contribute to coiled-coil structures and likely to be involved in intramolecular interactions between CC2 and CC3 of CAD (e.g., A369, L416, L423; FIGURE 5D) (64, 255). Thus evidence points to both the a1 and a3 helices of CC1 as part of the inhibitory clamp, although their sites of interaction with CC2 and/or CC3 of CAD remain to be identified.

Zhou, Hogan, and colleagues (455) have proposed a model to explain how store depletion leads to the release of the inhibitory clamp, based on studies of STIM1 cytosolic fragments in solution. They observed that bridging the NH2 termini (the ER insertion sites) of the CT-STIM1 peptide through cysteine crosslinking caused peptide elongation similar to that described by Muik et al. (253) upon Orai1...
Several studies support the model in which the CC1 monomer interacts with CAD to maintain it in a resting (closed) conformation, and store depletion and consequent dimerization of the EF-SAM domains releases the clamp by triggering formation of the CC1 coiled-coil dimer. CC1 α1 and specifically the residue Leu-251 is critical for maintenance of the inactive state, as truncation of the CC1 α helix or the L251S mutation elongates the STIM1 cytosolic domain and activates STIM1 in the absence of store depletion (94a, 255, 455). While L251S prevents coiled-coil formation it also weakens the interaction of CC1 with CAD, potentially explaining its ability to activate STIM1. Thus, in full-length STIM1, Ca\(^{2+}\) depletion has been proposed to release the inhibitory clamp on CAD by sequestering L251 within the newly formed CC1 coiled-coil (455). In a very recent extension of this work, Zhou and colleagues (214) suggest that the inhibitory clamp includes coiled-coil interactions of L258 and L261 of the CC1 α1 helix with V419 and L416 of the CC3 helix of CAD. Although these contacts have yet to be shown experimentally, and the insights from this work are derived from studies of isolated and mutagenized STIM1 fragments, they provide a strong basis for new studies to determine the structural interactions that maintain the resting state and how they change to trigger STIM1 activation, particularly for full-length STIM1 embedded in the ER membrane.

3. SOCE is a self-organizing process

The ability of STIM oligomerization to drive the process of SOCE was tested directly by studies in which the EF-SAM domains were replaced by FKBP and FRB protein modules (209). Heterodimerization of these two domains with a rapamycin analog triggered accumulation of FRB-STIM1 and FKBP-STIM1 in puncta as well as activation of Ca\(^{2+}\) entry and \(I_{\text{CRAC}}\) without affecting Ca\(^{2+}\) store content. These results are compatible with both the inter- and intradimeric STIM activation models described above; while the observed formation of high-molecular-weight forms of STIM1 by rapamycin is consistent with higher-order oligomers, it is also possible that rapalog activated STIM1 and SOCE by bringing together the NH\(_2\) termini within single STIM1 dimers. This study demonstrated that STIM1 oligomerization is the key triggering event that couples store depletion to the activation of SOCE, and that the underlying movements and interactions of STIM and Orai which culminate in CRAC channel activation are self-organizing.

Several studies support the view that the primary if not sole function of ER Ca\(^{2+}\) depletion in SOCE is to promote STIM oligomerization, and that once oligomers have formed, all further events leading ultimately to Ca\(^{2+}\) entry are autonomous. For example, mutations of the cEF hand that reduce the Ca\(^{2+}\) affinity of STIM1 as well as mutations in the SAM domain that promote STIM1 oligomerization (Table 1) evoke constitutive formation of STIM1 puncta (32, 199, 370). Because these mutations function without a change in \([\text{Ca}^{2+}]_{\text{ER}}\), their effects demonstrate the sufficiency of STIM proteins to act as the Ca\(^{2+}\) sensors for SOCE. One question from these studies is whether the EF-SAM domain is needed to impose a particular conformational change on the STIM1 luminal domains, or whether oligomerization is all that is necessary to activate SOCE. However, the ability of artificial dimerization of FRB/FKBP-STIM1 chimeras to evoke SOCE shows that oligomerization itself, independent of the EF-SAM domain, is the key triggering event. The \([\text{Ca}^{2+}]_{\text{ER}}\) dependence of STIM1 redistribution to peripheral puncta (\(K_{0.5} = 187 \mu\text{M}, n_{1/2} = 4\)) closely matched that of \(I_{\text{CRAC}}\) activation (209) (Figure 1B), adding further evidence that ER Ca\(^{2+}\) is critical only for controlling early events (i.e., oligomerization) leading to STIM1 activation, rather than directly modulating Orai channels at ER-PM junctions. Puncta formation for both STIM1 and STIM2 are highly nonlinear functions of \([\text{Ca}^{2+}]_{\text{ER}}\) (for STIM1, \(K_{0.5} = 210 \mu\text{M}, n_{1/2} = 8\); for STIM2, \(K_{0.5} = 406 \mu\text{M}, n_{1/2} = 5\); Ref. 32). The high cooperativity of puncta formation has not been fully explained, although it may reflect the release of two Ca\(^{2+}\) ions per dimer, the association of multiple dimers to form higher order STIM oligomers, or cooperative allosteric changes in STIM.

Importantly, these studies reveal that SOCE is a self-organizing process held in check by the state of STIM oligomerization. Self-organization is a prevalent driving force that contributes to many life processes, including the cell cycle and the induction of cell polarity and control of cytoskeletal structures and cell shape, among others (166). As described below, current evidence argues for a diffusion-trap mechanism as the basis for the self-organization of the SOCE machinery.

4. A diffusion-trap model for SOCE

After store depletion, STIM and Orai remain mobile in their membrane compartments and accumulate passively at ER-PM junctions by a two-part diffusion-trap mechanism. These events have been examined in detail using single-particle tracking techniques to follow single STIM1 oligomers and Orai1 channels as they enter ER-PM junctions (416) (Figure 5B). In store-depleted cells, STIM moves by diffusion in the ER membrane at an average rate \((D \sim 0.07 \mu\text{m}^2/\text{s})\) that is roughly half that of STIM in resting (i.e., store-replete) cells (64, 198). Orai also moves by diffusion in the PM with an average diffusion coefficient \(D\) of \(-0.1 \mu\text{m}^2/\text{s}\) (284, 416). Single-particle tracking shows that Orai channels are slightly subdiffusive, indicating interactions with proteins or lipids in their environment (416). STIM redistributes to ER-PM junctions independently of Orai, as STIM puncta form in store-depleted cells expressing only a nominal amount of endogenous Orai1 (e.g., HEK
A key region involved in STIM1 redistribution is the polybasic domain (PBD), a lysine-rich stretch of 15 residues at the extreme COOH terminus (Figure 2). Depletion of the PBD prevents redistribution but not oligomerization, consistent with the idea that oligomerization triggers conformational changes that expose the PBD to the PM (154, 198, 284). By promoting accumulation of STIM1 at junctions, the PBD enhances the rate at which STIM1 activates Orai1 (206). Several studies suggest that the by promoting accumulation of STIM1 at junctions, the PBD enhances the rate of which STIM1 activates Orai1 (206). PBD interacts electrostatically with negatively charged phospholipids in the plasma membrane, in particular PIP2 and phosphatidylinositol (3, 4, 5)-trisphosphate (PIP3). Depletion of PIP2 or PIP3 together in cells inhibits puncta formation and SOCE, and PBD-containing fragments from STIM1 and STIM2 bind to PIP2-containing liposomes in vitro (94, 400, but see Ref. 174). The trigger for PBD binding to PIP2 in the PM may involve changes in conformation (as extension of the STIM cytosolic domain moves the PBD towards the PM) as well as avidity (as STIM oligomerizes). The STIM1 PBD has only marginal affinity for PIP2-containing liposomes unless it is presented as a dimer or tetramer (26); in contrast, the STIM2 PBD has a higher affinity and can bind as a monomer, perhaps because of its ability to form an amphipathic helix. PIP2 has recently been detected at locations where STIM1 puncta form using PLCδ-PH domain-based indicators (343). Interestingly, PIP2 levels appear to decline within puncta as STIM-Orai complexes accumulate, possibly as a result of rearrangements of septins near junctions. These new findings raise the possibility that PIP2 could play a dynamic role in the formation and maintenance of signaling complexes at ER-PM junctions.

Like STIM, Orai moves by Brownian diffusion in the plasma membrane and accumulates at ER-PM junctions after store depletion (210, 418), but unlike STIM it is trapped solely through interactions with its partner protein (Figure 5, A and B) (284, 418). Figure 5B shows an example of a single Orai1 channel being trapped by STIM1 in a punctum before it eventually escapes. The requirement for STIM in trapping Orai is shown most clearly by observations that Orai1 alone fails to form puncta but colocalizes in puncta with STIM1-AK, which by itself is unable to form puncta without its PBD domain (284). Trapping at junctions occurs primarily by binding to the Orai1 COOH terminus, as the COOH terminus but not NH2 terminus appears to be necessary (206, 284). Close interactions between STIM and Orai in situ after store depletion have been shown by coimmunoprecipitation of Orai1 with STIM1 (287, 292, 396, 429) and STIM2 (287) and by FRET between STIM1-YFP and CFP-Orai1 (256, 260, 429). Orai1 is trapped through direct interactions with the C-terminal domain of STIM1 (284). Experiments in which the ratio of accumulated STIM:Orai at junctions was measured under conditions of excess Orai1 suggest that only 0.3–0.6 STIMs per Orai are needed to trap a channel (147). Given a hexameric channel stoichiometry, this result suggests that binding of two to four STIM1 proteins (1–2 STIM1 dimers) is sufficient for trapping, although this is not enough to trigger channel opening (147). Current views of how it opens the Orai channel are described in more detail below.

Most of the work on the choreography of SOCE has been done in cells overexpressing fluorescently labeled STIM and Orai at levels probably orders of magnitude greater than endogenous levels. To understand SOCE signaling in native cells, a question of interest is the number of endogenous CRAC channels that operate at the ER-PM junction. Electrophysiological and EM data collected from Jurkat T cells (299, 414) allows a rough estimate to be made (see also Ref. 145). From the maximal whole cell Na+/H exchange currents measured under divalent-free conditions (~190 pA), the unitary Na+ current amplitude (~110 fA), and an open probability of ~0.8, one can estimate roughly 2,000 functional CRAC channels in a typical Jurkat T cell, or ~2/μm² given the average surface area of the cell of 1000 μm² (299). Considering that 2-APB can increase the current by up to fivefold, the estimate increases to ~10,000 channels (10/μm²). EM measurements indicate the average size of the ER-PM junction profile in these cells is ~200 nm and in total they account for ~4% of the cell circumference (414). If one assumes that each profile is the cross-section of a circular disc (272), then the area of a junction is ~0.03 μm², and this will contain 0.03 μm² × 2–10 channels/μm² × 1/0.4 = 2–10 channels. While these are certainly rough estimates, they do indicate that the number of channels is likely to be quite small, far lower than the 1,300 channels/punctum estimated in HEK cells overexpressing STIM1 and Orai1 (162).

IV. CRAC CHANNEL GATING

A. Subunit Stoichiometry of Orai Channels

Given the small size of the Orai proteins (~300 amino acids), it was natural to assume that functional CRAC channels would have to be multimers of several Orai1 subunits. This expectation is supported by dominant negative effects of Orai1 pore mutants (E106Q and E190Q) on channel function (133, 396), coimmunoprecipitation of orthogonally tagged subunits, and FRET between CFP- and YFP-tagged Orai1 molecules (256, 260). The precise stoichiometry, however, has drawn considerable disagreement. Attempts to evaluate the stoichiometry of the channel from purely biochemical assays have not provided an easily interpretable answer, and as described below, early conclusions regarding the tetrameric structure of CRAC channels have been called into question by the hexameric structure reported for the Drosophila Orai channel.
Among early studies to directly address the issue of Orai channel stoichiometry, Shuttleworth and colleagues (246) used concatenated Orai1 subunits and the effects of over-expressing dominant negative Orai1 subunits to conclude that functional CRAC channel is a homotetramer of Orai1 subunits (246). Likewise, single-molecule photo-bleaching, diffusional analysis of labeled Orai1 proteins, and FRET measurements in tandem dimers and tetramers led to conclusions that STIM1-bound Orai1 channels are tetramers of Orai1 subunits (162, 213, 292). Interestingly, two studies reported that STIM1-free Orai1 channels are dimers (76, 292). These differing results underscore the need for further study to establish the stoichiometry of the resting and active states of the channel. Stoichiometry of channels is often difficult to determine based on a single method. Concatamer studies can be confounded by aberrant channel assembly from incomplete or degraded translation products, or subunit swapping between concatamers (235, 328). Reliable interpretation of single-molecule photobleaching experiments using GFP tags is limited to low numbers of subunits because of the flickery nature of GFP fluorescence which can make up to 30–80% of the single-particle bleaching events uninterpretable (76, 162, 292) and potentially bias the results towards lower numbers of bleaching steps (348). Stoichiometries greater than 4 cannot be ruled out from such studies, and in fact, larger complexes have been detected after oxidizing di-cysteine-substituted Orai1 monomers expressed in HEK cells (454) and crosslinking dOrai expressed in HEK cells (153).

### B. Overview of Orai Structure

Determining the molecular structures of ion channels is essential for understanding how they work, their roles in disease, and to guide development of small molecular therapeutics. Early efforts using cryoelectron microscopy did not yield easily interpretable structural models (227), but the recently solved structure of the *Drosophila* Orai channel by Long and colleagues (153) has enabled detailed visualization of the CRAC channel architecture. This model of a modified channel (with truncated NH₂ and COOH termini and a mutated III–IV loop) showed a complex composed of six dOrai subunits, whose transmembrane domains are arranged in concentric layers around a central aqueous pore [**FIGURE 6B**]. The TM1 helix directly flanks the length of the pore, while TM2 and TM3 surround TM1, shielding it from the surrounding lipid bilayer, and TM4 forms the outermost and presumably the most lipid-exposed segment. The pore is relatively long at 55 Å and is composed of both the TM1 segment as well as the membrane proximal Orai NH₂ terminus [**FIGURE 6C**]. The extracellular opening of the pore has a highly negative electrostatic potential derived from the presence of the six TM1 E178 (*Drosophila* equivalent of human E106) residues comprising the selectivity filter. The side chains of the Glu ring appear to point towards the central symmetry axis (pore) in the structure, with the oxygen atoms of the carboxylates separated by only ~6 Å. Crystal soaking experiments re-
The hexameric stoichiometry of the CRAC channel has been challenged by a report that hexameric hOrai1 concatemers lacked normal Ca$^{2+}$ selectivity (385). However, a subsequent study found that hexameric concatemers with longer intersubunit linkers exhibit normal selectivity and channel properties (428). Considering multiple lines of evidence, including the close correspondence of the general pore architecture, the agreement between predicted pore-lining residues and the results of cysteine scanning studies (238, 453), the crosslinking of dOrai protein in native membranes (153), and the general sequence similarity of Drosophila and human Orai proteins, it seems likely that the hexameric stoichiometry and overall architecture reported in the dOrai crystal structure is valid for human Orai1 channels. It is therefore puzzling that studies of concatemers containing four covalently linked subunits reported channels with apparently normal permeation and gating properties. One possibility is that functional channels with hexameric stoichiometry were formed through the combination of subunits derived from multiple tetramers, but this needs to be tested experimentally.

In addition to a hexameric stoichiometry, another unforeseen feature of the crystal structure is that the cytosolic termini of the TM4 helices are arranged in pairs, with each helix bending in opposite directions to form an antiparallel coiled-coil with its neighbor (FIGURE 6). This association appears to be maintained by hydrophobic interactions between the Drosophila equivalents of the residues L273 (I316) and L276 (L319) in human Orai1, creating a threefold symmetry at the channel periphery. Previous studies have shown that mutations at these sites that lower the coiled-coil probability of the COOH terminus, such as L273S/D (205, 256) and L276D (260), inhibit STIM1 binding and disrupt channel activation. It is currently unclear whether effects arise because L273 and L176 are needed to maintain the coiled-coil TM4 pairs or that they directly stabilize interaction with STIM. Hou et al. (153) have hypothesized that the individual Orai COOH termini may straighten during STIM1 binding, breaking the anti-parallel coiled-coil configuration and allowing the individual Orai COOH termini to interact with STIM1. In contrast, a recent NMR complex structure between STIM1 (amino acids 342–448; Ref. 284), also known as SOAR (STIM1–Orai activating region; amino acids 344–442; Ref. 434) or Ccb9 (amino acids 339–444; Ref. 167) (FIGURE 2A), CAD/SOAR/Ccb9 contains two putative coiled-coil regions (CC2 and CC3) and appears as a dimer in solution, although a tetrameric form has also been reported (284, 424). The CAD domain is both sufficient and necessary for Orai activation. CAD expressed by itself localizes to the plasma membrane where it binds to Orai and activates constitutive Ca$^{2+}$ entry. Conversely, CAD mutations or deletion abrogate Orai1 activity (see TABLE 2).

Several independent observations indicate that CAD binds directly to Orai1 rather than through an intermediary: GST-CAD pulls down affinity-purified Orai1, CAD interacts with Orai1 in a yeast split-ubiquitin assay, and it copurifies with coexpressed Orai1 (284). CAD interacts strongly with COOH-terminal and weakly with NH$_2$-terminal peptides from Orai1, but not detectably with the II–III loop (284); binding to the NH$_2$ and COOH termini is also direct as shown by GST pulldowns using purified STIM1 and Orai1 fragments (453). While we currently do not have a definitive picture of how the CAD engages the COOH-terminal helices of Orai, evidence based on mutagenesis and domain swapping between the CAD of

C. Activation Gating

Resolving the series of molecular steps from STIM binding to the activation of the Orai channel is a major challenge that has been approached using a range of techniques, especially mutational analysis. The interpretation of mutagenesis studies aimed at identifying and distinguishing domains involved in binding and gating is often not straightforward. Because gating of CRAC channels is allosterically coupled to STIM binding, mutations that affect the gating steps will also affect STIM binding (and vice versa), complicating attempts to distinguish between binding, gating, and coupling domains (62). However, recent studies that have provided structural snapshots of putative binding interfaces between STIM and Orai, and mutations causing constitutive channel activation in the absence of STIM offer promising leads to understand the molecular-structural basis of CRAC channel gating.

1. STIM binding to Orai

The initial observations that Ca$^{2+}$ influx occurs locally at sites where STIM and Orai are coclustered and separated only by the narrow gap of the ER-PM junction supported the idea that SOCE is driven by physical contact between STIM and Orai (210). Based on the finding that the isolated cytosolic domain of STIM1 (CT-STIM1; aa 233–685) was able to activate SOCE independently of store depletion (154), several groups truncated CT-STIM1 from both ends to identify a minimal region that when expressed by itself evoked constitutive SOCE and I$_{CRAC}$. This minimal activating region is the CAD domain (amino acids 342–448; Ref. 284), which has also been reported (284, 424). The CAD domain is both sufficient and necessary for Orai activation. CAD expressed by itself localizes to the plasma membrane where it binds to Orai and activates constitutive Ca$^{2+}$ entry. Conversely, CAD mutations or deletion abrogate Orai1 activity (see TABLE 2).
STIM1 and STIM2 supports an interaction of the CC2 domain of CAD with the cytoplasmic TM4 extension (40, 109, 402). Stathopulos, Ikura, and colleagues (368) have proposed a plausible model for this interaction based on the solution NMR structure of a STIM1 fragment including the distal (H92513) region of CC1 and most of CC2 in complex with purified Orai1 COOH-terminal helix (FIGURES 2 AND 7). In this structure, two Orai helices interact via a combination of polar and nonpolar contacts with CC2 in a pair of grooves (the STIM-Orai activation pocket, or SOAP). Binding of the Orai peptide is stabilized by a series of mostly hydrophobic interactions involving the residues listed at bottom left. Note that the paired binding of L273 to L276 in the Orai structure is disrupted and the two Orai helices are translated and rotated in opposite directions in the bound structure, potentially creating a force that could contribute to gating. (Adapted from Stathopulos et al. (368).) B: Surface representations showing the binding pocket of the SOAP completed with Orai1 COOH-terminal peptides. The middle and bottom views correspond to the helical representation in A, while the top model is a view from the left end of the SOAP. Surface electrostatic potential is colored from red (−5 kT) to blue (+5 kT) (dielectric constant 80).

Figure 7. A possible structural model for STIM-Orai binding. A: a dimer of subunits from the Orai crystal structure (153) is shown at top, with the cytosolic TM4 extension helices in yellow. The bottom two diagrams show an NMR solution structure of human Orai1 COOH-terminal peptides (yellow) bound to a dimer of human STIM fragments (amino acids 312–383) containing the distal portion of CC1 (a3 helix; blue) and the CC2 helix of CAD (teal) (2MAK.pdb) (368). The orientation of the TM4 extensions (top) is similar to that of the bound Orai1 COOH-terminal peptides. In the CC1-CC2-Orai structure, the two CC2 helices adopt an antiparallel orientation that creates a pair of symmetrical binding sites called the STIM-Orai Association Pocket (SOAP). Binding of the Orai peptide is stabilized by a series of mostly hydrophobic interactions involving the residues listed at bottom left. Note that the paired binding of L273 to L276 in the Orai structure is disrupted and the two Orai helices are translated and rotated in opposite directions in the bound structure, potentially creating a force that could contribute to gating. [Adapted from Stathopulos et al. (368).]

Although caution is warranted in extrapolating mechanisms from the binding of peptides removed from their full protein environment, the effects of STIM1 and Orai1 mutations on binding and SOCE support key aspects of the structure. For example, Orai1 residues L273 and L276 have long been known to be essential, as L273S/D and L276S mutations effectively quell STIM1 binding as well as SOCE (TABLE 2) (205, 256, 260), and these both make hydrophobic interactions with STIM1 side chains in the SOAP (FIG...
Likewise, mutations of other Orai1 residues predicted to interact with the SOAP (R281, L286, and R289) as well as STIM1 residues in the SOAP that interact with Orai1 (L347, L351, and Y362) are sufficient to disrupt Orai1-STIM1 interactions and SOCE (368) [TABLE 2]. Importantly, mutations of nearby hydrophobic residues in Orai1 (F279 and L282) that are not predicted to interact with STIM1 in the structure have no effect on I_Crac or STIM1-Orai1 FRET (256, 260). An intriguing aspect of the structure is that the SOAP domains engage two Orai helices at approximately the same angles shown by the antiparallel coiled-coil pairs of TM4 extensions in the dOrai structure (368) [FIGURE 7], suggesting that perhaps the extensions are maintained in a conformation that is optimal for binding by a STIM dimer. While this idea needs testing, recent results using Orai1 concatemers with variable arrangements of defective (L273D) STIM binding sites support the idea that STIM dimers bind to pairs of TM4 extensions (428). Furthermore, the disruption of STIM1 binding by Cys-cross-linking adjacent Orai1 COOH termini is rapidly reversed upon disulfide cleavage, consistent with a relatively small conformational rearrangement of the paired Orai1 COOH termini (387). It is apparent from the NMR structure that the L273 and L276 residues that form a paired interaction between adjacent Orai1 COOH termini are pulled apart laterally and rotated when bound within the SOAPs [FIGURE 7]; thus, based on this structure, the binding of STIM would be expected to exert torque on the COOH termini which could contribute to channel gating.

Table 2. Mutations that inhibit STIM1 activity or Orai1 activity/conduction

<table>
<thead>
<tr>
<th>Domain</th>
<th>Mutation or Deletion</th>
<th>Reference Nos.</th>
</tr>
</thead>
<tbody>
<tr>
<td>STIM1 SAM</td>
<td>E136X (E128RfsX9 frameshift)</td>
<td>296</td>
</tr>
<tr>
<td>STIM1 CC1</td>
<td>1538-1G&gt;A (splice site)</td>
<td>36</td>
</tr>
<tr>
<td>STIM1 CC2</td>
<td>L347A-G348A</td>
<td>434</td>
</tr>
<tr>
<td></td>
<td>L347A-W350A-L351A</td>
<td>424</td>
</tr>
<tr>
<td></td>
<td>A376K</td>
<td>64</td>
</tr>
<tr>
<td></td>
<td>K382E-K384E-K385E-K386E (4KE)</td>
<td>175</td>
</tr>
<tr>
<td>STIM1 CC2-CC3 linker</td>
<td>F394L/A/H</td>
<td>402</td>
</tr>
<tr>
<td>STIM1 CC3</td>
<td>R426L</td>
<td>255</td>
</tr>
<tr>
<td></td>
<td>R429C</td>
<td>112, 231</td>
</tr>
<tr>
<td></td>
<td>W430A-I433A-L436A</td>
<td>424</td>
</tr>
<tr>
<td></td>
<td>C437G</td>
<td>284</td>
</tr>
<tr>
<td>Orai1 NH2 terminus</td>
<td>Δ1-91, Δ1-85, Δ73-85, Δ1-88</td>
<td>402</td>
</tr>
<tr>
<td></td>
<td>L74A-L79A-L81A-L86A</td>
<td>206, 236, 256, 284, 448</td>
</tr>
<tr>
<td></td>
<td>L74S-W76S, L74R-W76R, L74E-W76E</td>
<td>80</td>
</tr>
<tr>
<td></td>
<td>K85A/E</td>
<td>201, 236</td>
</tr>
<tr>
<td>Orai1 TM1</td>
<td>R91W/F/I</td>
<td>77, 103</td>
</tr>
<tr>
<td></td>
<td>A103E</td>
<td>234</td>
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<tr>
<td></td>
<td>E106Q/C/A</td>
<td>133, 238, 396</td>
</tr>
<tr>
<td></td>
<td>D112X (A88SfsX25 frameshift)</td>
<td>234</td>
</tr>
<tr>
<td>Orai1 TM3</td>
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<td>234</td>
</tr>
<tr>
<td>Orai1 COOH terminus</td>
<td>Δ267-301, Δ261-301, Δ272-279</td>
<td>206, 236, 256</td>
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<tr>
<td></td>
<td>L273S/D, L276D</td>
<td>109, 205, 236, 256, 260</td>
</tr>
<tr>
<td></td>
<td>R281A, L286S, R289A, (partial inhib)</td>
<td>368</td>
</tr>
<tr>
<td></td>
<td>D284A-D287A-D291A (partial inhib)</td>
<td>368</td>
</tr>
</tbody>
</table>

Naturally occurring human mutations are indicated in bold.
formation is entirely derived from small STIM fragments, it will be necessary to use other approaches to establish whether the reported structures exist naturally in the full-length protein and to identify conformational intermediates in the activation process.

The Orai1 NH2 terminus, in particular the region 73–91, is also a critical determinant of activation gating, as shown by mutations and deletions that abrogate SOCE and $I_{\text{CRAC}}$ (TABLE 2). While Orai1 remains functional after deletion of residues 1–73 (206, 284, 448), activation is blocked by deletion of amino acids 73–84, a K85E mutation, paired hydrophilic substitutions for L74 and W76, or alanine substitutions for L74/L79/L81/L86 (80, 201, 236, 284, 402). Several studies have also noted functional changes in channel chimeras in which NH2-terminal regions were swapped between the three Orai isoforms (201, 380). While these experiments all affirm the importance of the NH2-terminal region in channel activation, its precise role remains unclear, in particular whether it is required for direct interactions with STIM1 rather than with adjacent protein domains within Orai1.

Evidence for weak interactions between CAD and Orai1 NH2-terminal peptides (amino acids 73–91) (284, 453) has led to a two-step model of STIM1 binding, wherein an initial binding event at the COOH terminus permits subsequent lower-affinity STIM1 interaction with the NH2 terminus and opening of the pore (448). In one version of this model, Gill and colleagues (402) have proposed that F394, a critical residue in the CC2-CC3 linker of STIM1, interacts with exposed leucines in TM1 to open the channel. However, direct binding of STIM1 to the Orai1 NH2 terminus in the intact channel has not yet been demonstrated, and isolated peptides often exhibit binding properties that are absent in the context of the full-length protein. Orai1 NH2-terminal mutations do reduce binding of full-length STIM1 to Orai1 in intact cells, as shown by diminished FRET and Orai1 puncta formation (80, 236) (TABLE 2). Still, it is not clear in these studies that the NH2 terminus promotes STIM1 binding through a direct interaction rather than through an allosteric coupling mechanism in which channel activation (which requires an intact NH2 terminus) increases the affinity of STIM1 for the Orai1 COOH terminus. Thus further studies, especially with full-length Orai1, will be needed to define more clearly the critical role of the NH2 terminus for STIM1 binding and gating.

2. Nonlinear stoichiometric requirements for Orai activation

The activation of Orai channels appears to be highly sensitive to the number of STIMs bound. Increasing the STIM-to-Orai heterologous expression ratio was first shown by Rychkov and colleagues (340) to increase current magnitude as well as enhance CDI, divalent cation selectivity, and reduce current potentiation by 2-APB. Hoover and Lewis (147) quantified these effects further and found that activation was a highly nonlinear bell-shaped function of the STIM-to-Orai ratio, as measured by the fluorescence of labeled proteins at ER-PM junctions. In cells expressing similar levels of STIM1, $I_{\text{CRAC}}$ increased with Orai1 expression as long as the STIM-to-Orai ratio was high, but plummeted when the ratio fell below ~2, even as Orai1 expression was increased; maximal Orai1 channel activation occurred at a STIM1-to-Orai1 ratio of ~2:1 (147). A similar conclusion was reached by Xu and colleagues (205), who reported that fusion of a tandem dimer of extended CAD domains (aa 336–485) to the Orai1 COOH terminus activates the channel much more effectively than a single domain. The conclusion from these two studies, that a dimer of STIMs binds to each Orai monomer, is apparently at odds with the model of Stathopoulos et al. (368) discussed above, which shows a STIM dimer engaging a pair of Orai TM4 extensions with a 1:1 STIM-to-Orai stoichiometry. Each approach has shortcomings: the former studies make indirect inferences about binding, while the latter involve isolated peptides that may interact differently than the intact proteins. New experimental approaches will be needed to resolve these important questions about the STIM:Orai binding stoichiometry, the structure of the bound state, and their relationship to gating.

The highly nonlinear dependence of CRAC current activation on the STIM-to-Orai ratio can be described by a modified Monod-Wyman-Changeux (MWC) kinetic scheme (147). However, although the MWC formalism offers a simple way of thinking about the origins of nonlinear gating, Orai channel activation appears to be more complex than the two-state open/closed mechanism the model assumes. Current noise analysis studies suggest that the slow activation of CRAC current by store depletion occurs by stepwise recruitment of single channels from a “silent” state to a high-open probability ($P_o$) state (~0.8 measured during brief, 200-ms intervals) (299). This process might be envisioned as the binding of channels to STIM1 as they enter ER-PM junctions. However, the ability of low doses of 2-APB to increase $I_{\text{CRAC}}$ by more than threefold implies that the $P_o$ is actually much lower, <0.3. One way to reconcile these results is that CRAC channels exhibit modal gating, such that the channel alternates between silent and high-$P_o$ states at a low enough frequency that the transitions evade detection as noise during brief measurement periods. According to such a scheme, STIM1 binding to Orai1 could activate the channels by increasing the amount of time spent in the high-$P_o$ mode. It is tempting to speculate that modal transitions of this sort are related to the high cooperativity of activation by STIM1 (147) or 2-APB (422).

3. Nature of the activation gate and hinge

The location, disposition, and regulation of the CRAC channel activation gate has attracted considerable attention. Structural, functional, solvent accessibility analysis as
well as disulfide crosslinking approaches have yielded a complex picture of gating with evidence for at least two potential gates, located at the extracellular and intracellular ends of the pore.

From the observed differences in the accessibility of thiol reagents to pore sites in closed versus open channels, McNally et al. (237) concluded that one gate is located towards the extracellular end of the pore in close proximity to the selectivity filter formed by E106. Mutational analysis subsequently showed that substitutions of V102 to more polar residues (Ala, Ser, Thr, or Cys) yielded constitutively open channels, leading to the proposal of V102 as a hydrophobic gate (FIGURE 6C) (237). This proposal is congruous with the dOrai structure, which reveals that the hydrophobic side chains of the six Val residues are well-packed in the pore and make extensive van der Waals contacts with each other (153), thus presenting a large desolvolysis barrier for ions in the closed channel (88). Moreover, molecular dynamics simulations confirm that the (modest) reduction of hydrophobicity caused by the V102A substitution does not appreciably perturb the pore structure, but significantly lowers the energy barrier for entry of water and ions, providing a possible explanation for constitutive conduction in the V102A mutant (88). A gate at V102 is also supported by a recent study that employed changes in the luminescence of Tb3⁺ bound in the pore, suggesting that STIM1 binding elicits conformational changes in the vicinity of E106 and V102 (130). How the native V102 side chains would regulate ion conduction remains unclear; presumably gating could occur through a rearrangement of V102 side chains, either by rotation of the TM1 helices or some other mechanism.

In a second gating model, the channel gate is presumed to be located at the cytoplasmic end of the pore, in TM1 or in the NH₂-terminal cytoplasmic extension of TM1 (325, 445). In this model, channel opening arises from bending of the TM1 helices at the conserved G98 residue and from dilation of the helices in the inner pore, in the region around R91. G98 was suggested to serve the role of a gating hinge, based on the constitutive opening of G98D and G98P mutants (445). R91 was proposed as the physical gate at the cytoplasmic rim of the pore based on the ability of R91C to form inter-subunit crosslinks that block ion conduction (445). However given evidence that Cys substitutions at all pore-lining residues (E106, V102, G98, L95, and R91) can form intersubunit disulfide bonds (454), the role of R91 as an inner gate requires further validation. More recently, the idea of the inner gate has been modified based on the structure of the dOrai crystal structure, which revealed that the cytoplasmic NH₂-terminal helix is contiguous with TM1 (153). Curiously, a prominent ion density attributed to the binding of anions between the basic residues of the inner pore (K155, R159 in dOrai) was observed (153) (FIGURE 6C). The triplet of basic residues in the lower pore could thus help stabilize the closed channel either through anion binding or through electrostatic repulsion of cation conduction in the inner pore.

The nature and mechanism of the activation gate in the CRAC channel thus remains an unsettled issue. The prevailing models come to different conclusions about the location of the Orai gate. Besides the possibility that there could indeed be gates at both ends of the pore, these conflicting models might also be resolved by postulating that gating occurs across an extended length of the pore due to energetic interactions such as hydrophobic exclusion of water and ions.

D. Fast Ca²⁺-Dependent Inactivation

Ca²⁺ entering through CRAC channels inhibits channel activity through a process called fast Ca²⁺-dependent inactivation, or CDI (105, 151, 152, 459). CDI is typically observed as a decline in ICₚ over tens of milliseconds during hyperpolarizing voltage steps. CDI is specific for Ca²⁺ over Ba²⁺ or Sr²⁺, and its apparent voltage dependence is accounted for by the voltage dependence of Ca²⁺ entry rather than any intrinsic voltage-dependent inactivation (459). A fast intracellular Ca²⁺ buffer (BAPTA) can reduce CDI, whereas a slower buffer (EGTA) cannot, consistent with feedback inhibition of channel activity by the high local [Ca²⁺] around individual CRAC channels (105, 152, 459). A quantitative analysis of these buffer effects suggested that CDI is evoked by the binding of at least two Ca²⁺ ions to a site located several nanometers from the pore, potentially on the channel or a nearby protein (459). Interestingly, for endogenous CRAC channels, the extent of CDI is constant over the time that ICₚ develops and global [Ca²⁺] rises, implying that the affinity of the CDI site is quite low and Ca²⁺ from one channel does not contribute to inactivation of others (459). This feature is especially striking, considering that Ca²⁺ from clusters of CRAC channels would be expected to spread throughout the small volume of the ER-PM junction.

Structure-function studies have implicated several players in the CDI mechanism, including STIM1, calmodulin (CaM), and Orai1. A role for STIM1 was first suggested by observations that the extent and rate of CDI increase steeply with the STIM-to-Orai expression ratio (340; see also Ref. 147) and that the isolated CAD domain (STIM1 342–448) does not support CDI (284). Residues 470–491 were identified as essential for CDI and were named the inactivation domain of STIM, or IDSTIM (257) (FIGURE 2). Within this domain, an acidic stretch (475DDVDDMDEE483) appears to be particularly critical, as neutralization of residues in this region reduces or eliminates CDI (78, 190, 257). By analogy to the acidic “Ca²⁺ bowl” sequence of Ca²⁺-activated BK channels (15), a possible role of the acidic region as a Ca²⁺ sensor for CDI was considered (257). In support
of such a role, the 470–491 fragment bound Ca\(^{2+}\) weakly in a \(^{45}\)Ca overlay, and several partial neutralizations of the region reduced CDI and \(^{45}\)Ca binding in parallel. However, one partial neutralization (E482A/E483A) reduced Ca\(^{2+}\) binding yet increased the apparent Ca\(^{2+}\) sensitivity and accelerated the kinetics of CDI, casting doubt on the acidic region as a Ca\(^{2+}\) sensor for CDI (257). More definitive tests will be needed to discriminate the possible roles of ID\(_{STIM}\) in CDI as a potential Ca\(^{2+}\) sensor or as a structural element that acts allosterically to stabilize or promote transitions of the channel to the inactivated state. These studies were the first to show that STIM1 was more than just an activating ligand for Orai1, but serves as an integral part of the channel, allowing it to respond to Ca\(^{2+}\) by inactivating; another example of this integral role, discussed below, is that STIM1 binding changes the ion selectivity of the Orai channel (237).

CaM has also been implicated as a Ca\(^{2+}\) sensor for CDI (202, 257). This notion was first suggested on the basis of partial inhibition of native CRAC CDI by overexpression of a Ca\(^{2+}\)-nonbinding CaM mutant (CaM1234) and a CaM inhibitory peptide (202). Later work showed that CaM binds in a Ca\(^{2+}\)-dependent manner to solubilized Orai1 and to an isolated Orai1 NH\(_{2}\)-terminal fragment (residues 68–91) (257). Consistent with a functional role in CDI, several mutations that eliminated CaM binding to the peptide (A73E, W76E, A, or S; Y80E) also suppressed CDI when introduced into full-length Orai1, and truncations within the same region in Orai3 produced a parallel loss of CDI and CaM binding to the NH\(_{2}\)-terminal peptide (19). Further validating a role in CDI, a crystal structure of the Orai1 peptide bound to Ca\(^{2+}\)-CaM confirmed an interaction of Orai1 W76 and Y80 with CaM, and showed that Orai1 W76 binds strongly within a hydrophobic pocket of CaM (204).

However, despite strong experimental support, the case for CaM as the CDI Ca\(^{2+}\) sensor also has weaknesses. First, mutant CaM that cannot bind Ca\(^{2+}\) virtually eliminates CDI of Ca\(_{\text{v}}\) channels where the role of CaM is well established (294), yet only modestly reduces CDI of CRAC channels (202). Second, in the dOrai structure the side chains that interact with CaM (e.g., W76 and Y80) are predicted to face the interior of the pore; if this holds for the human Orai1 channel in the open state, these sites would be inaccessible to CaM without an extreme conformational change in the pore-lining helices. Finally, the acceleration of CDI by Y80A and Y80S mutations are not easily explained by effects on CaM binding (257), but rather suggest a role of this residue in conformational changes in Orai1 leading to CDI. These issues raise the possibility that Ca\(^{2+}\) -CaM binding to the isolated Orai1 NH\(_{2}\)-terminal peptide or solubilized Orai1 may not reflect binding to the native channel and that NH\(_{2}\)-terminal mutations might instead inhibit CDI through CaM-independent mechanisms. Studies are underway to address these issues.

The cytoplasmic II–III loop of Orai1 has been suggested to function as a gate for CDI based on several lines of evidence (365). Alanine substitutions at four locations within a part of the II–III intracellular loop (151VSNV154) (FIGURE 3, TABLE 3) abolished fast inactivation while overexpression or intracellular perfusion of a short peptide encompassing this region diminished the steady-state \(I_{\text{CRAC}}\) (365). These results suggested that II–III loop could function as an intracellular blocking particle to inhibit ion conduction and produce inactivation. Such a mechanism is reminiscent of the hinged-lid inactivation model proposed for voltage-gated \(\text{Na}^{+}\) channels in which the intracellular loop connecting domains III and IV blocks the inner pore to produce fast inactivation (391). However, it is uncertain whether the II–III loop peptide functions strictly as a blocker or also elicits inhibition through allosteric pathways. Unlike in \(\text{Na}^{+}\) channels where short peptides containing the so-called IFM inactivation motif restore time-dependent inactivation (90), inhibition of \(I_{\text{CRAC}}\) by a soluble II–III Orai1 loop peptide is not time-dependent (365). Moreover, increasing

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**Table 3. Mutations that modulate STIM1 or Orai1 function**

<table>
<thead>
<tr>
<th>Domain</th>
<th>Effect of Mutation</th>
<th>Mutation</th>
<th>Reference Nos.</th>
</tr>
</thead>
<tbody>
<tr>
<td>STIM1 NH(_{2}) terminus</td>
<td>Altered [Ca(^{2+})](_{\text{leak}}) sensitivity</td>
<td>C58A</td>
<td>138</td>
</tr>
<tr>
<td>STIM1 COOH terminus (ID(_{STIM}))</td>
<td>Suppression of CDI</td>
<td>475DDVDMMDEE483 D, E -&gt; A or G</td>
<td>78, 190, 257</td>
</tr>
<tr>
<td>Orai1 NH(_{2}) terminus</td>
<td>Suppression of CDI</td>
<td>A73E, W76E/A/S, Y80E</td>
<td>257</td>
</tr>
<tr>
<td></td>
<td>Acceleration of CDI</td>
<td>Y80A/S</td>
<td>257</td>
</tr>
<tr>
<td>Orai1 TM1</td>
<td>Reduced Orai1 ion selectivity</td>
<td>G98D/E</td>
<td>445</td>
</tr>
<tr>
<td></td>
<td></td>
<td>V102C/A/S/T</td>
<td>237</td>
</tr>
<tr>
<td></td>
<td></td>
<td>E106D</td>
<td>297, 396, 429</td>
</tr>
<tr>
<td>Orai1 I–II loop</td>
<td>Reduced Orai1 lanthanide sensitivity</td>
<td>D110A/D112A/D114A</td>
<td>238, 396, 429</td>
</tr>
<tr>
<td>Orai1 II–III loop</td>
<td>Suppression of CDI</td>
<td>V151A/S152A/N153A/V154A</td>
<td>365</td>
</tr>
<tr>
<td>Orai1 TM3</td>
<td>Altered Orai1 ion selectivity</td>
<td>W176C</td>
<td>366</td>
</tr>
<tr>
<td></td>
<td></td>
<td>E190Q</td>
<td>297, 396</td>
</tr>
</tbody>
</table>
the number of intact II–III loops from one to four in tetrameric Orai1 concatemers did not accelerate CDI as might be expected from simple blockade. Additional kinetic and experimental approaches may help to better define the mechanistic role of the II–III loop in CDI.

Heterologous Orai3 and Orai2 currents generally inactivate more rapidly than Orai1 currents (110, 190, 200), but the reasons for this difference are not yet clear. A troublesome complication in many studies is that CDI appears to be strongly affected by the degree of STIM binding to Orai (147, 340), raising the possibility that Orai mutations or sequence differences among homologs may alter CDI indirectly by changing the STIM:Orai binding affinity and stoichiometry, which is not generally known or controlled. In one study, Orai1 current failed to inactivate, but substituting the Orai3 COOH terminus in Orai1 restored CDI (190). Neutralization of three glutamates in the COOH terminus of Orai3 (E281, E283, and E284) inhibited CDI, which was interpreted to indicate a role of these residues as a CDI Ca$^{2+}$ sensor. However, mutation of two of the three glutamates in Orai3 significantly increased fast inactivation, as did mutation of all three analogous residues in Orai2. In view of the role of analogous glutamate residues in STIM1-Orai1 interactions (39, 40), an alternative explanation is that these residues affect CDI through effects on STIM1 binding. Further complexity is indicated by a study of Orai1-Orai3 chimeras, suggesting that all three cytosolic domains may interact to determine CDI characteristics (110) although the nature of these interactions is not yet clear.

The gate for CDI is unknown, but the Orai1 E106D mutant exhibits little or no fast inactivation, suggesting that inactivation might be closely linked to conformational changes in the selectivity filter (420). Very rapid inactivation ($\tau <1 \text{ ms}$) was seen in a later study of Orai1$_{E106D}$ with Ca$^{2+}$ as the current carrier (341); however, this ultrafast inactivation differed from CDI in that it was insensitive to intracellular BAPTA, did not depend on the STIM-to-Orai ratio, occurred with CAD as the stimulus, and was supported by Sr$^{2+}$ as current carrier. A direct blocking effect of Ca$^{2+}$ has not been ruled out, and the precise role of E106 in the CDI process remains to be clarified.

A central remaining question concerns the physiological role of CRAC channel CDI. By limiting Ca$^{2+}$ entry, CDI is generally thought to protect against excessive Ca$^{2+}$ elevation, or make Ca$^{2+}$ entry relatively constant over a range of hyperpolarized potentials (459). However, CDI is typically only studied under artificial conditions that maximize its amplitude and speed (10–20 mM Ca$^{2+}$ and −80 to −120 mV), and there are no data to show whether it alters Ca$^{2+}$ entry at physiological levels of Ca$^{2+}$ and $V_m$. In the case of Ca$V$ channels, much of what is known about the functions of CDI comes from studies of channel mutants lacking CDI, such as the Timothy Syndrome mutation of Ca$V_{1.2}$ (288, 427). Analogous studies of transgenic animals lacking CRAC channel CDI may yield important insight into the functions of CDI in SOCE-mediated events.

V. CRAC CHANNEL PERMEATION AND SELECTIVITY

A. Ion Selectivity and Pore Size

CRAC channels select for Ca$^{2+}$ >1,000 times over Na$^+$ under physiological conditions, placing them among the most highly Ca$^{2+}$-selective channels known (152). This is an important property that enables the channels to conduct essentially only Ca$^{2+}$, minimizing the depolarizing effects of cation entry. Biophysical studies indicate that their exquisite Ca$^{2+}$ selectivity is not due to molecular sieving but arises from ion-ion and ion-pore interactions (13, 148, 152, 191, 299). While CRAC channels readily conduct a variety of small monovalent ions, including Na$^+$, Li$^+$, and K$^+$ in the absence of extracellular divalents, micromolar levels of extracellular Ca$^{2+}$ prevent monovalent permeation by binding to a site in the pore ($K_i \sim 20 \mu M$ at $-100 \text{ mV}$) (13, 152, 191, 299, 301, 378). Ca$^{2+}$ block of Na$^+$ flux is only mildly voltage dependent (299, 420), suggesting that the binding site is positioned near the external rim of the pore, consistent with the superficial location of the E106 Ca$^{2+}$ binding site (FIGURE 6C). Another key diagnostic feature of CRAC channels is their low permeability to the large monovalent Cs$^+$ ($P_{Cs}/P_{Na} \sim 0.1$) (191, 301). As discussed further below, this may be related to the narrow dimensions of the pore.

Early insights into the underlying mechanism of the CRAC channel’s high Ca$^{2+}$ selectivity came from observations that mutating conserved acidic residues in transmembrane segments TM1 and TM3 altered ion selectivity. On this basis, multiple acidic residues including E106 in TM1, E190 in TM3, and D110/112/114 in the I–II loop were implicated in regulating Ca$^{2+}$ selectivity (297, 396, 429) (TABLE 3; FIGURE 3). In particular, kinetic measurements revealed that the E106D substitution diminishes the association rate of Ca$^{2+}$ binding, thereby lowering Ca$^{2+}$ block affinity, consistent with the idea that the affinity of Ca$^{2+}$ binding at E106 is critical for Ca$^{2+}$ selectivity (420). These findings led to proposals that residues in both TM1 and TM3 flank the CRAC channel pore, with the acidic residues within these segments forming components of the CRAC channel selectivity filter. As described below, this view has been significantly revised by subsequent cysteine scan studies and the crystal structure of Drosophila Orai that revealed that TM3 does not flank the pore.
Measurements of the permeability of a series of organic monovalent cations indicate that the narrowest region across the pore for native CRAC as well as overexpressed recombinant Orai1 and Orai3 channels is \( \sim 3.8 \) Å (299, 420, 422). This is considerably narrower than the dimensions measured using comparable methods for Cs\(^+\)-permeable Ca\(_V\) channels and TRP channels (\( \sim 6 \) Å) raising the prospect that steric hindrance to the movement of Cs\(^+\) (dehydrated diameter \( \sim 3.4 \) Å) is responsible for its low permeability in CRAC channels. Consistent with this notion, pore mutations that increase Cs\(^+\) permeability also concomitantly widen the pore (420). Moreover, the 2-APB-gated Orai3 pore, which is readily permeable to Cs\(^+\) (\( P_{Cs}/P_{Na} \sim 1.0 \)), exhibits a considerably larger minimal diameter of \( \sim 5.6 \) Å (422). In general, decreases in Ca\(^{2+}\) permeability of pore mutants correlate well with increases in Cs\(^+\) permeability, suggesting that the structural changes that cause pore widening concurrently affect both Ca\(^{2+}\) and Cs\(^+\) selectivity in CRAC channels (420). What physical features limit the pore diameter? In the dOrai crystal structure the distance between the oxygen atoms of the carboxylates of the Glu ring, which appears to be the narrowest region of the pore, is \( \sim 6 \) Å, considerably larger than expected from pore sizing experiments. Thus the structural basis of the limiting pore diameter as measured by permeation of organic cations and its precise location along the ion conduction pathway remains to be determined.

Cysteine scanning studies that followed have broadened our understanding of what regions in Orai1 regulate ion conduction. In one study, Prakriya and colleagues (238) used probes differing in size, charge, and chemistry to probe the geometry, flexibility, and electrostatic potential of CRAC channel pore sites. In a second study, Hogan’s group (453) examined inter-subunit disulfide crosslinks of TM1 residues. These studies revealed that the CRAC channel pore is formed by the TM1 segments, with a reactivity pattern consistent with E106, V102, G98, L95, and R91 facing the pore (FIGURE 6C). There was negligible reactivity to bulky MTS reagents, yet significant coordination by these residues of the small probe, Cd\(^{2+}\), suggesting that the CRAC channel pore is narrow along much of the length of TM1 (238). This feature may produce heightened friction to diffusing ions, thereby accounting for the low permeability of CRAC channels to large cations (>3.8 Å) and their low unitary conductance. Interestingly, the I-II loop segments interact tightly with both large (>8 Å) and small (<3 Å) probes of different charge, suggesting that the loops form an outer vestibule with sufficient flexibility to accommodate ions of different size and charge (238). No significant reactivity was seen at E190 and the other residues of TM3, indicating that this segment is not pore-lining. Moreover, individual modification of D110, D112, and D114 in the I-II loop by Cys or positively charged compounds did not affect ion selectivity. Thus E190 and the acidic residues in the I-II loop do not form high-affinity Ca\(^{2+}\) binding sites as was originally thought (297, 396), but rather E106 in the centrally located TM1 segment solely underlies Ca\(^{2+}\) selectivity. Collectively, these studies provided the first step towards building a structural model of the CRAC channel pore and are quite congruent with the later dOrai crystal structure (153) which reveals Ca\(^{2+}\) and Ba\(^{2+}\) densities close to E178 (E106 in human Orai1) (FIGURE 6C).

How does E106 regulate ion permeation? Biophysical models indicate that a single Ca\(^{2+}\) binding site in the pore is inadequate to describe the movements of Ca\(^{2+}\) in Ca\(_V\) and CRAC channels (68, 334, 422). Rather, multiple closely spaced ion binding sites are required to accelerate Ca\(^{2+}\) conduction by creating electrostatic repulsion between closely spaced ions in the pore (68, 334, 422). This knock-on mechanism for Ca\(^{2+}\) permeation is supported by recent crystallographic analysis of a Ca\(^{2+}\)-selective bacterial Na\(_V\) channel with multiple Ca\(^{2+}\) binding sites in the selectivity filter (383). However, whether such a structural model can be to CRAC channels is unclear. The modified Ca\(^{2+}\)-selective Na\(_V\) channel was engineered with two closely spaced rings of Asp residues in the selectivity filter (383), yet the available structural evidence in CRAC channels shows only one obvious high-affinity Ca\(^{2+}\) binding site (E106), which is consistent with functional and mutational effects on Ca\(^{2+}\) block (299, 420, 422). One plausible scenario that could satisfy the requirement for multiple ion sites demanded by theoretical permeation models is that the side chains of the six Glu residues at E106 cluster into two groups along the axis of the pore to form two distinct binding sites. The close proximity of these sites could yield electrostatic repulsion between Ca\(^{2+}\) ions to enhance permeation, analogous to the scenario proposed for Ca\(_V\) channels (92).

Finally, it is worth noting that despite qualitative similarities in the Ca\(^{2+}\) selectivity and permeation between CRAC and Ca\(_V\) channels, the architecture of the two channel families is markedly different. Whereas CRAC channels display a long narrow pore flanked by the TM1 segment (153, 238), Ca\(_V\) channels have a wide pore with an inner vestibule large enough to accommodate very large thiol reagents and one or more hydrated ions (451). Moreover, blockade of Na\(^+\) conduction occurs at substantially lower Ca\(^{2+}\) concentrations in Ca\(_V\) channels (0.7 vs. 20 \( \mu \)M) (1, 299), suggesting that high-affinity Ca\(^{2+}\) binding to the selectivity filter may not be the sole mechanism by which CRAC channels achieve high Ca\(^{2+}\) selectivity. Consistent with this possibility, a recent study employing Eyring-rate theory analysis has postulated that in addition to Ca\(^{2+}\) binding at the selectivity filter, CRAC channels acquire high Ca\(^{2+}\) selectivity by restricting the rate of ion flow (for both preferred and nonpreferred ions) by high entry and exit energy barriers (422). High-energy barriers for ion flow are likely to be related to the narrow dimensions of the CRAC channel pore because conditions that enlarge the pore (mutations or
channel activation by 2-APB) elicit concomitant increases in ion flux rates and lower Ca\(^{2+}\) selectivity (422).

**B. Rectification**

Inward rectification of the current-voltage relationship is a consistent feature of CRAC channels. While not as steep as that of inwardly rectifying K\(^+\) channels (265), the rectification is nevertheless more prominent than in Ca\(_V\) channels (143) and is widely employed as an identifier for CRAC channels. It is worth noting that from an experimental standpoint, many studies have overemphasized inward rectification by applying voltage ramps (e.g., −100 to +100 mV) directly from a positive holding potential, which can trigger CDI at negative potentials and distort the current-voltage relation. Inward rectification is also apparent in the monovalent CRAC current recorded with symmetrical voltage relation. Inward rectification is nevertheless more prominent than in Ca\(_V\) channels (143) and is widely employed as an identifier for CRAC channels. It is worth noting that from an experimental standpoint, many studies have overemphasized inward rectification by applying voltage ramps (e.g., −100 to +100 mV) directly from a positive holding potential, which can trigger CDI at negative potentials and distort the current-voltage relation. Inward rectification is also apparent in the monovalent CRAC current recorded with symmetrical concentrations of Na\(^+\) in the internal and external solutions (13, 299), indicating that it is not simply due to the >10,000-fold concentration gradient of Ca\(^{2+}\) across the plasma membrane. One consequence of inward rectification is that it would be expected to enhance the effect of hyperpolarizing voltage changes on the rate of Ca\(^{2+}\) entry, which may be important for crosstalk between SOCE and pathways that influence the membrane potential.

The biophysical basis of CRAC channel rectification is largely unknown. Unlike many K\(^+\) channels, inward rectification in CRAC channels is not conferred by Mg\(^{2+}\) or polyamine blockade of outward currents (13, 177, 178, 301). Moreover, inwardly rectifying currents are also associated with pore mutants of Orai1 and Orai3 having altered ion selectivity and with 2-APB-activated Orai channels, especially under divalent-free conditions (236, 420, 422), indicating that the residues in question (such as E106, V102, R91) are not involved in conferring this biophysical attribute. Two conditions that do appear to alter inward rectification are permeation by ammonium derivatives such as hydroxyl ammonium or methyl ammonium (299), and the deletion of the Orai1 NH\(_2\) terminus (Orai1 Δ73–85 V102C) (236). These observations raise the possibility that asymmetry of the ion conduction pathway and the interaction of ions with the inner pore may contribute to rectification.

**C. Unitary Conductance**

One of the most distinctive features of CRAC channels and one that has been a cause both of fascination and frustration to CRAC channel biophysicists is its extremely low unitary conductance. CRAC channels produce very small current fluctuations, and the unitary currents are too small to resolve by the patch-clamp method. Fluctuation analysis of whole cell CRAC currents in T cells has yielded unitary conductance estimates of 9 fS in 2 mM extracellular Ca\(^{2+}\) and 24 fS in isotonic Ca\(^{2+}\) solution (458). These early measurements were not corrected for the channel P\(_o\), which subsequent nonstationary analysis showed to be high (P\(_o\) ≈ 0.8), at least under divalent-free conditions when the channels conduct Na\(^+\) ions (171, 297, 422). Normalization to Ca\(^{2+}\)-conducting solutions is difficult since direct measurements of P\(_o\) for Ca\(^{2+}\)-conducting channels are unavailable. However incorporating estimates of channel P\(_o\) in Ca\(^{2+}\)-conducting solutions (corrected for inactivation) would be expected to enhance the unitary conductances to 20 and 33 fS in 2 and 110 mM extracellular Ca\(^{2+}\) solutions, respectively (299). In divalent-free solutions, the unitary conductance of the Na\(^+\) current rises to ~0.7 pS, consistent with the increase of the macroscopic Na\(^+\) CRAC current in the absence of divalent ions (299). The single-channel Na\(^+\) current in divalent-free conditions (~0.1 pA at −110 mV) corresponds to an ion turnover rate of 7 × 10\(^{-5}\) Na\(^+\)/s (299).

How do these estimates of the unitary conductance compare to other channels? Unitary conductances of most K\(^+\) and Na\(^+\) channels are in the 4–30 pS range, with mammalian Ca\(^{2+}\)-activated BK channels achieving >200 pS (143). The closest functional relatives, the Ca\(_L\)-type channels, show unitary conductances of 4–10 pS for Ca\(^{2+}\) and 85 pS for Na\(^+\) (141), corresponding to ion turnover rates >100-fold larger than those of CRAC channels.

Kinetic measurements of Ca\(^{2+}\)-blockade of the Na\(^+\)-CRAC currents indicate that the association rate of Ca\(^{2+}\) for its block site is ~4 × 10\(^8\) M\(^{-1}\)s\(^{-1}\) (299, 420, 422). This rate, which is conserved both in native T-cell and in recombinant STIM1-gated Orai1 and Orai3 channels, is 100-fold slower than the association rate of Ca\(^{2+}\) in L-type Ca\(_V\) channels (~4 × 10\(^8\) M\(^{-1}\)s\(^{-1}\); Ref. 184) and the ion entry rates seen in K\(^+\) channels, which are commonly close to the diffusion limit ~10\(^9\) M\(^{-1}\)s\(^{-1}\) (143). This striking difference between CRAC and the other channels indicates the existence of a significant energy barrier for ion access from the extracellular space to the selectivity filter in CRAC channels, a feature that could in principle account for their low unitary conductance. While the molecular/structural basis of this barrier is currently unknown, one simple possibility is that it reflects a dehydration step that must occur before Ca\(^{2+}\) ions can interact with sites in the CRAC channel.

Curiously, a recent molecular dynamics simulation of ion permeation in the constitutively active V174A dOrai mutant has suggested that Na\(^+\) influx at hyperpolarized potentials is coupled to concomitant Cl\(^-\) efflux (89). Efflux of Cl\(^-\) ions was postulated to actively promote Na\(^+\) permeation by coordinating charge on Na\(^+\) ions, in effect constituting an
“anion-assisted” permeation mechanism. These simulations present interesting possibilities for the contributions of the basic residues in the inner hOrai1 pore (e.g., R91, K87, R83) for counter-ion movements and regulation of cation permeation. However, at present, the applicability of these conclusions to CRAC channels is uncertain, given the lack of experimental evidence for Cl− currents through CRAC channels.

From a physiological standpoint, the small unitary conductance of CRAC channels coupled with its high Ca2+ selectivity implies that CRAC channels function as a highly efficient and selective Ca2+ entry mechanism yielding negligible Na+ influx. A likely physiological benefit of such a feature is that it limits membrane depolarization, thereby diminishing the metabolic demands of pumping out Na+ over the long durations in which CRAC currents are generally active, while boosting specificity for activation of particular cellular effector functions. There are numerous examples of functional coupling of CRAC channels to downstream effectors, including c-fos activation (82), NFAT activation (164, 360), leukotriene production (46), and PMCA activation (18) driven by highly localized Ca2+ elevations that may be attributed to the low unitary conductance of CRAC channels.

D. Coupling Between Gating and Permeation in the CRAC Channel

1. Calcium-dependent potentiation

Although the primary signal for activation of native CRAC channels is depletion of intracellular Ca2+ stores, extracellular Ca2+ plays a crucial role in optimizing CRAC channel activity through a process referred to as calcium-dependent potentiation (CDP) (54, 299, 378, 461). This phenomenon is most clearly revealed when Ca2+ is readded to cells whose stores have been depleted in the absence of extracellular Ca2+. Upon the readdition of Ca2+, I_Crac appears in two kinetically different stages. A small fraction of the current appears instantaneously, representing channels active at the time of Ca2+ readdition (461). This instantaneous current is followed by a severalfold exponential increase in current over the following 10–20 s. Conversely, the removal of extracellular Ca2+ by perfusing divalent-free (DVF) solutions diminishes CRAC channel activity by ~80% over tens of seconds due to reversal of CDP, or depotentiation.

CDP and depotentiation are useful identifiers of native CRAC channels, but the molecular underpinnings of these related phenomena are largely unknown. As noted by Zhang and Cahalan (444), depotentiation of the Na+-I_Crac varies significantly between cells, suggesting that the process is regulated by components other than the channel itself, including the relative levels of STIM1 and Orai1. Noise analysis suggests that CDP occurs through the recruitment of CRAC channels from a “silent” state to one of high open probability, analogous to that seen during channel activation following the depletion of intracellular stores (299), suggesting that CDP and activation by store depletion may share common biophysical gating mechanisms. The Ca2+ binding site for CDP is probably not intracellular, because intracellular BAPTA and EGTA do not inhibit the process and extracellular Ni2+, a blocker of CRAC channels, can substitute for Ca2+ in keeping the channels potentiated (54, 461, but see Ref. 378). Several lines of indirect evidence hint that the CDP site (or a key regulatory mechanism) lies in the pore: the extent of CDP increases with hyperpolarization (461), and CDP is profoundly influenced by permeant ions (Prakriya and Lewis, unpublished data). Moreover, the E106D mutation, which affects ion selectivity, nearly completely eliminates depotentiation of Na+-I_Crac (420).

2. STIM1 binding affects Orai1 ion selectivity

A second example of the functional coupling between permeation and gating is revealed by the STIM1-mediated alterations in the ion selectivity of constitutively active Orai1 mutant channels. In particular, STIM1 markedly modulates the ion selectivity of the constitutively open V102X mutant channels (where X = C, A, S, or T) (80, 237). STIM1-free V102X mutant channels exhibit poor Ca2+ selectivity and allow permeation of Na+, Cs+ and several other large cations that are normally impermeable through CRAC channels (80, 237) (TABLE 3). Interaction of the mutant channels with STIM1 restores high Ca2+ selectivity while significantly narrowing the pore to more closely resemble the dimensions of wild-type Orai1 channels. The tuning of Orai1 ion selectivity by STIM1 is not unique to the V102X mutant channels, but is also seen in wild-type Orai1 channels as the amount of STIM1 bound to Orai1 is increased (237), suggesting that the V102X mutations may mimic a native intermediate channel activation state. Although the connection between STIM1 binding and the alterations in ion selectivity remains to be clarified at a structural level, one possibility is that movement of the NH2 terminus consequent to STIM1 binding allosterically affects the conformation of the selectivity filter located a short distance away in TM1.

The coupling of permeation and gating in CRAC channels contradicts conventional assumptions on the separation of gating and selectivity in ion channels and suggests that there is much more happening in the vicinity of the selectivity filter than originally thought. The presence of a gating structure (V102) located in proximity to the selectivity filter (E106) may enable conformational coupling between the two structures during gating. From a physiological standpoint, the ability of CRAC channels to conduct Na+ under conditions of low STIM1 occupancy may expand their potential functions when activated by subsaturating concen-
trations of STIM1 (although their activity under these conditions would likely be greatly reduced).

VI. CRAC CHANNEL MODULATION

In accordance with the multistep nature of the CRAC channel activation process, a panoply of influences regulate the activity of CRAC channels. Below, we summarize some key modes of CRAC channel modulation that have attracted attention, highlighting the molecular mechanisms of the underlying process wherever known.

A. Slow Ca$^{2+}$-Dependent Inactivation and Mitochondria

During prolonged elevations in [Ca$^{2+}$]$_i$, $I_{\text{CRAC}}$ declines from a combination of deactivation due to store refilling and a process called Ca$^{2+}$-dependent slow inactivation. Store refilling triggers deactivation of the channel as STIM1 binds luminal Ca$^{2+}$ and reverts to its resting state. Slow inactivation is mechanistically distinct from deactivation and can be observed when store refilling is prevented by SERCA inhibitors like TG (276, 460). It appears during whole cell recordings as a decline in current over tens of seconds as [Ca$^{2+}$]$_i$ rises to levels above ~200 nM. The site of action of Ca$^{2+}$ is global rather than local to CRAC channels, because slow inactivation is strongly inhibited by slow intracellular Ca$^{2+}$ buffering conditions that can only capture incoming Ca$^{2+}$ effectively at distances >100 nm from the channel (460). Thus, while its molecular mechanism is not known, slow inactivation is easily distinguished from fast CDI by its slow kinetics and its dependence on global [Ca$^{2+}$]$_i$, and is distinct from deactivation by its independence of Ca$^{2+}$ store content.

Interestingly, mitochondria can modulate the sensitivity of CRAC channels to slow inactivation. Conditions that deenergize mitochondria also reduce CRAC channel activity (149, 150). In whole-cell recordings, slow inactivation can be attributed to mitochondrial depolarization, as it can be prevented by supplying the cell with metabolic substrates and under these conditions be reinstated by mitochondrial inhibitors (118, 149). The molecular basis of mitochondrial maintenance of CRAC channel activity is not entirely clear. However, it is generally thought that, by efficiently buffering Ca$^{2+}$ (320), mitochondria prevent the inactivation of SOCE by competing with the slow inactivation sites for intracellular Ca$^{2+}$ (149, 150). In addition, mitochondria may also facilitate SOCE by releasing pyruvate or ATP (14, 249, 277, 278).

The ability of mitochondria to prolong SOCE may depend on their proximity to CRAC channels. Hoth and colleagues (311) have reported that during periods of prolonged Ca$^{2+}$ entry, mitochondria move closer to the PM, and preventing this translocation by nocodazole enhances slow inactivation (311). An attractive mechanism to explain the relocalization of mitochondrial towards sites of active CRAC channels involves the detachment of the mitochondria-attached kinesin motor from microtubules through its Ca$^{2+}$-dependent binding to the EF-hand protein miro (403, 431). A similar translocation to the immune synapse that forms between T cells and antigen-presenting cells may enhance Ca$^{2+}$ signaling during T-cell activation (312, 339). However, the importance for SOCE of mitochondrial motility and distribution relative to Ca$^{2+}$ influx sites in general has been questioned in several other studies (108, 176, 259). While the basis for the different outcomes is not yet clear, a major complication appears to be that mitochondria modulate SOCE through multiple interacting pathways, none of which is well understood mechanistically (75). Mitochondria have been reported to increase SOCE by enhancing IP$_3$-mediated store depletion (117), but they also can promote store refilling (6), and mitochondrial depolarization may further inhibit SOCE by hindering the translocation of STIM1 to ER-PM junctions (350). Additionally, mitochondria may indirectly regulate CRAC channel activity through their ability to produce reactive oxygen species (see below).

Unraveling the basis for these and other effects remains a major challenge.

B. Phosphorylation and Trafficking

An increasing number of studies implicate phosphorylation as an important inhibitory influence on SOCE (282, 356, 436, 437). Early studies using antagonists of PKC suggested that PKC-mediated phosphorylation of a SOCE protein inhibits $I_{\text{CRAC}}$ (281). Kawasaki et al. (168) subsequently showed that Orai1 itself can be phosphorylated by PKC in a reaction that requires calcium, didecanolglycerol, and phosphatidylserine (168). Alanine substitutions at several potential phosphorylation sites (S25/S27 and S30/S25 in the Orai1 NH$_2$ terminus) modestly enhanced steady-state $I_{CRAC}$, consistent with an inhibitory action of phosphorylation as well as observations that the sites are phosphorylated both constitutively and following store depletion (168). PKC-mediated inhibition of Orai1 may be a widespread mechanism to limit CRAC channel activity through Ca$^{2+}$-dependent feedback. However, this remains an underexplored question, and in particular, the physiological conditions under which PKC phosphorylation of Orai1 dynamically regulates CRAC channel activity have not been identified.

Phosphorylation of STIM1 has also been linked to inhibition of SOCE. Store depletion induces a rapid Tyr phosphorylation of STIM1 in platelets, and based on effects of kinase antagonists, Lopez et al. (207) suggested that this is likely mediated by Src/Abl kinases and may be central for STIM1-Orai1 association. More recently, Putney and colleagues (356) found that phosphorylation of...
multiple sites in STIM1, in particular S486 and S668, potently inhibits STIM1 translocation to the ER-PM junctions and SOCE, which could explain longstanding observations that SOCE is suppressed during mitosis (303). Interestingly, the loss of SOCE during mitosis does not appear to be necessary for normal cell-cycle progression, but the mitosis-specific phosphorylation of STIM1 reduces affinity for EB1 and is needed to release the ER from the mitotic spindle during cell division (355). The suppression of SOCE observed during meiosis is thought to occur through a different mechanism, involving internalization of Orai1, likely through a caveolin- and dynamin-dependent endocytic pathway (436, 437). Thus changes in both STIM1 and Orai1 proteins are likely involved in modulating SOCE during the cell cycle.

C. Redox Modulation of STIM and Orai Function

Cysteine thiol groups make STIM1 and Orai1 sensitive to multiple modes of redox modulation (28). The ensuing modulation has implications for a wide range of cellular effector functions, especially under pathological conditions such as hypoxia (20, 131, 220, 258). In one mode of regulation, oxidation is reported to activate STIM1 and elicit Ca\(^{2+}\) influx even when stores are replete (138). Activation was attributed to S-glutathionylation of a Cys residue located close to EF-SAM (Cys 56), resulting in reduction of the [Ca\(^{2+}\)\]\(_{\text{ER}}\) sensitivity of the EF-hand and consequent unfolding of this domain. However in the same study, a mutation predicted to eliminate S-glutathionylation (C56A) was also reported to activate STIM1 (but see Ref. 305). Further work will be needed to resolve these contradictory findings; mechanisms of redox modulation are often complex, as they reflect the balance between ROS and antioxidant scavenger levels (317), as well as the potential contributions of additional reactive cysteines located elsewhere in the protein.

In a second mode of redox-dependent regulation, Niemeyer and colleagues (29) discovered that Orai1 is directly inhibited by oxidation of a Cys residue (C195 in hOrai1) located in the extracellular III–IV loop. This residue is absent in Orai3, making it insensitive to H\(_2\)O\(_2\)-induced inhibition. Interestingly, the differential sensitivity of Orai1 and Orai3 channels to oxidative inhibition may confer resistance to oxidizing environments as naive T lymphocytes differentiate into effector cells and increase their expression of Orai3 (29). The opposing effects of hypoxia and oxidative stress on STIM1 activation and Orai1 function suggest that the overall effect of redox modulation on Ca\(^{2+}\) signaling is likely to be complex and shaped by several feedback and feedforward loops between oxidant and Ca\(^{2+}\) signaling pathways (425).

D. pH

Acidification of the extracellular milieu commonly occurs in normal tissues during episodes of intense metabolic activity, hypoxic insults, and inflammation, as well as during neoplastic transformation where pH decline is correlated with tumor aggressiveness (185, 405). Acidification of the extracellular pH strongly inhibits \(I_{\text{CRAC}}\) in macrophages with a \(pK_a\) of ~8.2 (217). CRAC channels are also inhibited by intracellular acidification with a \(pK_a\) of 6.8, resulting in an approximately fivefold inhibition between pH 8.2 and 6.2 without a noticeable change in ion selectivity (169). Intracellular acidification also speeds the rate and extent of depotentiation of Na\(^{+}\)-\(I_{\text{CRAC}}\) (169). Interestingly, the \(pK_a\) of the extracellular and intracellular pH effects are very similar to those reported for L-type \(Ca_V\) channels, raising the possibility that the molecular mechanisms of pH regulation could be similar in the two classes of channels. In \(Ca_V\) channels, the glutamates at the selectivity filter were suggested as a possible molecular target of extracellular pH regulation (49). Likewise, Scrimgeour et al. (341) discovered that the E106D mutation in Orai1 strikingly reduced the extracellular pH dependence of Orai1, suggesting that as Ca\(_V\) channels, the Glu residues controlling Ca\(^{2+}\) selectivity in Orai1 also account for the block by extracellular protons. Although Glu has a \(pK_a\) of ~4.3 in solution, hydrogen bonding of carboxylates within proteins can elevate the \(pK_a\) to as high as 8.8 (106), potentially explaining the modulation of CRAC channels by high pH. pH modulation could provide a feedback regulatory mechanism to limit Ca\(^{2+}\) overload during inflammation or hypoxia and control cell damage under pathological conditions (220).

E. Temperature

It has long been known that the activation of \(I_{\text{CRAC}}\) is steeply temperature dependent, displaying an abrupt decline around 21°C (361). This nonlinearity appeared specific to the channel activation process, because it was not observed once channels were already activated (361). The nonlinear dependence on temperature was suggested to result from dependence of the activation machinery on the lipid environment, a premise that remains to be tested. Based on current knowledge of STIM1 and Orai1 and the channel activation process, it is possible that cooler temperatures may impede unfolding of STIM1 to the active form (see above), but once a functional STIM-Orai connection is made, cooling would be expected to have a smaller effect.

More recently, native CRAC channels and heterologous STIM1 and Orai1 have been shown to activate in response to heating without changes in ER Ca\(^{2+}\) content (417). STIM1 appears to be the locus of this effect, as heating cells above 35°C causes STIM1 clustering in the absence of store depletion. Interestingly, the heat-induced clusters of STIM1 do not trap or activate Orai1 at ER-PM junctions and Ca\(^{2+}\)
influx only occurs during a cool-down phase as the temperature is lowered below 37°C. These interesting effects provide new insights into the energetics of STIM1 and Orai1 activation: formation of STIM1 puncta may result from the unfolding of STIM1, which allows plasma membrane binding through its PBD, but heat induced unfolding may not activate the CAD domain or high temperature may destabilize CAD-Orai1 binding such that cooling is required for trapping and activation of Orai1 to occur. Xiao et al. (417) speculate that this pathway of STIM1 gating may contribute to lymphocyte activation during fever, as lymphocytes cycle between central areas where they are exposed to the higher core body temperature and cooler peripheral sites.

F. Diffusible Activator

Of the many early candidates invoked to explain the link between store depletion and CRAC channel activation, the release of a diffusible activator from the ER, termed calcium influx factor (CIF), attracted the most attention (316). The existence of CIF has not been formally confirmed, nor has the activity been purified. Ultimately, the discovery that STIM1 and Orai1 bind directly to each other (284) and that this binding is sufficient to activate Orai1 (453) argue against the necessity for such a messenger. However, a modulatory role for CIF or one that operates in parallel with direct coupling to potentiate SOCE under physiological conditions cannot be formally ruled out. A detailed scheme has been proposed in which STIM1 controls the release or synthesis of CIF, which would displace CaM from iPLA2, releasing the enzyme from inhibition and allowing it to generate lysolipids in the PM that activate CRAC channels (30, 66, 135, 353). Bolotina (30) has suggested that STIM1 and Orai1 may be sufficient for SOCE only when both are overexpressed, but that iPLA2 is required for activation at endogenous levels of expression. One difficulty in testing this model is that iPLA2 has multiple isoforms that are involved in many functions, including homeostasis of intracellular organelles like the ER and Golgi (35, 318). A number of fundamental challenges remain to establish a role for a diffusible messenger such as CIF: isolation and purification of an active substance capable of activating or modulating Orai channels, the demonstration that it is produced and/or released in response to store depletion, identification of the enzymatic pathway that generates it, identification of its biochemical target, and identification of the conditions under which it is active (i.e., does it work in isolation, or only in concert with STIM1).

G. ATP

Several early studies showed that CRAC channel activity is highly dependent on cellular ATP (115, 157, 225). More recent work by Tepikin and colleagues (56) has extended upon these findings by tracking the movements of STIM1 and Orai1 during ATP depletion. They show that as acute inhibition of mitochondrial and glycolytic ATP production causes ATP and PIP2 levels in the PM to decline, Ca2+ leaks from the ER, stimulating the accumulation of STIM1 and Orai1 at ER-PM junctions. As in the earlier studies, the influx of Ca2+ was significantly reduced under ATP-depletion conditions. These results demonstrate that ATP is apparently not required for the events leading up to formation of STIM-Orai complexes (consistent with the diffusion-trap mechanism discussed in sect. III) but that the function of the channel is ATP sensitive in some way, perhaps related to the loss of PIP2 or other phospholipids from the PM. The identification of the molecular targets of ATP regulation may not only reveal additional events in the activation of CRAC channels but also improve our understanding of the biological role of this regulation for cells. ATP regulation of CRAC channel activity could potentially serve to decrease metabolic load, in essence providing protection against excessive Ca2+ influx under ATP-depleting conditions such as hypoxia.

H. STIM/Orai-Interacting Proteins

While STIM1 is able to activate Orai1 without additional proteins in vitro (453), this does not rule out the possibility that these functional interactions are modulated in vivo by auxiliary proteins or environmental conditions. An apt analogy can be made to the membrane fusion process, which can be driven by SNARE proteins alone in vitro, but which in vivo involves a large number of additional proteins that confer essential properties and control (such as high speed, low noise, Ca2+ dependence, etc.) (319). There is indirect evidence that STIM and Orai interact with other proteins; for example, store depletion slows the diffusion of STIM1 even at locations distant from the PM (416), and Orai1 is excluded from cross-bridged ER-PM junctions having a gap of 8–9 nm, which would be expected to otherwise accommodate the extension of the Orai1 cytoplasmic domains (394). An increasing number of STIM- or Orai-binding proteins have been isolated through tandem affinity purification, mass spectrometry/proteomics, and RNAi screens. These include STIM1-binding proteins such as CRACR2A (364), P100 (412), junctate (363), Golgi (97, 98, 401), POST (180), SARAF (274), the ER oxidoreductase ERP57 (305), calnexin (330), and CaM (17, 266). Orai-binding proteins include CaM (257), CRACR2A (364), POST (180), and SPCA2 (99). These regulators and interaction partners are in many cases thought to fine tune the activity of CRAC channels, but the mechanistic details have not yet been worked out. For more information, we refer the reader to the original papers; here we discuss several of the more well-characterized modulator proteins.

Septins were identified as important regulators of SOCE from a genome-wide siRNA screen for inhibitors of SOCE-mediated NFAT signaling in HeLa cells (343). Knockdown
experiments demonstrated that septins 2, 4, and 5 are necessary to maintain the diffuse organization of inactive Orai1 in the PM when ER stores are full. Septins also facilitated the translocation of STIM1 to ER-PM junctions and stabilized Orai1 clusters after store depletion, in line with their requirement for robust SOCE. Interestingly, septins are also required for the local depletion ofPIP2 from ER-PM junctions that occurs when Orai1 is recruited to puncta, perhaps playing a role in enhancing stability of the STIM-Orai complex. Thus septins appear to coordinate the movements and interaction of STIM1 and Orai1 at several different stages of SOCE (343).

Junctate, a Ca2+-binding ER membrane protein, was identified through affinity purification as a STIM1 binding partner with a role in recruiting STIM1 to ER-PM junctions (363). Overexpression of an EF hand mutant of junctate with reduced luminal Ca2+ binding facilitates STIM1 clustering without store depletion and recruits STIM1 to ER-PM junctions without phosphoinositide or Orai1 interaction, leading to activation of CRAC channels. Srikanth et al. (363) suggest that formation of STIM1 clusters via binding to junctate indicates an additional pathway for STIM1 accumulation at the ER-PM junction, and under physiological conditions where Orai1 and STIM1 concentrations are low, it may be required to ensure efficient assembly and activation of CRAC channels.

CRAC regulatory protein 2A (CRACR2A) is a cytoplasmic, EF-hand containing protein that was discovered as an Orai1 binding partner in a large-scale affinity screen (364). The protein has been shown to bind directly to STIM1 and Orai1 NH2 terminus and suggested to form a ternary complex to stabilize the interaction between Orai1 and STIM1. Complex formation facilitates clustering of STIM1 and Orai1 at the ER-PM junction and enhances SOCE (19, 364). Ca2+ binding to the EF hand of CRACR2A promotes its dissociation from STIM1 and Orai1. Thus CRACR2A may participate in the initial formation of STIM-Orai complexes, but may dissociate once channels open and the local [Ca2+]i in the junction rises to high (μM) levels.

SARAF (SOCE-associated regulatory factor), unlike the three proteins described above, is thought to promote the disassembly of STIM1-Orai1 complexes upon store refilling. SARAF was discovered serendipitously from a screen for cDNA candidates that influence mitochondrial Ca2+ homeostasis (274). SARAF is an ER membrane protein that binds to both STIM1 and STIM2 and translocates to ER-PM junctions in a STIM-dependent manner following store depletion. Its luminal domain is proposed to sense [Ca2+]ER (though the mechanism is unknown) (274), while its cytosolic region exerts an inhibitory action on SOCE possibly by binding to the CAD region and preventing it from binding to and activating Orai1 (161). A recent study suggests that SARAF binding to STIM is complex and regulated by a number of other molecules including PIP2, septins, and the mammalian extended synaptotagmin protein, E-Syt1 (218). The requirement for PIP2 may restrict SARAF modulation of STIM1-Orai1 primarily to PIP2-rich domains (218). These features may explain SARAF’s ability to facilitate dissociation of STIM1 from the ER-PM junctions upon store refilling, hence playing a key role in negative regulation of SOCE and preventing the ER from overloading with Ca2+ (274).

Partner of STIM1 (POST) is a protein with 10 putative transmembrane-spanning segments that was identified by affinity purification of Orai1 (180). Like STIM1, the majority of POST protein is located in the ER membrane with 5–10% residing in plasma membrane. POST is reported to associate with STIM1 and a number of transporters (SERCA, PMCA, Na+–K+–ATPase, and nuclear transporters) following store depletion and migrate to ER-PM junctions. Although POST is not required for STIM1-Orai1 interaction and does not influence CRAC channel activation, it appears to inhibit PMCA activity to some degree, possibly enhancing local [Ca2+]i within the ER-PM junction. An intriguing possible function based on its association with karyopherins is that POST may mediate STIM- and [Ca2+]ER-dependent modulation of nuclear transport (180).

VII. PHARMACOLOGY

The complexity of the CRAC channel activation process, involving a choreographic sequence of protein-protein and ER-PM interactions, offers an abundance of potential targets for pharmacological regulation of channel activity (211). Historically, inhibitors such as SKF96365, La3+, and 2-APB were widely used to identify and assign functions to CRAC channels in various tissues. However, the selectivity of these SOC inhibitors is generally weak, and with the exception of La3+, the mechanisms by which they affect CRAC channel function are unclear. Newer reagents have been identified that appear to be much more selective for CRAC channels, including some purported to be selective for particular isoforms (e.g., Orai1) (196). Still, the molecular pharmacology of most compounds is inadequately understood, and the site of action, in most cases, remains unclear. The pharmacology of CRAC channels has been reviewed in detail elsewhere (79, 160, 280, 309, 379).

A. Lanthanides

The trivalent ions La3+ and Gd3+ block endogenous CRAC currents with high affinity (Ki ≈ 20-60 nM) (9, 324, 430).
Although the use of La\(^{3+}\) as a selective CRAC channel blocker is undermined by its ability to also block voltage-gated Ca\(^{2+}\) (Ca\(_V\)) channels, TRP channels, as well as PM-CAs (albeit with much lower affinity) (41, 60, 183), La\(^{3+}\) block at low concentrations has proven useful for identifying CRAC channel-mediated Ca\(^{2+}\) signals (131, 360) as well as to reveal the structural features of the CRAC channel pore. For example, La\(^{3+}\) and Gd\(^{3+}\) blockade in CRAC channels appears to depend on interactions with acidic residues in the outer mouth of the pore formed by the I–II loop region (238, 429). A recent report has suggested that there may be a second, lower affinity lanthanide site at the selectivity filter that is revealed when the high-affinity lanthanide binding in the outer loops is mutated (422). Consistent with this notion, when soaked in millimolar concentrations of Gd\(^{3+}\), the dOrai crystal structure reveals Gd\(^{3+}\) bound by the sextet of glutamate side chains in the selectivity filter (153). These findings suggest that lanthanides block Orai channels by interfering with the access of permeant ions to the selectivity filter and pore, and thus they can reasonably be considered to be pore blockers of CRAC channels. Finally, it is worth noting that La\(^{3+}\) sensitivity of overexpressed Orai1 and Orai3 channels (\(K_i = 0.2–0.4\) M) is reported to be

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<td></td>
<td>46 nM, (n_H =1.0) (IC(_{RAC})-S2 cells)</td>
<td>430</td>
</tr>
<tr>
<td>2-APB</td>
<td>Enhancement of STIM-activated current</td>
<td>Unknown</td>
<td>3 (\mu)M, (n_H =4) (IC(_{RAC}))</td>
<td>300</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>4 (\mu)M, (n_H =3) (Orai1)</td>
<td>290</td>
</tr>
<tr>
<td>2-APB</td>
<td>Inhibition</td>
<td>Unknown</td>
<td>10 (\mu)M, (n_H =4) (IC(_{RAC}))</td>
<td>300</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>8 (\mu)M, (n_H =3) (Orai1)</td>
<td>290</td>
</tr>
<tr>
<td>2-APB</td>
<td>Activation</td>
<td>Direct channel activation?</td>
<td>24 (\mu)M, (n_H =8) (Orai3, IC(_{RAC}))</td>
<td>422</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>15 (\mu)M, (n_H =2) (Orai3, SOCE)</td>
<td>290</td>
</tr>
<tr>
<td>2-APB analogs (DPB162-AE and DPB163-AE)</td>
<td>Inhibition</td>
<td>Unknown</td>
<td>90–170 nM (IC(_{RAC}))</td>
<td>121</td>
</tr>
<tr>
<td>ML-9</td>
<td>Inhibition</td>
<td>Inhibits STIM translocation</td>
<td>16 (\mu)M (SOCE)</td>
<td>354</td>
</tr>
<tr>
<td>BTP2 (YM584883)</td>
<td>Inhibition</td>
<td>Unknown</td>
<td>100–150 nM (SOCE; acute)</td>
<td>159</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>6–12 nM (SOCE; 24 h preinc)</td>
<td>456</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>0.5–2.2 (\mu)M (IC(_{RAC}); acute)</td>
<td>382</td>
</tr>
<tr>
<td>Synta66</td>
<td>Inhibition</td>
<td>Unknown</td>
<td>3 (\mu)M (IC(_{RAC}))</td>
<td>263</td>
</tr>
<tr>
<td>Econazole</td>
<td>Inhibition</td>
<td>Unknown</td>
<td>0.6–14 (\mu)M (IC(_{RAC}))</td>
<td>55, 107</td>
</tr>
<tr>
<td>SK&amp;F96365</td>
<td>Inhibition</td>
<td>Unknown</td>
<td>4 (\mu)M (IC(_{RAC}))</td>
<td>107</td>
</tr>
<tr>
<td>GSK-7975A GSK-5503A</td>
<td>Inhibition</td>
<td>Unknown</td>
<td>4 (\mu)M (Orai1)</td>
<td>81</td>
</tr>
<tr>
<td>R02959</td>
<td>Inhibition</td>
<td>Unknown</td>
<td>25 nM (Orai1)</td>
<td>47</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>530 nM (Orai3)</td>
<td>456</td>
</tr>
<tr>
<td>AnCoA4</td>
<td>Inhibition</td>
<td>Inhibits Orai1</td>
<td>1–10 (\mu)M (Orai1)</td>
<td>329</td>
</tr>
<tr>
<td>m2C1.1 (monoclonal Ab)</td>
<td>Inhibition</td>
<td>Inhibits Orai1 function (by endocytosis?)</td>
<td>&lt;1 nM (SOCE)</td>
<td>65</td>
</tr>
<tr>
<td>Anti-Orai1 mAb</td>
<td>Inhibition</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Inhibitory activity measured from endogenous CRAC current (IC\(_{RAC}\)) or SOCE, or from heterologous currents (Orai1, 2, and 3) as indicated.
lower than that found for endogenous CRAC currents (238, 422). This difference may be related to the higher extracellular Ca\(^{2+}\) concentration used in the Orai measurements (20 mM rather than 2 mM Ca\(^{2+}\)) which could compete more effectively with La\(^{3+}\) for access to its blocking site in the pore.

### B. 2-APB

2-APB, a noncompetitive antagonist of IP\(_3\) receptors (226), is a widely used SOCE/IC\(_{\text{CRAC}}\) inhibitor. Its ability to antagonize IP\(_3\) receptors (IP\(_3\)Rs) was used initially to probe the possible involvement of IP\(_3\)Rs in the gating of SOC\(_{\text{Ca}}\). Based on the inhibition of SOCE elicited by 2-APB, one early study concluded that gating of SOCs occurred through conformational coupling with IP\(_3\)Rs (215). However, subsequent work using DT40 pre-B cells deficient in IP\(_3\)R expression found that the effects of 2-APB on SOCE are unrelated to IP\(_3\)R expression. Nevertheless, these studies also uncovered a dose-dependent bimodal effect of the compound on SOCE, with strong enhancement of IC\(_{\text{CRAC}}\) and SOCE at low doses (<5 \(\mu\)M) and transient enhancement following inhibition by high concentrations (>20 \(\mu\)M).

2-APB is relatively nonselective, as it activates or inhibits many classes of ion channels and receptors including IP\(_3\)Rs, TRPC, TRPM, and TRPV channels, and mitochondrial Ca\(^{2+}\) efflux (156, 195, 300, 399). While this limits its usefulness in identifying activity of endogenous CRAC channels in native cells, it is nonetheless useful for probing gating and permeation mechanisms for heterologously expressed Orai channels (335, 422, 444).

2-APB inhibits IC\(_{\text{CRAC}}\) with an IC\(_{50}\) of \(\sim 10 \mu\)M and reduces fast Ca\(^{2+}\)-dependent inactivation (CDI) of IC\(_{\text{CRAC}}\) in parallel with inhibition of the current amplitude (300). The precise mechanisms by which 2-APB inhibits CRAC channel activity is unclear. 2-APB could affect STIM1, Orai1, or the coupling between the two proteins. Based on observations that high doses of 2-APB dissociate STIM1 puncta, some reports have suggested that 2-APB inhibition of the store-operated current is mediated by disruption of STIM1-Orai1 coupling (73, 290). However, reversal of STIM1 puncta is observed only when STIM1 is overexpressed alone; when Orai1 and STIM1 proteins are coexpressed, puncta are unaffected by 2-APB even as IC\(_{\text{CRAC}}\) is inhibited (73, 260). Furthermore, FRET measurements indicate that the STIM-Orai interaction is maintained even though the current is strongly inhibited (260, 421). Thus, although 2-APB clearly affects STIM1, the mechanism of IC\(_{\text{CRAC}}\) inhibition is unlikely to be related to its ability to inhibit STIM1 translocation into the ER-PM junctions. Still, there are hints that 2-APB affects the functional coupling of STIM1 on the CRAC channel. For example, as noted above, 2-APB eliminates CDI (300, 421). Because CDI requires the presence of STIM1, the inhibition of CDI by 2-APB may indicate the loss of functional coupling between STIM1 and Orai1 (421). One possibility is that 2-APB could either competitively or allosterically displace STIM1 from the NH\(_2\) terminus of Orai channels, thereby inhibiting store-operated gating without interrupting tight STIM1 binding at the COOH terminus.

2-APB enhances STIM-activated IC\(_{\text{CRAC}}\) with an EC\(_{50}\) of 3–4 \(\mu\)M. The underlying mechanism is not known but may involve increased binding of STIM1 to CRAC channels. Such a mechanism is qualitatively consistent with findings that 2-APB modestly increases STIM1-Orai1 FRET (260) and enhances FRET between the cytoplasmic domain of STIM1 (CT-STIM1) and Orai1 (404). In addition, high doses of 2-APB can transiently activate mutants of STIM and Orai1 that are otherwise poorly responsive (402). Noise analysis indicates that potentiation by 2-APB and activation by store depletion both reflect the recruitment of CRAC channels from a silent state to one of high \(P_o\) (299), as if both modes of regulation share common mechanisms, either increased binding of STIM1 to Orai or enhanced coupling between STIM1 binding and channel opening. Interestingly, studies using overexpressed proteins indicate that the degree of potentiation is larger in cells with low STIM-to-Orai ratios compared with those with high STIM1-to-Orai1 ratios (340). Thus the degree of 2-APB potentiation may be related to the extent of initial channel activation by STIM1: lower STIM1-Orai function stoichiometry leading to stronger 2-APB potentiation possibly due to the lower initial channel \(P_o\) at this condition. Furthermore, because STIM1-Orai1 puncta are unaffected by 2-APB, these effects may occur through allosteric modulation of Orai1 gating.

Finally, 2-APB can also directly activate Orai3 (and to a lesser extent, Orai1) independently of ER Ca\(^{2+}\) depletion and STIM1 (73, 290, 335, 422, 444). These 2-APB-evoked currents are only seen when Orai3 is overexpressed and are abolished by Orai3 TM1 mutations such as R66W and G73A (5, 335), indicating that they are not due to 2-APB stimulation of endogenous TRP or other channels. Interestingly, 2-APB-activated Orai3 currents exhibit strikingly different biophysical properties than STIM1-activated currents, including low Ca\(^{2+}\) selectivity and ability to conduct Cs\(^{+}\) and large monovalents normally impermeable through STIM1-activated Orai3 channels, indicative of a wider pore (335, 422).

How 2-APB gates Orai3 channels is unknown. Zhang and colleagues (444) used chimeras of Orai1 and Orai3 to determine that an extended region spanning TM2 through TM3 is critical for 2-APB gating of Orai3 channels. Moreover, examination of gating in a G158C Orai3 mutant suggests that G158 in TM3 moves close to C101 in TM2, thereby trapping Orai3 channels in a distinct intermediate open state (4). A key issue is whether 2-APB and STIM1
gating mechanisms occur through similar or distinct pathways. Noise analysis has indicated that both gating modes appear to involve the stepwise recruitment of closed channels to a long-lasting, high \( P_o \) state (422). Furthermore, the STIM1- and 2-APB-dependent modes of Orai3 activation appear to be mutually exclusive: Orai channels bound to STIM1 resist 2-APB gating, and 2-APB antagonizes STIM1 gating (421). These findings suggest that despite different pore structures, the two modes of channel activation may share common gating steps.

Despite its weak selectivity for CRAC channels, the ability of 2-APB to potentiate and inhibit \( I_{\text{CRAC}} \) has generated interest in using 2-APB as a basis for future drug development. From the parent chemotype, two related stereoisomers, DPB-162AE and DPB-163AE, were constructed as dimers of 2-APB (121). These compounds are over 100-fold more potent than 2-APB itself in inhibiting SOCE (IC\(_{50}\) of ~0.6 \( \mu \)M), but do not affect IP\(_3\)R function at these concentrations (121). DPB-162AE elicits bimodal modulation of SOCE, potentiating at low doses and inhibiting at high doses, whereas DPB-163AE only inhibits. The myriad nonspecific effects of 2-APB on other channels and its many effects on the CRAC channel itself present formidable challenges as a platform for developing specific CRAC channel therapeutics. Still, the ability of 2-APB to modulate key aspects of CRAC channel behavior such as permeation and inactivation is likely to be useful in understanding the molecular mechanisms underlying these properties.

C. ML-9

ML-9 [1-(5-chloronaphthalene-1-sulfonyl)homopiperazine HCl] is a potent inhibitor of MLCK, and early evidence of its ability to inhibit SOCE suggested a role for MLCK in the SOCE mechanism, prior to the discovery of STIM and Orai (for references, see Ref. 354). In store-depleted cells expressing exogenous STIM1, ML-9 was found to disperse STIM1 puncta at doses comparable to those that inhibited SOCE, and slightly preceding the reduction in SOCE (354). These results led Putney and colleagues (354) to propose that ML-9 inhibits SOCE by preventing the accumulation of STIM1 in puncta. Inhibition of STIM1 translocation appears to be independent of MLCK inhibition, as it persists in cells in which MLCK is knocked down, and MLCK inhibition by wortmannin did not affect SOCE (354). Interestingly, overexpression of STIM1 reduces the sensitivity of SOCE to inhibition by ML-9 (354), and ML-9 is essentially ineffective when Orai1 is also coexpressed (73); the reasons for this are unclear, but could potentially be related to the increased stability of STIM1 at the ER-PM junctions caused by STIM1-Orai1 overexpression. Because ML-9 inhibits MLCK by competing with ATP binding, one possibility is that its effects on SOCE are related to the requirement for ATP in the induction of CRAC channel activation (157, 225). However, STIM1 puncta formation occurs independently of global ATP concentrations (56), suggesting that this is not the most straightforward explanation. Thus, although its target and mechanism of action are unknown, ML-9 is the only inhibitor so far to inhibit SOCE by interference with STIM1 localization. Further work to define its mechanism of action, as well as to identify related SOCE inhibitors that lack effects on MLCK, may cast new light on the mechanisms of STIM function, including clustering at the ER-PM junctions.

D. Imidazoles

The imidazole compound SKF96365 and related antimycotic compounds including econazole, miconazole, and clotrimazole inhibit all SOCs with IC\(_{50}\) values in the range of 0.6–14 \( \mu \)M (55, 107). However, these compounds also independently block TRP channels (31), voltage-gated Ca\(^{2+}\) channels (241, 351), and K\(^+\) channels (338), as well as the cytochrome P-450 enzyme (55). It is worth noting that although some reports raised the possibility that P-450 may be involved in the control of SOCE based on the inhibition of both P-450 and SOCE (188), Christian et al. (55) showed that application of econazole through the recording pipette does not inhibit \( I_{\text{CRAC}} \) while a less membrane-permeant derivative applied extracellularly blocked \( I_{\text{CRAC}} \) with normal efficacy and kinetics, suggesting that econazole works through an extracellular interaction as a true CRAC channel inhibitor (55). SKF96365 has been used in many cell-based and in vivo studies (432, 457), but the inadequate specificity, slow kinetics, and incomplete reversibility of these compounds limit their usefulness. The effects of these compounds on recombinant, overexpressed STIM1 and Orai1 have not been reported, and the mechanism by which these compounds exert their inhibitory effects is unknown.

E. BTP2

Another class of agents that inhibits CRAC channel activity are the bis(trifluoromethyl)pyrazoles (BTPs) which potently inhibit cytokine release from human lymphocytes and suppress T-cell proliferation (52, 159, 390). The best-studied member of this group is the compound BTP2 (also called YM-58483), which inhibits TG-evoked Ca\(^{2+}\) influx and \( I_{\text{CRAC}} \) in Jurkat T cells (159, 382, 456). BTP2 inhibits \( I_{\text{CRAC}} \) in Jurkat T cells with a \( K_D \) of \( \sim 10 \) nM following overnight incubation, and CRAC current inhibition is essentially irreversible when preapplied (456). However, the specificity and effectiveness of BTP2 for \( I_{\text{CRAC}} \) has been challenged by findings showing that the compound’s potency is ~100-fold lower when applied acutely and that it potently activates the Na\(^{+}\)-permeable channel TRPM4 at low nanomolar concentrations (382). Based on these findings, Takezawa et al. (382) have proposed that a key mechanism of BTP2 inhibition of Ca\(^{2+}\) influx and cytokine release is related to its ability to depolarize the cell membrane via
TRPM4 activation, thereby reducing the driving force for Ca\(^{2+}\) entry. BTP2 exhibits specificity for CRAC channels over K\(^+\) channels (456), Ca\(_{\text{V}}\) channels (159), and TRPV6 channels (139) but inhibits overexpressed TRPC3 and TRPC5 channels (139), raising the question of whether inhibition of both classes of channels occurs through a common mechanism. Although the compound’s effects have been extensively characterized in many immune-based cellular and even animal models of autoimmune diseases for asthma and delayed-type hypersensitivity (47, 267, 268, 433), the precise mechanisms by which the BTP compounds inhibit Ca\(^{2+}\) influx are not fully understood.

F. Synta 66

Synta 66 [3-fluoropyridine-4-carboxylic acid (2’5’-dime-thoxybiphenyl-4-yl)amide, Synta Pharmaceuticals, Lexington, MA] is structurally similar to BTP2 but contains a biphenyl group rather than the pyrazole ring in BTP2. The compound is reported to inhibit \(I_{\text{CRAC}}\) in RBL cells with an IC\(_{50}\) of \(\sim 3\) \(\mu\)M and appears to be selective at least in so far as it does not inhibit K\(^+\) channels or Ca\(^{2+}\) pumps (263). As with BTP2 and the GSK compounds (see below), the speed of inhibition by Synta 66 is slow, requiring cells to be preincubated for long periods (>1 h), and its effects are poorly reversible, if at all, making a pore blocking mechanism unlikely. Synta66 has no effects on STIM1 puncta formation, suggesting that it does not inhibit the early steps of STIM1 activation and its translocation to junctional ER sites (194). Despite limited information on its mechanism of CRAC channel inhibition, however, a growing number of studies have employed this compound to probe the physiological contributions for CRAC channels for effector function, including antigen-induced signaling, cytokine production, and transcriptional regulation (7, 85, 331).

G. GSK-7975A and GSK-5503A

Romanin and colleagues (81) have recently described two pyrazole derivatives, GSK-5503A [2,6-difluoro-N-(1-(2-phenoxybenzyl)-1H-pyrazol-3-yl)benzamide] and GSK-7975A [2,6-difluoro-N-(1-(4-hydroxy-2-(trifluoromethyl) benzyl)-1H-pyrazol-3-yl)benzamide], that inhibit recombinant Orai1 and Orai3 currents with an IC\(_{50}\) of \(\sim 4\) \(\mu\)M (81). As with many other inhibitors described above, the inhibition is slow to develop, requiring several minutes of preincubation. FRET studies show no inhibition of STIM1-Orai1 coupling, suggesting that the compounds do not affect the proximal steps of the CRAC channel activation process. The IC\(_{50}\) for inhibition increased 10-fold in the E106D Orai1 mutant and in 2-APB-activated Orai3 channels (81), both of which are poorly selective for Ca\(^{2+}\) and which exhibit wider pores than the wild-type Orai1 channel (420, 422). Although these results were interpreted to imply that the compounds act as Orai1 pore blockers, the slow onset of inhibition argues against such a mechanism, and other possibilities including stabilization of closed channels and allosteric effects on STIM1 function cannot be ruled out. Interestingly, along with Orai inhibition, these compounds also inhibit TRPV6 channels, an effect that was attributed to possible structural similarities between CRAC and TRPV6 channels in the target site (81). More studies are clearly needed to define the mechanism and site of antagonism and selectivity of these compounds before they can be employed as tools to probe CRAC channel mechanisms and functions.

H. RO2959

RO2959 (Roche) is another recently developed compound with functional properties analogous to BTP2, the GSK compounds, and Synta66 in that it requires preincubation for suppression of SOCE and exhibits a modest affinity for suppression of \(I_{\text{CRAC}}\) (47). However, a key notable feature of this compound is its apparent selectivity for Orai1 channels over Orai2 or Orai3 channels (IC\(_{50}\) values for recombinant Orai1 and Orai3 channels were 25 and 530 nM, respectively). SOCE in CD4 T cells is highly sensitive to RO2959 (IC\(_{50}\) \(\sim 40\) nM), and the compound potently inhibits a variety of effector functions including gene expression, cytokine production, and T-cell proliferation (47). Puzzlingly, however, currents in RBL cells exhibited a much lower sensitivity (IC\(_{50}\) \(\sim 400\) nM), raising the possibility that the CRAC channels in RBL cells have a somewhat different molecular composition (Orai1-Orai2 or Orai1-Orai3 heteromers), or that the compound acts at a different target site that is only indirectly coupled to CRAC channel activation. RO2959 does not antagonize a variety of other channels, including TRPM2, TRPM4, TRPC1, Ca\(_{\text{V}}\)1.2, and various KV channels (47). The molecular basis of drug action, including whether it affects the function and choreography of STIM1, remains unclear.

I. AnCoA4

Sadaghiani et al. (329) employed a novel strategy to enhance drug specificity for the Orai1 and STIM1 proteins by screening for compounds that bound to peptide fragments of Orai1 and/or STIM1 immobilized in microarrays. The resulting library screen yielded several promising hits, one of which (AnCoA4) was characterized in detail in biophysical and functional tests. AnCoA4 inhibits CRAC channels at concentrations in the low micromolar range and attenuates T-cell activation in vitro and in vivo assays. The identification of AnCoA4 represents a different methodology of screening compounds based on mechanism—in this case, the coupling of STIM1 to Orai1. The drug reduces the recruitment of Orai1 into puncta and also directly inhibits the activity of the constitutively active Orai1 V102C chan-
nels independently of STIM1, suggesting that binding to the COOH terminus of Orai1 inhibits STIM1-Orai1 binding and also delivers an inhibitory signal to Orai. This novel drug screening strategy may yet prove useful to arrive at different classes of drugs based on a highly focused step of the activation mechanism rather than the entire target protein or the end result of target activation.

J. Monoclonal Antibodies

As an alternative to small-molecule antagonists, Lin et al. (196) employed a novel approach to generate high-affinity fully human monoclonal antibodies (mAbs) against human Orai1 (hOrai1). These recombinant mAbs exhibited strong and specific binding to human Orai1 with \( K_d \) values of 20–100 pM. Analysis of binding selectivity to the human and rodent homologs revealed high specificity to human Orai1. Epitope mapping revealed amino acid residues 210–217 in the second extracellular loop of Orai1 as the interaction site for the antibody. The recombinant residues 210–217 in the second extracellular loop of human Orai1. Epitope mapping revealed amino acid residues 210–217 in the second extracellular loop of Orai1 to human Orai1 with \( K_d \) values of 20–100 pM. Analysis of binding selectivity to the human and rodent homologs revealed high specificity to human Orai1. Epitope mapping revealed amino acid residues 210–217 in the second extracellular loop of Orai1 to human Orai1 with \( K_d \) values of 20–100 pM. Analysis of binding selectivity to the human and rodent homologs revealed high specificity to human Orai1. Epitope mapping revealed amino acid residues 210–217 in the second extracellular loop of Orai1 to human Orai1.

Taking a similar approach, Cox et al. (65) reported that a specific anti-Orai1 monoclonal antibody targeting the second extracellular loop inhibited the proliferation of T cells and cytokine production in vitro and in vivo. Their analysis indicated that the inhibition of T-cell effector functions occurred through the internalization of Orai1. Examination of Orai1 expression on subsets of immune cells from patients with rheumatoid arthritis (RA) suggested strong up-regulation of Orai1 expression in RA patients, and the mAb was found to be effective in inhibiting T-cell mediated graft-versus-host disease in a mouse model. Collectively, these antibody-based approaches support Orai1 as a potential target for the treatment of autoimmunity and other inflammatory immune diseases.

The findings of these carefully executed studies validate anti-CRAC channel antibodies for therapeutic applications for immune diseases, in particular for autoimmune diseases to regulate runaway activation of immune cells. It is worth noting, however, that attempts to translate the therapeutic potential of these mAbs in vivo have not yet succeeded. In vivo tests in cynomologus monkeys injected with the 2C1.1 mAb revealed unexpected generation of antigen-specific antibodies, despite robust inhibition of T-cell cytokine production (IL-2) ex vivo (114). This result was interpreted to indicate that full inhibition of the CRAC channel is required to suppress the antibody response, rather than the partial suppression achieved by the administration of anti-CRAC channel mAbs. These unforeseen results highlight the growing need for a better understanding of the effects of partial inhibition of CRAC channels and the pitfalls of extrapolating expected results from the phenotypes of human SCID patients with complete loss-of-function mutations in Orai1.

K. Miscellaneous

In addition to the compounds described above, CRAC channels are also inhibited by endogenous regulators of cell growth and proliferation such as sphingosine, ceramide, and related analogs at micromolar concentrations (IC\(_{50}\) values \( \sim 1–10 \) \( \mu \)M) (229). Sphingosine and ceramide are bioactive lipid metabolites generated by the breakdown of the widespread lipid sphingomyelin and can directly bind proteins, activate signaling pathways, and affect a variety of cellular responses. Ceramide and sphingosine are generated in T cells by cross-linking of Fas (CD95) on the cell surface. Thus inhibition of CRAC channels by these lipid mediators may represent a mechanism for conferring immunosuppression by tumor cells expressing high levels of Fas ligand, enabling these cells to evade immune surveillance in vivo. Although sphingosine has been suggested to inhibit CRAC channels in mast cells through a “direct” effect (229), the precise mechanism of sphingosine inhibition remains unknown.

The biotechnology firm CalciMedica has described two structurally related inhibitors, CM2489 and CM3457, that inhibit CRAC current in lymphocytes and T cell-derived cytokine production (314). They report that CM2489 has completed phase 1 clinical trials for the treatment of moderate-to-severe plaque psoriasis. This is the first CRAC channel inhibitor to be tested on humans and represents a promising lead for the development of novel therapeutics for allergic autoimmune disorders.

Some compounds that have attracted wide attention for their therapeutic and/or beneficial effects on human health have been found to potently inhibit CRAC channel activity. For example, diethylstilbestrol, a synthetic estrogen and endocrine disruptor that was widely manufactured and clinically prescribed until 1971 to pregnant women (when it was banned), was found to inhibit \( I_{\text{CRAC}} \) in RBL cells (IC\(_{50}\) \( \sim 0.5 \) \( \mu \)M) and SOCE in several other cell types through an extracellular mechanism (440). Likewise, the plant-derived polyphenol, curcumin, which has drawn attention for potential therapeutic effects based on its anti-inflammatory properties, reportedly inhibits \( I_{\text{CRAC}} \) in Jurkat T cells (IC\(_{50}\) \( \sim 6 \) \( \mu \)M) and recombinant \( I_{\text{CRAC}} \) in HEK293 cells overexpressing STIM1 and Orai1 (IC\(_{50}\) \( \sim 0.5 \) \( \mu \)M) (346). This effect was suggested to contribute to curcumin’s reported anti-inflammatory effects. The mechanism(s) underlying the inhibitory effects of these compounds are unknown.
Additionally, Sirolimus (rapamycin), a bacterial byproduct that blocks T- and B-cell activation through inhibition of mTOR (mammalian target of rapamycin), is widely used to reduce transplant rejection. A recent report finds that rapamycin inhibits both recombinant STIM1/Orai1-mediated currents as well as SOCE in human arterial smooth muscle at clinically relevant concentrations (179). This finding raises the interesting possibility that its immunosuppressive effects may be mediated at least in part through inhibition of effector functions driven by CRAC channels (gene expression and cytokine production). Thus rapamycin may represent the first CRAC channel inhibitor to modulate immune functions in human patients. Finally, N-arachidonoyl glycine (NAGly), a derivative of the endocannabinoid anandamide that functions as a ligand for the cannabinoid receptors CB1 and CB2, inhibits SOCE by interfering with STIM1-Orai1 association (71). This finding raises the interesting notion that the well-known pleiotropic effects of endocannabinoids might be mediated at least in part through modulation of SOCE in the brain.

VIII. PHYSIOLOGICAL FUNCTIONS AND DISEASE

In light of the widespread tissue distribution of Orai and STIM proteins, many specific roles are to be expected for CRAC channels in different organ systems. Studies in human patients and genetically engineered mice with loss- or gain-of-function STIM1 and Orai1 genes have revealed serious functional abnormalities in several systems including the immune system, musculature, and the skin, supporting the concept that STIM1 and Orai1 are important mediators of many cellular functions and pathologcal events. Several excellent reviews (63, 84, 100, 101, 304, 326) have described a diverse set of cell behaviors that are regulated by STIM1/Orai1. In this section, we present an overview of key functions attributed to CRAC channels in a narrow range of cells and tissues, with the expectation that many more functions are likely to be recognized in the future. In particular, the roles of the noncanonical homologs, STIM2, Orai2, and Orai3, remain largely unknown. Identification of human mutations in these proteins should further our understanding of their physiological and pathophysiological roles. Mouse knockout models for these less studied homologs will also be informative, but if the immune system is a guide, the usage of particular STIM and Orai isoforms is likely to differ between humans and mice, which may complicate attempts to extrapolate to their roles in human physiology.

A. Spatiotemporal Control of Gene Expression

A critical and widespread function of SOCE is to control gene expression through Ca\(^{2+}\)-sensitive transcription factors such as NFAT (102). Ca\(^{2+}\) activates calcineurin which dephosphorylates NFAT, exposing a nuclear localization sequence that enables its translocation into the nucleus where it combines with other transcription factors to control a wide variety of genes in many cells (145a). Although [Ca\(^{2+}\)]\(_{i}\) oscillations most often occur through repetitive release of Ca\(^{2+}\) from the ER (21), in T lymphocytes global [Ca\(^{2+}\)]\(_{i}\) oscillations also arise through cyclical activation and deactivation of CRAC channels (87, 193). STIM1 movements toward the PM in HEK cells also fluctuate in synchrony with [Ca\(^{2+}\)]\(_{i}\), oscillations, consistent with the oscillatory SOCE seen in T cells (27). [Ca\(^{2+}\)]\(_{i}\) oscillations have been shown to increase both the efficiency and the specificity of transcriptional activation among NFAT, NFκB, and Oct1-OAP pathways (86). Interestingly, Parekh and colleagues (164) have reported that activation of NFAT in HEK cells occurs preferentially at ER-PM junctions, based on the inability of slow Ca\(^{2+}\) buffers (which reduce global but not local [Ca\(^{2+}\)]\(_{i}\)) to inhibit NFAT translocation to the nucleus. Similarly, local Ca\(^{2+}\) near CRAC channels activates NFAT in neural stem cells (360). Thus one attractive idea is that pulsatile Ca\(^{2+}\) influx through CRAC channels, which may be too small to contribute significantly to global [Ca\(^{2+}\)]\(_{i}\), may nonetheless serve to effectively and selectively drive NFAT activation by elevating [Ca\(^{2+}\)]\(_{i}\) in the restricted volume of the junctional cleft. The molecular basis for local NFAT activation may involve the scaffold AKAP79 that brings calcineurin to the vicinity of CaM bound to Orai1 at ER-PM junctions (165). Related mechanisms may underlie the ability of Orai channels to activate Fos (264), leukotriene C\(_4\) production (46), and TRPC1 insertion (48) and to modulate enzymes like adenyl cyclase 8 (410) and the plasma membrane Ca\(^{2+}\)-ATPase 4b (18) through local elevation of [Ca\(^{2+}\)]\(_{i}\).

B. Immune System

Human and mouse studies by Feske and colleagues show that Orai1 deficiency or loss-of-function diminishes \(I_{CRAC}\) in T cells and impairs the production of key cytokines including interleukin (IL)-2, IL-4, interferon (IFN)-γ, and IL-10 (102, 134) (reviewed in Refs. 100 and 101). Likewise, STIM1 knockout mice exhibit severely reduced SOCE and \(I_{CRAC}\) in various immune cells including T cells, mast cells, B cells, and macrophages (12, 25, 33, 269). These effects on CRAC channel function are accompanied by striking defects in effector function including impaired degranulation and cytokine production in mast cells and production of cytokines such as IL-2, IFN-γ, and IL-4 by T cells (12, 25, 33, 269). Proliferation of B cells in response to antigenic stimulation is also suppressed (134). These impairments collectively lead to the loss of host defense pathways and a devastating immunodeficiency in human patients (101). However, the same reduction in T-cell effector function may in some cases be beneficial in ameliorating pathological responses associated with graft rejection, multiple sclerosis, and inflammatory bowel disease (233, 337).
Although deficiency in STIM2 does not significantly impair CRAC channel function and SOCE in T cells when measured immediately following store depletion, a marked deficit in Ca\(^{2+}\) levels and activation of the Ca\(^{2+}\)-dependent transcriptional regulator NFAT was noted at longer times (>1 h) following stimulation, suggesting that STIM1 alone is not sufficient to maintain prolonged Ca\(^{2+}\) signals (269). The basis for this phenotype is not entirely clear, but as noted above, STIM2 exhibits a lower affinity for Ca\(^{2+}\) than STIM1 and as a consequence is activated by lower levels of store depletion near the resting [Ca\(^{2+}\)]\(_{ER}\) (32). Thus one possibility is that as IP\(_3\) levels decline and stores slowly refill during the late stages of the prolonged stimulation, STIM2 knockout T cells are unable to generate the minimal level of Ca\(^{2+}\) entry that is required for cytokine induction. STIM2 deficiency also results in a reduction in effector T-cell function and cytokine production, although to a milder extent than in STIM1 KO cells (269).

Given the immunosuppressive effects described above, it is somewhat surprising that mice (and humans) lacking critical CRAC channel proteins also exhibit autoimmunity, characterized by hemolytic anemia, thrombocytopenia, and lymphoproliferative disease (296). Studies in STIM1/STIM2 double knockout mice indicate that this phenotype may arise from a reduction in the number of regulatory T cells (Tregs) (269), a subpopulation of T cells with suppressor functions required for maintaining tolerance to self-antigens. The autoimmunity is surprising because mice (and humans) without functional SOC proteins should lack immune effector cell function. The paradox suggests that low levels of T-cell effector function may be retained even in the absence of SOC proteins, and when left unchecked by Tregs, lead to autoimmunity. However, autoimmunity and Treg deficiencies are not always seen in SOCE-deficient animal models, for example, in the Orai1\(^{-/-}\) or STIM1\(^{-/-}\) mice where Treg numbers are in the normal range (233, 269). These phenomena reveal the complexity of STIM and Orai function in immune cells and deserve further study.

The Orai isoforms may be differentially regulated during development. It has been reported that Orai2 is expressed in mouse naive T cells and may underlie the seemingly normal T-cell effector responses and CRAC currents seen in the naive T cells of Orai1-deficient mice (233, 397). Given that mature T cells lacking Orai1 also lack \(I_{\text{CRAC}}\) and functional responses, these data point to a switch from Orai2 to Orai1 expression during T-cell maturation in mice. The prevalence of such phenotypic switching and its functional relevance for Ca\(^{2+}\) signaling remain largely unknown.

An unexpected result from the knockout studies is that loss of Orai1, STIM1, and STIM2 does not grossly impair the development and differentiation of T cells (CD4 and CD8), B cells, and mast cells (12, 25, 134, 269). These observations are consistent with the normal levels of CD4 and CD8 T cells seen in Orai1-and STIM1-deficient patients (100) and indicate that the CRAC channel machinery is not critical for the development of immune cells in the bone marrow and thymus. However, a recent study has found that pharmacological suppression of CRAC channel function or deletion of Orai1 impairs the differentiation of a subclass of proinflammatory CD4 T cells, the Th17 cells (172). This finding is reminiscent of the lack of Tregs in STIM1/STIM2 DKO mice (269) and highlights the complexity of SOCE contributions to the development of subpopulations of T cells. Thus it would appear that in thymocytes, Ca\(^{2+}\) influx pathways unrelated to SOCE are responsible for stimulation of the calcineurin-NFAT pathway, which has long been implicated in effector T-cell development.

An intriguing finding is that STIM and Orai localize to the immune synapse that forms between T cells and antigen-presenting cells during the immune response (16, 197). At later times following contact, STIM and Orai are also reported to accumulate at the distal pole of the cell (16). STIM-Orai FRET suggests a close interaction between the proteins at both locations (16), while Ca\(^{2+}\) influx appears to be concentrated at the synapse (197). These findings raise a number of important questions: what mechanisms determine the redistribution of STIM and Orai to these locations, and what are their functions there (181)?

C. Platelets

STIM1 and Orai1 are reported to be the primary Ca\(^{2+}\) influx pathway for agonist-evoked Ca\(^{2+}\) influx (thrombin and ADP-induced signaling) in platelets, and STIM1-deficient mice exhibit impaired agonist-stimulated platelet SOCE and impaired thrombus formation, resulting in a mild increase in bleeding time following injury (393). These mice are significantly protected from arterial thrombosis and ischemic brain infarction. Likewise, Orai1-deficient mice exhibit defective SOCE in platelets, impaired thrombus formation, and resistance to downstream consequences including pulmonary thromboembolism, arterial thrombosis, and ischemic brain infarction (34). A key role for CRAC channels in platelet function is further highlighted by recent reports identifying missense gain-of-function mutations in Orai1 (P245L) and STIM1 (R304W). These mutations boost Ca\(^{2+}\) signals including resting Ca\(^{2+}\) levels by activating Orai1 and STIM1 through mechanisms that are likely related to destabilization of their closed or inactive states (250, 262, 275). The ensuing defect causes thrombocytopenia characterized by preactivation of platelets, coagulation, and fibrinolysis (224, 248, 250, 262). Thus CRAC channels appear to be critical regulators of platelet effector function, raising the possibility that modulation of CRAC channel activity could be beneficial for the treatment of diseases associated with vascular thrombosis including cerebrovascular disorders.
D. Skeletal Muscle

An important physiological role for SOCE in skeletal muscle was historically considered unlikely because Ca\(^{2+}\) entry is not directly required for contraction, being instead mediated by Ca\(^{2+}\) release through RyRs in the SR that are mechanically coupled to Ca\(_v\) L-type channels in the T tubules. Several studies, however, identified an SOCE mechanism of Ca\(^{2+}\) entry in skeletal muscle fibers that requires the canonical STIM1 and Orai1 proteins (373, 406). STIM1 appears to be essential for myotube development; loss of STIM1 causes defects in muscle differentiation both in vitro and in vivo, and has been proposed to underlie the high perinatal mortality of STIM1-deficient mice (373). In humans, loss of STIM1 or Orai1 expression is associated with a congenital nonprogressive myopathy attributed to loss of the fast twitch type II muscle fibers (101, 234). In addition, mouse studies have revealed that loss of STIM1 increases the propensity for rapid muscle fatigue during prolonged stimulation (373). Interestingly, patients with gain-of-function mutations in STIM1 and Orai1 (e.g., R304W in STIM1 and G98S, L138F, and P245L in Orai1) exhibit significant muscle abnormalities, including tubular aggregate myopathy, which results in muscle weakness and propensity for fatigue (93a, 224, 250, 262). These studies indicate that the proper regulation of SOCE is required to support the normal development and homeostatic function of skeletal muscle.

STIM1 is localized to the muscle SR at triadic junctions where Ca\(^{2+}\) is released by RyR1, and it appears that STIM1 senses the gradual depletion of SR stores during tetanic activity, thereby stimulating SOCE and ultimately stores refilling to maintain the SR Ca\(^{2+}\) content (373). Skeletal muscle expresses high levels of both STIM1 and Orai1 and the muscle defects seen in the KO mice are consistent with the congenital myopathy observed in patients deficient in Orai1 and STIM1. More recent work from Dirksen and colleagues (406) indicates that in skeletal muscle store depletion triggers SOCE via rapid conformational coupling between STIM1 luminal Ca\(^{2+}\) sensor proteins located in the SR and Orai1 channels present in the transverse (t)-tubule membrane. Interestingly, unlike in nonexcitable cells, STIM1 and Orai1 proteins appear to be prelocalized in close proximity to each other within the triad junction in skeletal muscle under resting conditions, thus permitting extremely fast activation and deactivation of SOCE and presumably, efficient trans-sarcotomal Ca\(^{2+}\) influx during SR depletion (91). Depletion of SR Ca\(^{2+}\) stores and accompanying activation of SOCE under conditions of repetitive stimulation has, however, not been measured directly. Launikonis and colleagues (91, 186) have argued that the level of store depletion required for activation of SOCE in skeletal muscle is far greater than in nonmuscle cells, raising questions about precisely how the Ca\(^{2+}\) concentration in the SR changes and how this relates to CRAC channel activation during tetanus. Regardless of their possible role in acute muscle contractility, it is becoming clear that the store-operated and excitation-coupled Ca\(^{2+}\) pathways reflect two distinct molecular channel complexes within the triad junction that may enable trans-sarcolemmal Ca\(^{2+}\) entry across a wide range of transmembrane voltages. These results have challenged preexisting notions about the importance of SOCE for skeletal muscle function and indicate the importance of SOCE for the development and function of skeletal muscle fibers (173).

E. Cell Migration and Cancer

STIM1 and Orai1 have emerged as potential new targets for treatments of several types of cancers. In fact, before the discovery that STIM1 is the ER Ca\(^{2+}\) sensor, one study implicated STIM1 in cancer metastasis and suggested that STIM1 could be a tumor suppressor (377). In several types of cancer, SOCE has been associated with tumorigenesis, tumor growth, and metastasis. One study found that Orai1 and STIM1 are essential for breast tumor cell migration in vitro and tumor metastasis in mice (423), with pharmacological inhibition or knockdown of Orai1 or STIM1 reducing metastasis. These protective effects were attributed to blockade of the assembly and disassembly of focal adhesions, which are crucial for cellular migration. Another study (232) reported that human breast cancer lines displayed increased levels of Orai1, and microarray analysis of 295 breast cancers showed that women with a transcriptional profile characterized by high STIM1 and low STIM2 expression had the poorest prognosis. Interestingly, tumorigenesis is reportedly accompanied by a switch in expression of the Orai isoform, from Orai1 to Orai3 functioning in a store-independent fashion (252). Whether the switch to Orai3 is the cause or consequence of enhanced tumorigenesis is not clear. Along the same lines, there is evidence that tumorigenesis in breast cancer cells may be driven by constitutive activation of Orai1 channels through a mechanism that involves the Golgi Ca\(^{2+}\)-ATPase SPCA2. Aberrant expression of SPCA2 in breast cancer cells in the ER was postulated to constitutively activate plasma membrane Orai1 channels through a direct interaction, resulting in enhanced Ca\(^{2+}\) entry and promoting tumorigenesis by increasing the expression of cell cycle proteins (99).

STIM1-dependent Ca\(^{2+}\) signaling is reported to be important for cervical cancer cell proliferation, migration, and angiogenesis (50, 51). The level of STIM1 expression was associated with the risk of metastasis and survival. Analogous to the findings in breast cancer cell lines described above, STIM1 expression was found to regulate focal adhesion dynamics, through modulation of the activity of the protease calpain and the kinase Pyk2 (50), which regulates focal adhesion turnover. Another recent study has found that Ca\(^{2+}\) oscillations driven by STIM1 and Orai1 promoted melanoma metastasis by driving the assembly of in-
plasticity (10), Ca\(^{2+}\) transitions, including neurotransmitter release (93), synaptic studies implicate SOCE in several neuronal cellular functions in the brain, including the hippocampus, dentate gyrus, and cerebellum as well as in proliferating regions such as the subventricular zone and the olfactory bulb (20, 127, 352).

Consistent with the above expression studies, functional studies implicate SOCE in several neuronal cellular functions, including neurotransmitter release (93), synaptic plasticity (10), Ca\(^{2+}\) oscillations (349), and gene expression (182, 360). Moreover, aberrant SOCE has been implicated in hypoxia-mediated neuronal death (20), epilepsy (372), and the response to axonal injury (116). More recently, Konnerth and colleagues (136) have reported that STIM1 is a key regulator of mGluR1-dependent intracellular Ca\(^{2+}\) signals and synaptic potentials. Ablation of STIM1 suppressed mGluR1 evoked slow synaptic potentials and impaired motor coordination, indicating a role for STIM1 in cerebellar homeostasis and function. This finding is consistent with reports that intact SOCE is vital for the firing of flight motorneurons in Drosophila (393). On the postsynaptic side, Sun et al. (376) found that SOCE activated by STIM2 and the ensuing activation of Ca\(^{2+}/\)calmodulin-dependent kinase II (CaMII) is a key regulator of spine maturation. They further reported that familial Alzheimer’s disease mutations compromised the SOC activation, resulting in impaired spine maturation, a defect that could be rescued by overexpression of STIM2. These interesting results suggest that impairments in SOCE in the brain may be linked to neurodegenerative diseases and impaired neuronal function.

In the developing nervous system, CRAC channels may have a particularly important role for neurogenesis and proliferation given the neuroepithelial origin and nonexcitable nature of neural stem cells. Indeed, CRAC channels composed of Orai1 and STIM1 appear to be a major route of Ca\(^{2+}\) entry in neural stem/progenitor cells and regulate key effector functions including gene expression and proliferation (360). Because recent findings indicate that neurogenesis persists into adulthood (240) and may assume special importance for recovery following brain injuries, the regulation of NPC proliferation by CRAC channels may have implications for manipulating neurogenesis after brain injury and the treatment of neurodegenerative diseases.

**F. Brain Development and Function**

A potential role for CRAC channels in regulating effector functions in the brain is of particular interest since Ca\(^{2+}\) regulates critical neurobiological processes including neuronal excitability, synaptic transmission, and neuronal development (322). Although voltage- and ligand-gated Ca\(^{2+}\) channels have been widely studied for contributions to these functions, the role of CRAC channels for neuronal physiology remains poorly understood. Expression studies show high levels of Orai2 and Orai3 in the brain and moderate levels of Orai1 (128, 132, 133). mRNA and immunohistochemistry studies have also shown the presence of STIM1 and STIM2 in several regions of the brain, including the hippocampus, dentate gyrus, and cerebellum.

In terms of mechanism, we need to know more about the conformational transitions of STIM proteins following unbinding of Ca\(^{2+}\) from the cEF hand. What controls these transitions, and how does the stoichiometry of STIM complexes change during activation? How does STIM bind to Orai, where is the channel gate, and how does STIM binding lead to its opening? How are ER-PM junctions formed, maintained, and disassembled, and what factors regulate these critical processes? Finally, how do accessory proteins...
shape the SOCE response under different conditions and in specific cell types to optimize it for particular functions?

There are also many functional questions to address, including the functions of Orai2 and Orai3, and how their unique properties may contribute to tissue- or cell-specific responses. How are the unique features of the CRAC channel adapted for physiological responses, and can these be revealed by engineering animal models with altered function rather than simple loss-of-function channel mutations? To what extent does SOCE signaling occur locally at the ER-PM junction rather than globally throughout the cell, and does STIM have additional functions besides the control of store-operated channels? If the pace of progress over the past several years is any indication, we should expect answers to these questions in the not so distant future.

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REFERENCES


STORE-OPERATED CALCIUM CHANNELS


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