Published in final edited form as:
Immunol Rev. 2009 September ; 231(1): 88–98. doi:10.1111/j.1600-065X.2009.00820.x.

The molecular physiology of CRAC channels

Murali Prakriya[1]
[1]Department of Molecular Pharmacology and Biological Chemistry, Northwestern University School of Medicine, Chicago, IL, USA

Summary

The Ca^{2+}release-activated Ca^{2+} (CRAC) channel is a highly Ca^{2+}-selective store-operated channel expressed in T cells, mast cells, and various other tissues. CRAC channels regulate critical cellular processes such as gene expression, motility, and the secretion of inflammatory mediators. The identification of Orai1, a key subunit of the CRAC channel pore, and STIM1, the endoplasmic reticulum (ER) Ca^{2+} sensor, have provided the tools to illuminate the mechanisms of regulation and the pore properties of CRAC channels. Recent evidence indicates that the activation of CRAC channels by store depletion involves a coordinated series of steps, which include the redistributions of STIM1 and Orai1, direct physical interactions between these proteins, and conformational changes in Orai1, culminating in channel activation. Additional studies have revealed that the high Ca^{2+} selectivity of CRAC channels arises from the presence of an intrapore Ca^{2+} binding site, the properties of which are finely honed to occlude the permeation of the much more prevalent Na^{+}. Structure-function studies have led to the identification of the potential pore-binding sites for Ca^{2+}, providing a firm framework for understanding the mechanisms of selectivity and gating of the CRAC channel. This review summarizes recent progress in understanding the mechanisms of CRAC channel activation, pore properties, and modulation.

Keywords

CRAC channel; SOC; Orai1; STIM1; Ca^{2+} signaling

Introduction

In virtually all animal cells, stimulation of cell surface receptors coupled to the generation of 1,4,5-inositol trisphosphate (IP_3) causes the release of Ca^{2+} from endoplasmic reticulum (ER)-derived Ca^{2+} stores, followed by Ca^{2+} influx across the plasma membrane (1). This ubiquitous Ca^{2+} entry pathway is named store-operated Ca^{2+} entry to reflect the view that it is regulated by the level of free Ca^{2+} in the lumen of the ER (2). The store-operated channels (SOCs) of T lymphocytes and mast cells were the first to be studied using patch-clamp techniques and have been extensively characterized in terms of their biophysical properties and downstream functions. This category of SOCs, known as Ca^{2+} release-activated Ca^{2+} (CRAC) channels, is distinguished by an extremely high selectivity for Ca^{2+} and low conductance (2). Opening of CRAC channels enables the refilling of ER Ca^{2+} stores and sustains long-lasting Ca^{2+} oscillations and plateau signals, critical for the proliferation of T cells and production of cytokines following antigenic stimulation (3). For nearly two decades following the discovery of CRAC channels, their molecular composition and the

Correspondence to: Murali Prakriya, Department of Molecular Pharmacology and Biological, Chemistry, Northwestern University School of Medicine, 303 E. Chicago Ave, Chicago, IL 60611, USA, Tel.: +1 312 503 7030, Fax: +1 312 503 5349, m-prakriya@northwestern.edu.

mechanisms linking store depletion to channel activation remained obscure. However, recent studies using genetic approaches have identified a gene family that encodes CRAC channels as well as the sensor for ER Ca^{2+} depletion (4), propelling rapid progress in our understanding of the molecular physiology and modes of regulation of these channels. Here, we review progress in understanding the regulation and molecular physiological properties of CRAC channels.

Historical overview

The earliest descriptions of Ca^{2+} currents that came to be later identified with CRAC channels were reported two decades ago in mast cells and T cells. Using a combination of patch-clamp and Ca^{2+} imaging techniques, Penner and coworkers (5, 6) 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 peritoneal mast cells. This small current developed in parallel with a rise in $[Ca^{2+}]_i$ and led the authors to suggest that it is responsible for the rise in $[Ca^{2+}]_i$ by agonists and IP_3. Likewise, Lewis and Cahalan (7) described a small, highly selective Ca^{2+}-current that developed slowly during whole-cell recordings in lectin-stimulated T cells with low Ca^{2+}-buffered internal pipette solutions. They described several unique aspects of the Ca^{2+} current, including a lack of voltage-dependent gating, an inwardly rectifying current–voltage relationship, and blockade by Ni^{2+} and Cd^{2+}. This study (7) also showed a tight temporal correlation between rises in $[Ca^{2+}]_i$ and the Ca^{2+} conductance, supporting a causal role for this conductance in generating $[Ca^{2+}]_i$ oscillations in mitogen-activated T cells. Interestingly, at this juncture, these studies did not identify the proximal cause of the activation of the Ca^{2+} current.

The identification that ER Ca^{2+} store depletion activates SOCs arose, in large part following the discovery of thapsigargin, a plant-derived lactone that inhibits the SERCA [sarcoendoplasmic reticulum calcium adenosine triphosphatase (ATPase)] ATPase in the ER without concomitantly generating IP_3 (8). With this tool, Putney and coworkers (9) showed in rat parotid cells that Ca^{2+} influx across the plasma membrane (i.e., store-operated Ca^{2+} entry) was triggered following the depletion of an intracellular Ca^{2+} pool. They also showed that IP_3 and thapsigargin activated the same Ca^{2+} influx process (9). Likewise, several groups used fluorescence measurements of $[Ca^{2+}]_i$ to demonstrate that ER Ca^{2+} store depletion by thapsigargin triggers Ca^{2+} influx across the plasma membrane in various cell types, including T cells and mast cells (10–15). This was followed by the well-known study of Hoth and Penner (16, 17) that described a Ca^{2+} current activated by various stimuli that depleted Ca^{2+} stores in mast cells, which was presumably the same current that they had previously studied (5, 6). These authors termed the Ca^{2+} current I_{CRAC} for Ca^{2+} release-activated Ca^{2+} current. Using thapsigargin, Zweifach and Lewis (18) similarly showed that the Ca^{2+} current identified in the earlier study of Lewis and Cahalan (7) was activated by ER Ca^{2+} store depletion. Significantly, they showed that same current is also induced by stimulation of endogenous T-cell antigen receptors (TCRs), suggesting an important role for CRAC channels in mediating Ca^{2+} influx in response to physiological stimulation of T cells (18). In the ensuing decade, extensive investigations of the functions and biophysical characteristics of I_{CRAC} firmly established CRAC channels as a model for Ca^{2+}-selective SOCs.

The CRAC channel is essential for gene expression in activated T cells

In the years following the discovery of CRAC channels, substantial evidence accumulated showing that Ca^{2+} influx through these channels is essential for activation of Ca^{2+}-dependent gene transcription in T cells. SKF96365, an imidazole compound that inhibits CRAC channels, was also found to block interleukin-2 (IL-2) production in T cells with

similar efficacy (19). Likewise, nanomolar concentrations of La^{3+} were found to block I_{CRAC}, the $[Ca^{2+}]_i$ rise, and the induction of T-cell activation markers such as CD25 and CD69 in response to CD3 or thapsigargin stimulation (20). However, in the absence of selective pharmacological inhibitors, genetic studies have provided the most compelling evidence for the involvement of CRAC channels in T-cell activation. In mutant Jurkat T cells that exhibit <10% of the normal I_{CRAC}, T-cell activation as measured by the production of cytokines such as IL-2 was found to be severely attenuated (21). Additionally, several studies of human patients have shown that severe immunodeficiencies arise from mutations in CRAC channels that render them inactive (22–26). The abrogation of CRAC channel function in these cells results in the elimination of Ca^{2+} elevations necessary to drive nuclear translocation of nuclear factor of activated T cells (NFAT) (22), an important and widely expressed transcription factor involved in cytokine gene expression (27). Collectively, these studies established the critical role of CRAC channels for antigen-mediated T-cell activation and generated strong interest in understanding the regulation and identity of the CRAC channel at a molecular level.

The molecular components of the CRAC channel pathway

The decade following the discovery of I_{CRAC} saw an intense quest to determine its molecular composition and identify the signal linking store depletion to channel activation. Mammalian homologs of the *Drosophila* TRP channel were proposed as the likely pore-forming subunits of CRAC channels, and several competing hypotheses were proposed for the nature of the activation signal (28). These hypotheses included a diffusible activator released from the ER following store depletion, the insertion of active channels into the plasma membrane through a vesicle fusion mechanism, and direct physical coupling of CRAC channels with proteins in the ER membrane (28). Many of these models were highly controversial, and genuine progress in resolving these issues was stalled for years due to a lack of clearly defined proteins in the pathway. This era of uncertainty ended recently with the successful application of forward and reverse genetic approaches. In 2005, STIM1 (stromal interaction molecule 1) was identified as the mammalian ER Ca^{2+} sensor, followed closely in 2006 by the identification of Orai1 (also known as CRACM1) as a key subunit of the CRAC channel pore (23, 29–34). The discovery of these proteins has propelled rapid progress in illuminating the molecular mechanisms of this pathway and its functions in various tissues.

STIM1 is a 77 kDa single pass transmembrane protein with several functional domains including a luminal EF-hand motif that senses the ER Ca^{2+} concentration and multiple protein–protein interaction motifs (4). Knockdown of STIM1 in mammalian cells or in *Drosophila* S2 cells results in suppressed I_{CRAC} (29, 30). STIM1 is expressed in nearly all mammalian tissues and is conserved from *Drosophila* to humans, consistent with the widespread prevalence of SOCs in mammalian tissues and other organisms. Homozygous nonsense mutations in the *STIM1* gene produce symptoms of severe immunodeficiency, autoimmune disease, and myopathy in human patients (35). These symptoms are remarkably similar to the indications seen in patients with Orai1 mutations (23), underscoring the critical role of CRAC channels in immune function and host defense. STIM1 is distributed throughout the ER network in resting cells with replete ER Ca^{2+} stores. Remarkably, ER Ca^{2+} store depletion induces a rapid redistribution of STIM1 from the bulk ER into discrete puncta that accumulate near the plasma membrane (30, 36, 37). EF-hand mutants of STIM1 with impaired Ca^{2+} binding do not display the wildtype distribution in the ER but instead form puncta near the plasma membrane, mimicking the store-depleted state (30, 36). As if tricked into reporting the emptying of Ca^{2+} stores, cells expressing these mutants exhibit constitutive opening of CRAC channels without store depletion (30, 36). Moreover, careful measurements of $[Ca^{2+}]_{ER}$, STIM1 redistribution, and I_{CRAC} activation show that the

$[Ca^{2+}]_{ER}$ dependence of I_{CRAC} activation is identical to the $[Ca^{2+}]_{ER}$ dependence of STIM1 translocation to the plasma membrane, indicating that CRAC channels derive their dependence on store depletion from the ER Ca^{2+} dependence of STIM1 redistribution (38). Collectively, these properties firmly established STIM1's role as the Ca^{2+} sensor involved in coupling ER Ca^{2+} store depletion to CRAC channel activation.

Although STIM1 is essential for CRAC channel function, with one report even speculating on a possible role for STIM1 in forming Ca^{2+} channels in the plasma membrane (36), it is clearly not sufficient to reconstitute all aspects of store-operated Ca^{2+} entry. An early investigation of the possible molecular basis of defective CRAC channel function in human patients with the SCID syndrome failed to reveal any defects in STIM1 expression or its function (26). Moreover, overexpression of STIM1 in the mutant cells from the patient failed to rescue the defect, pointing to another component that is responsible for the inactivity of CRAC channels (26). Feske *et al*. (23) subsequently employed linkage analysis and positional cloning to identify a region in chromosome 12 containing approximately 74 genes as the locus of the defect. Simultaneously, a genome-wide RNA interference (RNAi) screen in *Drosophila* S2 cells led to the identification of a previously uncharacterized molecule that had a human homologue within the 74-gene region on chromosome 12 (23). Two other screens of the same RNAi library for suppression of SOC function identified the same gene in S2 cells (34, 39). This molecule, termed Orai1, is a widely expressed 33 kDa cell surface protein with four predicted transmembrane domains, intracellular N- and C-termini (Fig. 1), and no significant sequence homology to other previously identified ion channels. The human SCID defect was found to arise from a single point mutation in Orai1 (R91W) that abrogated CRAC channel activity (23).

Two important lines of evidence indicated that Orai1 is an essential pore subunit of the CRAC channel. First, overexpression of Orai1 together with STIM1 in HEK293 cells produced enormous CRAC currents similar to native I_{CRAC} in its biophysical and pharmacological characteristics (40–43). These characteristics included high Ca^{2+} selectivity and low permeability to Cs^{+}, a narrow pore diameter of approximately 3.8 Å, kinetic properties of Ca^{2+} block of Na^{+} currents, and pharmacological responses to 2-APB and La^{3+} (40, 41, 43). More definitive evidence came from the finding that mutations of highly conserved acidic residues in Orai1 significantly alter the ion selectivity of the CRAC channels (31–33). Together these studies definitively identified Orai1 as a key component of the CRAC channel pore and provided the first glimpse into the molecular mechanisms of ion selectivity in the CRAC channel. Mammalian cells express two other closely related homologues, Orai2 and Orai3. All three isoforms appear to function similarly in producing store-operated Ca^{2+} entry when co-expressed with STIM1 in HEK293 cells (44–46) and are widely expressed in most tissues (44, 46, 47).

Biophysical characteristics of the CRAC channel pore

Ion selectivity and permeation

A defining feature of the CRAC channel is its extremely high selectivity for Ca^{2+} over monovalent ions. Using the fura-2 overload method to estimate the fractional Ca^{2+} current (48, 49), Hoth and Penner estimated that CRAC channels conduct $Ca^{2+} > 1000$ times better than Na^{+} under physiological conditions, placing CRAC channels among the most Ca^{2+} selective channels known (17). Biophysical studies indicated that the pore of the CRAC channel does not intrinsically exclude monovalent ions; instead, the high Ca^{2+} selectivity seen under physiological conditions arises via ion-pore and ion–ion interactions. This is readily seen in the absence of extracellular divalents: CRAC channels readily conduct a variety of small monovalent ions, such as Na^{+}, Li^{+}, and K^{+} in divalent-free solutions, but the addition of micromolar concentrations of Ca^{2+} blocks the permeation of the more prevalent

monovalent ions by high-affinity Ca^{2+} binding in the channel (K_i approximately 20 μM at −100 mV) (17, 50–54). Occupancy by a single Ca^{2+} ion appears sufficient to block the large monovalent conductance, and, as expected for a binding site within the pore, Ca^{2+} block is voltage-dependent (43, 52). These characteristics are qualitatively reminiscent of the properties of voltage-gated (Ca_v) L-type channels, which have historically served as prototypes for studies of Ca^{2+} selectivity (55). In these highly Ca^{2+}-selective channels, Ca^{2+} ions bind tightly to a high-affinity binding site within the selectivity filter, a narrow region of the pore that controls ion selectivity, to occlude Na^+ flux. However, at millimolar concentrations of extracellular Ca^{2+}, the Ca^{2+} binding site is thought to be sufficiently flexible to enable interactions with multiple Ca^{2+} ions, permitting rapid Ca^{2+} flux (55–57).

The identification of Orai1 provided the impetus and tools to investigate the molecular underpinnings of the CRAC channel's high Ca^{2+} selectivity. Guided by past studies on L-type Ca_v channels, wherein four glutamate residues, one in each of the four pore-loops converge to form a single high-affinity Ca^{2+} binding site (the EEEE locus) (57, 58), it was reasoned that the Ca^{2+} binding site(s) in the CRAC channel pore may similarly arise from the carboxylate side-chains of pore-lining acidic residues. Indeed, sequence comparisons of various Orai proteins revealed the presence of several highly conserved acidic residues in the first and third predicted transmembrane (TM) segments and in the loop connecting TM1 and TM2 (Fig. 1), setting the stage for mutagenesis studies. A Glu → Asp substitution at position 106 in the predicted TM1 segment of human Orai1 strongly diminished the high Ca^{2+} selectivity of CRAC channels (31–33) and altered the affinity and voltage-dependence of Ca^{2+} block of the monovalent current (43). A Glu → Gln mutation at position 190 in the TM3 segment similarly compromised the Ca^{2+} selectivity of mutant channels (31, 33, 43). In addition, mutations of three Asp residues D110A/D112A/D114A in the predicted TM1–TM2 loop segment strongly diminished La^{3+} block of CRAC channels, while also causing modest changes in ion selectivity (32, 33, 43). More recently, several groups have reported that CRAC channels are formed from the homomeric assembly of four Orai1 subunits (59–61). Collectively, these studies provided the foundation to understand the molecular determinants of ion selectivity and permeation in CRAC channels. Current models of the tertiary structure of the channel posit that residues in the TM1 and TM3 segments line the ion conduction pathway, with the acidic residues identified from the mutations, in particular E106 and E190, constituting key components of the CRAC channel selectivity filter (33, 62, 63).

To understand additional features of the pore that govern ion selectivity, measurements of permeability ratios for a series of ions that differ in size, charge, and chemical properties such as hydration energy are often employed in studies of ion channels. For CRAC channels, however, obtaining information on the selectivity of various ions has been difficult, because the removal of Ca^{2+} causes CRAC channels to lose activity by a process referred to as depotentiation (51, 64). Thus, measurements of the divalent ion selectivity from steady-state currents have led to the mistaken notion that the order of divalent ion conductance is $Ca^{2+} > Sr^{2+} > Ba^{2+}$ (16, 18). However, more recent measurements of peak currents immediately following solution exchange indicate that the conductance series is, in fact, the reverse: $Ba^{2+} > Sr^{2+} > Ca^{2+}$ (53, Prakriya and Lewis, unpublished data). This series is identical to the selectivity series seen in Ca_v channels and suggests that despite the strikingly different molecular compositions of CRAC and Ca_v channels, the core mechanisms of ion permeation and selectivity are similar in the two channel types.

The Orai1 mutagenesis studies also provided insight into a puzzling hallmark of CRAC channels: their unusually low permeability to Cs^+. CRAC channels are practically impermeable to Cs^+ (P_{Cs}/P_{Na} approximately 0.1) (50–52, 54), which stands in sharp contrast to L-type Ca_v channels that readily pass Cs^+ under divalent-free conditions (P_{Cs}/P_{Na}

approximately 0.6) (65) as well as other Ca^{2+}-selective channels such as TRPV6 channels (P_{Cs}/P_{Na} approximately 0.6) (66). One clue to explain this difference has come from estimates of the minimal pore diameter of these channels. By examining the channel permeability for a series of monovalent organic cations of varying sizes, the diameter of Ca_v channels has been estimated to approximately 6 Å (67, 68) and that of the TRPV6 channels to be approximately 5.5 Å (69). By comparison, the narrowest region of the CRAC channel pore is only approximately 3.8 Å (43, 52). Given that the diameter of a naked Cs^+ ion is approximately 3.6 Å, the finding that CRAC channels have a narrow pore suggests that the low permeability to Cs^+ reflects steric hindrance to its permeation. In agreement with this hypothesis, mutations of key acidic residues such as E106 and E190 in Orai1 significantly widen the pore and concomitantly increase the Cs^+ permeability of mutant CRAC channels (43). Intriguingly, decreases in Ca^{2+} selectivity caused by these mutations correlate well with increases in Cs^+ permeability, suggesting that pore geometry has a powerful influence on both Ca^{2+} and Cs^+ selectivity (43).

Altogether, the mutagenesis studies provided a first glimpse into the structural elements controlling Ca^{2+} selectivity, although this work has also contributed to its share of outstanding questions. For example, it is clear that the acidic residues identified in these studies do not by themselves account for all aspects of permeation in CRAC channels, such as its low conductance (see below). What other parts of the channel are involved, and how do they interact with the conducting ions? Additionally, why are mutations of E106 are so poorly tolerated for channel function? While a conservative substitution of E106 to aspartate, as described above, is tolerated and produces channels with diminished Ca^{2+} selectivity, other substitutions including alanine, glutamine, or cysteine produce non-functional CRAC channels (31, McNally and Prakriya, unpublished data). This is in sharp contrast to the quadruple alanine or glutamine mutations of the EEEE locus in Ca_v channels, which exhibit robust monovalent permeation (57). Exactly how these mutations exert their profound effects on ion permeation remains unknown. An additional area of uncertainty is whether the residues implicated in these studies truly line the pore (as hypothesized) or whether the mutations alter ion selectivity because of indirect, allosteric effects. This concern may be especially valid for E190: although a glutamine substitution of this residue alters the ion selectivity of mutant channels, substitutions to alanine or aspartate have no detectable effect (31). These specific questions more broadly reflect the scarcity of information on the pore architecture, including the identity, number, and locations of the ion-binding sites. Ultimately, progress in understanding these issues will require elucidation of the structural characteristics of the pore.

Unitary conductance

A key distinctive feature of the CRAC channel is its extremely small apparent unitary conductance. Whole-cell I_{CRAC} generates little visible current noise and the activity of single CRAC channels is yet to be directly measured. Estimates from noise measurements of whole-cell I_{CRAC} in T cells indicate a unitary conductance of 9–24 fS in 2–110 mM Ca^{2+} (18, 51). Measurements in mast cells similarly resulted in a conductance estimate of < 1 pS based on the low level of visible current noise in whole-cell recordings of I_{CRAC} (17). As expected from the increased macroscopic Na^+ currents in divalent-free solutions, the monovalent conductance of Na^+-conducting CRAC channels is estimated to be at least an order of magnitude larger, or approximately 0.7 pS (52). The unitary Na^+ current amplitude (0.11 pA at −110 mV) corresponds to an ion flux rate of approximately 7×10^5 Na^+/s, much faster than most ion carriers but compatible with a channel-like transport mechanism (52).

By comparison, measurements of the single channel conductance of Ca_v L-type channels yield values of 4–10 pS for Ca^{2+} and 85 pS for Na^+ (65), indicating a > 100-fold larger rate of ion flux. This difference is all the more striking in light of the many similarities in the

core mechanisms of Ca^{2+} selectivity and permeation profile of the two channel types noted above. Interestingly, we find that the association rate of Ca^{2+} for its pore blocking site is approximately 4×10^6 M^{-1} s^{-1} in the CRAC channel (43, 52). By contrast, the corresponding rate determined for L-type Ca_v channels from single channel recordings is approximately 4×10^8 M^{-1} s^{-1} (56), a value close to the diffusion limit. The sharply lower association rate of Ca^{2+} for its blocking site in CRAC channels indicates that Ca^{2+} access from the extracellular space to the selectivity filter is severely limited, a factor that could explain the small conductance of CRAC channels. Although we do not yet know the reasons for this difference, the narrower pore of the CRAC channel noted above may be an underlying factor, especially if it presents a rate-limiting barrier for the flux of ions through the pore.

The small conductance and high Ca^{2+} selectivity of CRAC channels have important implications for their function. Given that the kinetics of activation and deactivation of CRAC channels occurs on a slow time scale of seconds to tens of seconds, the high efficiency of Ca^{2+} entry with little or no accompanying Na^+ influx prevents membrane depolarization that could compromise the driving force for Ca^{2+} entry over such long durations. At the same time, the small conductance of CRAC channels ensures strong specificity for the activation of Ca^{2+}-dependent downstream functions. A striking example of this specificity is seen in the gene expression of *c-fos* by Ca^{2+} oscillations triggered by stimulation of mast cells with the leukotriene LTC_4 (70). *c-fos* gene expression was observed only following Ca^{2+} influx through CRAC channels, even when Ca^{2+} oscillations of the same amplitude could be artificially maintained in the absence of CRAC channel-mediated Ca^{2+} entry (70). Likewise, activation of Ca^{2+}-dependent enzymes and the production of leukotrienes in mast cells are selectively coupled to Ca^{2+} influx through CRAC channels (71). These studies directly illustrate a key point: that the role of CRAC channels for cellular Ca^{2+} signaling extends significantly beyond the mere maintenance of ER Ca^{2+} store homeostasis.

Rectification

Another prominent hallmark of I_{CRAC} noted in nearly all electrophysiological studies of CRAC channels is the steep inward rectification of the whole-cell I_{CRAC}. The rectification is not simply because most patch-clamp studies of CRAC channels employ Cs^+, which is largely impermeable through CRAC channels, as the main charge carrier in internal solutions. The sharp inward rectification is also observed under a range of conditions with various permeant ions in internal solutions, including in symmetrical Na^+ ions (52, 54). Currently, little is known of the mechanisms underlying this feature and the inability to resolve single CRAC channel currents has severely hampered efforts to determine whether it arises because of block, intrinsic channel gating, or inherent asymmetry in the ion conduction pore. Rectification is unaffected by intracellular Mg^{2+} or polyamines suggesting that internal blockade by these charged compounds is not the cause (51, 54, 72, 73). Inward rectification of I_{CRAC} is also seen in Orai1 mutants with altered Ca^{2+} and Cs^+ selectivity under divalent-free conditions (43), indicating that the molecular determinants of this feature are unrelated to the elements involved in ion selectivity. Interestingly, rectification is strongly reduced when ammonium derivatives such as hydroxyl ammonium or methyl ammonium are employed as charge carriers (52), suggesting that ion-pore interactions may contribute to this feature. The molecular underpinnings of inward rectification and its consequences for cellular function remain obscure.

Molecular mechanisms of channel gating

Activation by store depletion

The identification of STIM1 and Orai1 provided the molecular tools to illuminate the nature of the signal linking ER Ca^{2+} store depletion to channel activation. As noted above, STIM1 senses the depletion of ER Ca^{2+} through its luminal EF-hand domain. STIM1 is distributed diffusely throughout the ER membrane in resting cells. ER Ca^{2+} store depletion triggers a coordinated series of events beginning with STIM1 oligomerization followed by its redistribution into discrete puncta at the junctional ER sites where the ER tubules and the plasma membrane are within 20 nm of each other (30, 36, 38, 74). The puncta of over-expressed STIM1 accumulate at the sites of Ca^{2+} influx through active CRAC channels (75). Orai1, which is also diffusely distributed in the plasma membrane at rest, aggregates and colocalizes with STIM1 at the same puncta following store depletion (75–79). Collectively, these studies revealed a fascinating complexity of signaling in the activation of CRAC channels involving coclustering of STIM1 in the junctional ER directly opposite to Orai1 in the plasma membrane.

The finding that STIM1 and Orai1 accumulate in overlapping clusters in closely apposed membranes raised a key molecular question central to resolving the historical debate on the nature of the CRAC channel activation mechanism: does STIM1 directly interact with Orai1? Support for a direct physical interaction between STIM1 and Orai1 was provided by findings showing that overexpressed STIM1 and Orai1 co-immunoprecipitate with each other (32, 33) and that the redistribution and formation of overlapping STIM1–Orai1 puncta occurs in parallel with increases in fluorescence resonance energy transfer (FRET) between these proteins (78, 79). An amphipathic domain in the C-terminus of Orai1 was identified as the likely STIM1 interaction site (76), and two hydrophobic Orai1 residues, L273 (78) and L276 (79), were determined to be critical for this interaction. More recently, evidence supporting a direct physical interaction of STIM1 and Orai1 has come from structure-function studies that have discovered a minimal domain of STIM1 that can activate CRAC channels independently of ER store depletion. Several groups have identified an approximately 107 amino acid region in a coiled-coiled domain of the C-terminus of STIM1 that activates CRAC channels independently of ER Ca^{2+} store depletion when over-expressed in HEK293 cells (80–83). Park *et al*. (80) tested whether this minimal activation region of STIM1 directly binds Orai1 using GST-pulldown assays and size-exclusion chromatography. Their results indicated that the GST-tagged STIM1 domain coprecipitates Orai1 and co-elutes with Orai1, indicating that this region of STIM1 directly binds to Orai1 *in vitro*.

These results indicate that the cytoplasmic portion of STIM1 activates CRAC channels by direct binding to Orai1 and argue against the hypothesis that a diffusible messenger released by ER Ca^{2+} store depletion activates CRAC channels. The ability of the entire C-terminus of STIM1 to activate CRAC channels was found to be significantly lower than that of the 107-amino acid peptide region in several studies, suggesting that this region of STIM1 is normally hidden and that a conformational change, possibly caused by Ca^{2+} unbinding at the luminal EF-hand domain, is needed to expose the activation domain of STIM1 (80, 82, 83). Thus, CRAC channel activation occurs via a conformational coupling mechanism involving direct physical associations between the channel and its sensor in closely apposed membranes.

Although these studies have established the framework for understanding the signal linking ER Ca^{2+} store depletion to the opening of CRAC channels, how STIM1 binding subsequently causes the gating conformational changes that culminate in the opening of the pore remains unclear. Elucidating the nature and identity of these gating movements is

critical for understanding the many modes of regulation of CRAC channels: by ER Ca^{2+} stores, second messengers, extracellular Ca^{2+}, and pharmacological agents. To address this issue, we have identified a conformational rearrangement in Orai1 triggered by store depletion by monitoring FRET between cyan fluorescence protein (CFP) and yellow fluorescence protein (YFP) probes attached to the C- or N-termini of Orai1 (79). ER Ca^{2+} store depletion causes a small but significant decline in FRET between the probes of adjacent Orai1 subunits, suggesting that STIM1 binding to Orai1 leads to conformational changes in the intracellular C- and N-termini of Orai1 (79). These rearrangements may be coupled to movements of the gate and provide the energy required for channel opening.

Other modes of CRAC channel regulation

In addition to ER Ca^{2+} store depletion and refilling, the CRAC channel exhibits various other modes of channel regulation. These include (i) Ca^{2+}-dependent potentiation (CDP) of CRAC channels in store-depleted cells after the readdition of extracellular Ca^{2+} and the reverse process of depotentiation after removal of divalent cations, (ii) fast inactivation, arising from feedback inhibition of I_{CRAC} during brief hyperpolarizing steps by the high local [Ca^{2+}] around individual CRAC channels, and (iii) enhancement of channel activity by low doses of 2-APB (<5 μM) and inhibition by higher doses (>10 μM). These forms of regulation and our understanding of their underlying mechanisms are discussed below.

CDP

CDP was first described by Zweifach and Lewis (64, 84) and is indicative of a process wherein the activity of CRAC channels is strongly facilitated by extracellular Ca^{2+}. Conversely, the removal of extracellular Ca^{2+} causes the CRAC channel activity to drop, a process referred to as depotentiation (51). The mechanism of CDP, including the location of the Ca^{2+}-binding site, remains unknown. Our recent studies indicate that CDP is directly dependent on the degree of pore occupancy by divalent ions, and ions with higher permeability support a greater degree of potentiation (Prakriya and Lewis, unpublished data). These results are compatible with the idea that the CDP binding site is located within the CRAC channel pore. However, how Ca^{2+} binding is coupled to changes in channel activity remains unclear. Noise analysis suggests that CDP occurs from the recruitment of CRAC channels from a ‘silent’ state to one of high open probability (52). A similar stepwise recruitment of silent channels to a high open-probability mode also occurs during the slow activation of CRAC channels by store depletion (52), raising the possibility that the conformational changes underlying these events are identical. In support of this idea, examination of Orai1 conformational changes by FRET reveal that readdition of extracellular Ca^{2+} to store depleted cells causes a qualitatively similar change in Orai1–Orai1 FRET as ER Ca^{2+} store depletion (79). Thus, there may be common gating steps in the process by which store depletion and CDP increase channel activity.

Fast inactivation

Fast inactivation is a prominent hallmark of CRAC channels and is observed as a decline in I_{CRAC} over 200–500 ms during hyperpolarizing voltage steps. Fast inactivation is thought to be mediated by feedback inhibition of channel activity by the high local $[Ca^{2+}]_i$ around individual CRAC channels (17, 85, 86). Key aspects of the fast inactivation mechanism such as the identities of the Ca^{2+} binding sites, the nature of the inactivation gate, and whether inactivation occurs by a change in open probability or unitary Ca^{2+} conductance remain unknown. Past experiments have shown that the Ca^{2+} binding to the inactivation site is sensitive to chelation of intracellular Ca^{2+} by fast buffers such as BAPTA, suggesting that the Ca^{2+} binding site is probably located on the intracellular face of the channel (17, 85, 86), but whether the binding site resides directly on Orai1 or on another protein is unclear. Interestingly, mutations in the putative selectivity filter of Orai1 that alter the ion selectivity

of CRAC channels also strongly diminish fast inactivation, suggesting that the structural elements regulating ion selectivity and inactivation gating may be coupled (43). The nature of this coupling will require elucidation of critical structural elements of the pore such the pore-lining residues and the location of the channel gate.

Modulation by 2-APB

Of the various modes of CRAC channel regulation, the modulation of channel activity by the pharmacological agent 2-aminoethyldiphenyl borate (2-APB) has perhaps been the most puzzling and garnered widest attention. 2-APB elicits complex effects including a two-to fivefold persistent enhancement of I_{CRAC} at low concentrations (1–5 μM) and transient enhancement followed by strong inhibition of I_{CRAC} at higher concentrations (>10 μM) (87). The multiplicity of 2-APB effects has long intrigued SOC investigators, and many explanations have been invoked over the years for its likely mode of action. Some early studies concluded that the inhibition of SOC activity by 2-APB occurs through the drug's known ability to inhibit IP_3 receptors, and this effect was cited in strong support for a role for IP_3 receptors in transducing the activation of SOCs (88, 89). However, subsequent studies indicated that 2-APB inhibits CRAC channels directly and even in cells lacking IP_3 receptors, casting doubts about this interpretation (87, 90, 91). More recently, studies in HEK293 cells overexpressing STIM1 found that 2-APB (50–100 μM) reverses STIM1 puncta in store-depleted cells (92, 93). However, this effect is largely eliminated when Orai1 is co-expressed together with STIM1, suggesting that disruption of the STIM1–Orai1 aggregates is not the underlying mechanism of CRAC channel inhibition by 2-APB (79, 93).

Noise analysis suggests that the potentiation of I_{CRAC} seen at low concentrations of 2-APB arises from the recruitment of CRAC channels from a silent state to one of high open probability (52). As noted earlier, a similar stepwise recruitment of silent channels to a high open-probability mode also occurs during the slow activation of CRAC channels by store depletion (52). Thus, it is tempting to speculate that the conformational changes in CRAC channels occurring during 2-APB-induced potentiation of I_{CRAC} and store depletion-induced channel activation may be identical. In support of this idea, two studies employing FRET have found that 2-APB facilitates the association between STIM1 and Orai1 (79, 94). Thus, 2-APB-induced potentiation of I_{CRAC} and the opening of CRAC channels by store depletion may share common mechanisms, both involving increased binding of STIM1 to Orai1 culminating in channel activation. By contrast, the inhibition of I_{CRAC} by high concentrations of 2-APB does not appear to involve obvious decreases in STIM1–Orai1 interaction. In fact, by monitoring conformational changes in CRAC channels with Orai1–Orai1 FRET, a recent study has found that high (but not low) concentrations of 2-APB produce conformational changes in Orai1 (79). Based on these findings, one model of 2-APB action proposes that the drug induced potentiation of I_{CRAC} arises from strengthening of STIM1–Orai1 contacts, whereas the inhibition, which is specific to high drug concentrations, arises from alterations in CRAC channel gating (79). A direct effect of high concentrations of 2-APB on CRAC channel gating is also supported by electrophysiological measurements of I_{CRAC} revealing that 2-APB-induced current inhibition is accompanied by the loss of fast Ca^{2+}-dependent inactivation, a gating process probably mediated by conformational changes in the channel (87).

Admittedly, these descriptions of the molecular mechanisms of channel modulation are still rudimentary and many puzzling questions remain. However, the studies of CDP and 2-APB described above project a dynamic picture of channel regulation wherein the interaction of STIM1 with Orai1 and the downstream conformational changes in Orai1 can be independently modulated to tune CRAC channel activity. These checkpoints might also be targets for modulation *in vivo* by second messengers and may be attractive targets for designing drugs to alter CRAC channel activity in diseased cells.

Future perspectives

The past few years have seen a remarkable transformation in our understanding of the molecular basis of store-operated Ca^{2+} entry. Two key molecular components of the CRAC channel pathway have been identified and the nature of the signal linking store depletion to channel activation has been elucidated. A large body of knowledge also exists about the fundamental biophysical properties of CRAC channels, providing a firm foundation to explore the molecular mechanisms of Ca^{2+} selectivity and channel gating. Thus, a major goal of future efforts will be the illumination of the molecular and structural basis of the intriguing properties of the CRAC channel. What is the structure of the CRAC channel pore? Where is the channel gate? How is STIM1 binding to Orai1 coupled to the opening of the pore? These questions will require a combination of approaches, from X-ray crystallography to solve the channel structure to traditional molecular and electrophysiological approaches to determine the functions of particular protein domains. There is great anticipation that the inner workings of this important and enigmatic channel will finally be understood.

Acknowledgments

Work in the author's laboratory was supported by grants from the NIH and AHA. The author is grateful to the members of the laboratory for helpful discussions and Dr. T. Hornell for a critical reading of the manuscript.

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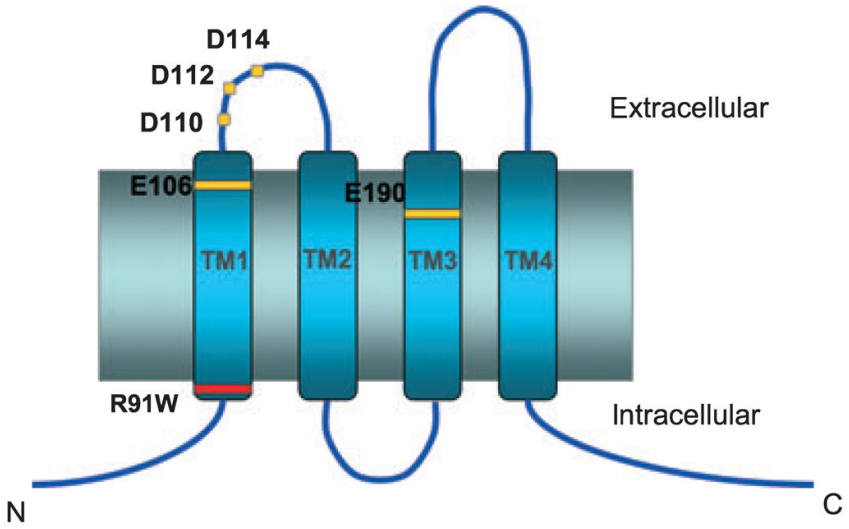

Fig. 1. Predicted topology of Orai1

Critical amino acids residues identified from structure-function and human linkage-analysis studies are highlighted. Mutations of E106 in TM1 and E190 in TM3 (yellow) affect ion selectivity and permeation, whereas mutations of the aspartate residues in the TM1-TM2 loop segment (D110/112/114, yellow) affect La^{3+} block while producing relatively small changes in ion permeation. An inherited mutation (R91W, red) produces non-functional CRAC channels and immunodeficiency in human patients.