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Pore opening mechanism of CRAC channels

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Abstract

Three decades ago, James W. Putney Jr. conceptualized the idea of store-operated calcium entry (SOCE) to explain how depletion of endoplasmic reticulum (ER) Ca^{2+} stores evokes Ca^{2+} influx across the plasma membrane. Since the publication of this highly influential idea, it is now established that SOCE is universal among non-excitable and probably even many types of excitable cells, and contributes to numerous effector functions impacting immunity, muscle contraction, and brain function. The molecules encoding SOCE, the STIM and Orai proteins, are now known and our understanding of how this pathway is activated in response to ER Ca^{2+} store depletion has advanced significantly. In this review, we summarize the current knowledge of how Orai1 channels are activated by STIM1, focusing on recent work supporting a hydrophobic gating mechanism for the opening of the Orai1 channel pore.

1. Introduction

Of the several known mechanisms for eliciting Ca^{2+} signals in cells, store-operated calcium entry (SOCE) is one of the most widespread pathways. James W. Putney Jr. first described the idea of SOCE in 1986, noting that intracellular inositol triphosphate (IP_3) triggers a biphasic mobilization of Ca^{2+} in rat salivary and lacrimal glands that involves an initial release of Ca^{2+} from intracellular endoplasmic reticulum (ER) stores followed by a more sustained Ca^{2+} influx from the extracellular compartment [1]. He hypothesized that intracellular Ca^{2+} store depletion in these exocrine glands triggers activation of a plasma membrane Ca^{2+} influx pathway which refills depleted stores, naming it capacitative calcium entry (CCE) [1]. Later studies showed that this Ca^{2+} influx pathway elevates cytosolic [Ca^{2+}] in a wide variety of cells and was renamed SOCE to designate its dependence on the ER Ca^{2+} store content [2,3]. The best-studied SOCE channel is the “calcium release-activated calcium” (CRAC) channel, a highly Ca^{2+} selective channel with a very low unitary conductance [4]. CRAC channels are widespread, perhaps universally expressed among all animal cells, and are typically activated following stimulation of G-protein coupled receptors or receptor tyrosine kinases that produce IP_3 to deplete ER Ca^{2+} stores [4–6]. Apart from refilling ER Ca^{2+} stores, the opening of CRAC channels also causes a rise in intracellular Ca^{2+} that regulates a variety of effector cell responses including gene expression, cell proliferation, exocytosis, and motility [4]. Human studies have shown that loss of CRAC channel function leads to a devastating immunodeficiency along with additional symptoms

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of autoimmunity, ectodermal dysplasia, and muscle defects, highlighting the vital importance of CRAC channels for human health [7–9]. Conversely, constitutive channel activation from gain-of-function mutations in CRAC channel proteins are linked to pathologies such as tubular aggregate myopathy and thrombocytopenia [10–12]. In several instances, the molecular mechanisms of how these loss- and gain-of-function mutations disrupt CRAC channels remains unclear, underscoring the need for a better understanding of the operational mechanisms of CRAC channels including their gating mechanism.

The prototypical CRAC channel is formed by two subunits: Orai1, the pore-forming protein in the plasma membrane [13–16], and STIM1, the Ca^{2+} sensor in the ER [17,18]. Orai1 contains four transmembrane domains (TMs) with cytosolic N- and C-termini that serve as interaction sites for STIM1 (Fig. 1A). STIM1 makes a single pass through the ER membrane and contains several functional domains including a luminal EF-hand motif that acts as the ER Ca^{2+} sensor [17–19] and two coiled-coil domains in the cytoplasmic side that comprise the CRAC-activation domain (CAD) [20] or STIM1-Orai1 activating region (SOAR) [21]. CAD/SOAR is the catalytic region of STIM1 that binds to and activates Orai1 channels [20–22]. In response to store depletion, STIM1, which is diffusely distributed in the bulk ER membrane at rest, exhibits a complex molecular choreography in which it first oligomerizes and subsequently migrates to ER-PM junctions [19,23,24], where it then physically interacts with the cytoplasmic tails of Orai1 to gate Orai1 channels [20,25–30]. This physical interaction causes the two previously diffusely localized proteins to accumulate into distinct puncta at ER-PM junctions to form active CRAC channels [20,31].

2. Permeation, selectivity, and pore architecture of CRAC channels

CRAC channels are characterized by two biophysical features which provide a relatively easy way to identify them in native cells: high Ca^{2+} selectivity and low unitary conductance. As measured by the proportion of current carried by Ca^{2+} in a physiological mix of Na^+ and Ca^{2+} ions, CRAC channels select for Ca^{2+} over the more prevalent Na^+ ions by a ratio >1000 ($P_{\text{Ca}}/P_{\text{Na}} > 1000$) [14,32,33], making them amongst the most Ca^{2+} -selective channels known. Early studies using mutational analysis and measurements of binding kinetics and voltage-dependence revealed that a ring of glutamates (E106) at the external mouth of the pore function as the selectivity filter [14–16,34]. These glutamates form a Ca^{2+} binding site to block permeation of Na^+ ions through the pore ($K_i \approx 20 \mu\text{M}$ [34–36]), analogous to the mechanism of Ca^{2+} selectivity described for voltage-gated Ca^{2+} (Ca_v) channels [37]. More recently, this explanation for the CRAC channel's high Ca^{2+} selectivity has been modified based on the observation that Ca^{2+} selectivity in Orai3 channels is controlled not only by the binding of Ca^{2+} ions to the selectivity filter (formed by E81), but also by the overall rates of ion entry and exit from the selectivity filter, with slower ion flux rates yielding higher selectivity [38]. This latter feature likely explains why CRAC channels exhibit comparable levels of Ca^{2+} selectivity as Ca_v channels despite 20-fold weaker Ca^{2+} binding in the pore ($K_i < 1 \mu\text{M}$ in Ca_v channels [37] vs. $20 \mu\text{M}$ in CRAC channels [4]). In effect, a slow ionic flux rate in CRAC channels essentially eliminates the need for tight Ca^{2+} binding to achieve high Ca^{2+} selectivity. Interestingly, studies in constitutively active Orai1 channels indicate that the Ca^{2+} selectivity of CRAC channels is strongly regulated by STIM1 binding [39,40], suggesting that the selectivity of the CRAC channel is not a fixed property of the open state

structure, but a dynamic property coupled to channel gating. The details of how this occurs remain to be formally determined, but as discussed below, one possibility is that Ca^{2+} binding to the selectivity filter is altered by the conformational motions in the pore triggered by STIM1 binding.

The second functional characteristic of CRAC channels, their low unitary conductance, is less well understood. Estimates from analysis of the whole-cell current noise indicate that the unitary conductance is only 10–30 fS in 2–110 mM Ca^{2+} containing solutions [35,38,41], which suggests the pore contains significant energy barriers for ion conduction in the active state.

An important breakthrough in our understanding of the molecular basis of ion conduction in CRAC channels came from identification of the pore-lining residues [42,43]. In one approach, the substituted cysteine accessibility method (SCAM) was used to examine the extent of current inhibition after thiol-reactive reagents were applied to the extracellular side of Orai1 channels bearing cysteine mutations [42]. In a second approach, the fraction of inter-subunit disulfide cross-linking of Orai1 cysteine mutants was quantified based on the prediction that cysteine residues introduced at pore-lining positions should be able to form disulfide bonds across the pore upon protein oxidation [43]. The results of these studies, which were consistent with one another, indicated that residues in TM1 flank the pore and ruled out a contribution for TM3 and, specifically, E190, a residue in TM3 that was previously thought to regulate Ca^{2+} selectivity [14,15]. In the TM1 domain, the key pore-lining residues found from these studies included E106, V102, G98, L95 and R91 (Fig. 1B). The SCAM study used Cd^{2+} ions to form metal ion bridges between introduced cysteine residues across the pore [42]. Because the length of Cd^{2+} -S bridges is short (~ 2.5 Å) [44,45], the strong reactivity to Cd^{2+} at several pore-lining residues indicated that TM1 helices are close to one another and therefore line a narrow pore, a feature that has been proposed to account for the CRAC channel's low permeability to large cations (>3.8 Å) and its low unitary conductance. Moreover, differences in the accessibility of probes of different sizes showed that the pore narrows sharply at the base of the outer pore vestibule, near E106 [42]. These results provided the first step toward building a structural model of the open pore.

The pore architecture suggested by the electrophysiological and biochemical studies was later corroborated by the crystal structure of *Drosophila* Orai [46], which revealed that each channel is formed by six Orai molecules, with a narrow central pore flanked by TM1 helices and the transmembrane helices arranged in concentric rings around the pore (Fig. 1B, C). The innermost TM1 segment lines the pore, TMs 2 and 3 are in the next layer, and TM4 forms the most external, lipid-exposed segment. Among the many interesting features of the Orai channel complex revealed by this structure, one unanticipated finding was that the N-terminus, which was previously thought to lack a secondary structure, instead forms a cytosolic helix continuous with the TM1 domain, termed the “TM1 extension helix” [46] (Fig. 1B).

3. Gating mechanism of CRAC channels

The identification of pore-lining residues provided the structural and functional framework to next address the pore opening mechanism of CRAC channels. Where is the location of the channel gate and what is its nature? And what conformational changes in the pore underlie the activation of the gate? Two models have been proposed. One model has postulated that the gate is in the inner pore [47]. This viewpoint is based on the loss-of-function phenotype of R91W human mutation [13] and the ability of R91C to be cross-linked across the pore [47]. R91 was suggested to constitute at least a part of the gate, with G98 functioning as a hinge to kink the lower portion of the TM1 segment and induce dilation in the region encompassing R91 to open the gate [47,48] (Fig. 2). Moreover, based on the presence of anions detected in the crystal structure bound to the basic residues (K163, K159, R155) in the TM1 extension helix, the crystal structure study went further and suggested that these positively charged residues may function as a part of an inner gate to block ion conduction either via steric occlusion or electrostatic repulsion [46]. As postulated in the earlier study [47], gating was hypothesized to occur through widening of the inner pore to release the anions or decrease the electrostatic barrier [46] (Fig. 2). However, there have been no functional studies determining whether the N-terminal extension constitutes part of the ion conduction pathway, and the idea of an inner pore gate has been challenged by recent studies showing that a channel with a truncated N-terminal helix still retains a barrier to ion conduction [49], presumably a gate that lies elsewhere in the channel pore.

An alternative model has argued that the channel gate is located near the outer end of the pore at V102 [39,49] (Fig. 1B). An important clue for this viewpoint came from attempts to localize the channel gate from analysis of state-dependent blockade of a cysteine introduced at G98, located near the midpoint of the membrane. 2-Aminoethyl methanethiosulfonate (MTSEA), a thiol reagent that covalently modifies the side-chains of cysteine residues, blocked G98C in open, but not closed Orai1 channels when administered externally [39]. This result indicated that there exists a barrier to ion flow located extracellular to G98 in a region which includes the pore residue V102 (Fig. 1B). Subsequent mutational analysis revealed that substitutions of V102 to polar amino acids yield constitutively permeant channels that allowed ion conduction to occur in the absence of STIM1 [39]. Further study of these mutations pointed to a model wherein V102 likely presents a hydrophobic free energy barrier to prevent water and ions from entering the pore in the closed channel state [39]. These ideas received support from molecular dynamics (MD) simulations, which showed that the V102A mutant reduces the energetic cost of water entering the pore, especially in the hydrophobic region encompassing V102 and F99 [50], potentially explaining its constitutive permeation phenotype. In addition, lanthanide resonance energy transfer experiments using purified Orai1 in liposomes found that STIM1 binding at the cytosolic surface of Orai1 elicits a gating movement at the outer mouth of the pore in the region encompassing V102 [49]. These investigators also found that a mutant lacking the N-terminal cytosolic helix, which presumably should also lack an inner gate, remained closed, yet could be opened by introducing a V102C mutation, leading them to conclude that a barrier to ion flux exists outside the inner pore, most likely in the hydrophobic zone [49]. Collectively, the results from differing approaches converged towards a model wherein the

hydrophobic V102 presents a free energy barrier that is substantive enough to occlude ion conduction in closed channels.

Although the studies described above imply that V102 functions as a hydrophobic barrier, exactly how this barrier is relieved upon channel opening was not explained. Ion sizing experiments in the constitutively conducting V102C mutant showed that STIM1 binding causes the Orai1 channel pore to narrow [39], which suggests that the gating mechanism in CRAC channels does not involve simple pore dilation to permit ion flow. Paradoxically, direct examination of the conformational change during gating using the accessibility of pore-applied thiol reagents revealed that access of the reagents Cd^{2+} and Ag^+ to V102C is not altered by STIM1 binding, indicating that V102C remains pore-facing in both the resting and activated conformations [51]. This surprising finding implies that although V102 likely forms part of the hydrophobic barrier to stabilize closed channels, it alone does not constitute the channel gate given its constant exposure into the pore.

What then is the identity of the gate and how does it dynamically regulate ion conduction? One clue came from the subtle distinction between the pore-lining residues in the hydrophobic region identified in the STIM1-activated channel by SCAM versus those observed in the closed dOrai structure, which showed G98 and F99 respectively as the pore-lining residues (Fig. 3A). This difference led us to hypothesize that STIM1 binding evokes a conformational change that alters the accessibility of these residues upon pore opening, thereby exposing G98 towards the pore and concealing F99 away from it. Indeed, analysis of the accessibility of cysteine mutants of key pore residues in the V102A background (to confer constitutive ion conduction in the absence of STIM1) indicated that F99C is blocked strongly by Cd^{2+} , but this block decreases dramatically when STIM1 is added [51]. Conversely, the G98C/V102A current is weakly inhibited by Cd^{2+} , but the addition of STIM1 greatly increases its sensitivity to Cd^{2+} blockade [51]. These functional results imply that STIM1 induces a modest ($\sim 20^\circ$) counterclockwise rotation of the pore helix that moves F99 away from the pore axis but which brings G98 towards a more pore-facing orientation (Fig. 3C).

The finding that the bulky non-polar F99 rotates away from the pore axis following STIM1 binding raised the strong possibility that this residue may regulate ion conduction. Moreover, examination of the dOrai crystal structure indicates that the orientation of F99 side-chains in this configuration would not be expected to allow permeation of ions (Fig. 3B). Indeed, consistent with its role as a potential gate, substitutions of F99 to more polar residues (Tyr, Ser, Thr, Cys, Met) yielded leaky channels in the absence of STIM1 [51]. Channels with substitutions to Ile, Leu, or Val, however, remained closed. This observation is consistent with a scenario wherein F99 functions as an energy barrier for water and ion occupancy, with manipulations that lower hydrophobicity at this position evoking leaky channels, whereas maintaining the barrier with hydrophobic amino acid substitutions stabilize the non-conducting state. Surprisingly, the Ile, Leu, and Val mutants remained closed even in the presence of STIM1, but could be opened by decreasing the hydrophobicity at the upstream Val through a V102A substitution [51]. These data indicated that increasing the overall hydrophobicity in this region produces an insurmountable energetic barrier even when STIM1 is bound, thereby stabilizing the closed channel state. Overall, these functional

results indicated that the hydrophobicity at F99 is finely tuned such that mutations that decrease hydrophobicity lead to a leaky gate whereas increases in hydrophobicity abrogate STIM1-mediated gating.

Molecular dynamics simulations using models constructed from the crystal structure of the *Drosophila* Orai channel reaffirmed these conclusions. The MD simulations showed a striking increase in pore hydration compared to WT Orai in the constitutively conducting F171Y mutation (equivalent to the F99Y gain-of-function mutation in human Orai1) [51]. By contrast, the more hydrophobic F171V mutation (equivalent to the closed F99V mutation in human Orai1) lowered water occupancy in the pore. These opposing effects of the F171V and F171Y on water occupancy agree with the experimental findings of gain- and loss-of-function effects in the equivalent human Orai1 mutants and support the idea that two rings of closely spaced hydrophobic pore-lining residues (V102 and F99) contribute to a desolvation barrier that prevents ion conduction in closed channels.

In sum, the results of this study lead to a model in which STIM1-mediated opening of Orai1 channel pore occurs through a mechanism involving rotation of the pore helix, leading to displacement of the F99 side-chains away from the pore axis to increase water occupancy and allow ion flow through the hydrophobic gate formed by V102 and F99 (Fig. 3B, C). Such a mechanism potentially also explains why STIM1 binding alters the selectivity of the constitutively active V102X and F99X mutant channels [39,40,51], as rotation of the outer pore during gating would naturally be expected to reorient the E106 side chains of the selectivity filter thereby altering Ca^{2+} binding. The energetic cost of reorienting TM1, which would be expected to modify interactions of the pore helix with other parts of the channel, is unknown. One possibility is that water molecules around the pore helices could lower the free energy required for rotating the helices during channel opening. The hydrophobic gating mechanism proposed for Orai1 is similar to the activation mechanisms of several other ion channel families, including the nicotinic acetylcholine receptor [52], the magnesium channel CorA [53], and the mechanosensitive channel MscL [54], which also involve a ring of hydrophobic residues forming an energetic barrier in the pore.

4. Future directions

Although we have come a long way in the three decades since James Putney conceptualized the SOCE pathway, there is still much to learn about the molecular and structural basis of how CRAC channels are gated. In the context of the gating model presented in this review, a major unanswered question is how STIM1 binding leads to Orai1 pore helix rotation. One straightforward possibility is that this motion is induced by STIM1 binding to the non-pore facing surface of the nearby N-terminus, which could rotate the pore helix above the STIM1 binding site. Recent studies have, however, cast doubts that STIM1 binds to the Orai1 N-terminus, arguing instead that the only site of STIM1 interaction is at the Orai1 C-terminus [55]. Thus, an alternate possibility is that helix rotation is induced by reversible interactions between the non-pore facing residues of TM1 with residues in TM2 and/or TM3. The role of these non-pore lining transmembrane helices in gating Orai channels currently remains poorly understood. However, intriguing new evidence [55], including the identification of a constitutive active Orai1 human mutation in TM4 [11], suggests that these distal domains

may have a larger role in transmitting the gating signal than previously appreciated. Of course, these mechanisms need not be exclusive and gating could involve several overlapping allosteric processes. Whatever the final answers, the story that James Putney began in 1986 before a store-operated calcium current was measured, and long before the molecules were known continues today at an ever-accelerating pace.

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Fig. 1.

(A) Topology diagram of Orai1 showing four transmembrane domains and cytosolic N- and C- termini that serve as interaction sites for STIM1. TM1 lines the channel pore with the selectivity filter formed by E106. Potential inner and outer channel gates are proposed to be near V102 and R91, respectively. Residue numbering corresponds to human Orai1. (B) Two diagonally facing subunits in the crystal structure of *Drosophila* Orai showing predicted pore-lining TM1 residues. dOrai residue numbering is indicated with equivalent hOrai1 residues shown in parentheses. (C) Top-down view of the complete crystal structure of dOrai. Each channel is formed by six Orai proteins arranged in concentric rings surrounding the narrow pore flanked by TM1.

Fig. 2.

Schematic of the inner pore dilation gating model based on the dOrai crystal structure. In this model, STIM1 binding to Orai1 causes the inner pore formed by the cytoplasmic N-terminal extension helix to widen, relieving steric and electrostatic barriers formed by the basic residues (R91, K87, R83) to allow ion conduction. The left and right panels represent the closed and open states, respectively. Pore lining residues in the inner pore correspond to those in dOrai with hOrai1 residue numbering shown in parentheses.

Fig. 3.

(A) Top-down helical wheel representation of TM1, indicating pore-lining residues of hOrail identified in the open channel by SCAM (lined in orange) and those identified through the crystal structure of the closed dOrail channel (lined in yellow). (B) Top-down view of the human Orail pore based on the dOrail crystal structure. Space-filling representations of F99 (yellow) and a barium ion in the pore are shown. Based on its orientation towards the pore in the crystal structure, F99 is not expected to permit ion flow in this configuration. The orientations of V102 (maroon sticks) and G98 (orange) are also displayed. (C) Schematic of the pore helix rotation model. In this scheme, gating occurs through a slight rotation of TM1, which moves F99 away from the pore axis. This is

predicted to decrease the free energy barrier in the V102/F99 hydrophobic region to allow ion permeation.

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