Control of cardiac ryanodine receptor by sarcoplasmic reticulum luminal Ca^{2+}

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Introduction

Cardiac myocyte contraction is driven by the coordinated release of Ca^{2+} from the sarcoplasmic reticulum (SR). This release of Ca^{2+} occurs through the cardiac RyR2. Physiologically, Ca^{2+} release occurs in response to an influx of Ca^{2+} through L-type Ca^{2+} channels, via a mechanism termed CICR. This transient increase in cytosolic Ca^{2+} activates the RyR2 channel as a result of Ca^{2+} binding to the cytosolic Ca^{2+} activation site of the channel (Bers, 2002). However, RyR2 is also known to be activated in the absence of cytosolic Ca^{2+} elevation, under conditions of SR Ca^{2+} overload. This phenomenon is commonly known as spontaneous Ca^{2+} release, or store overload–induced Ca^{2+} release (SOICR), because of its dependence on SR Ca^{2+} load (Jiang et al., 2004, 2005; Jones et al., 2008). SOICR occurs when SR Ca^{2+} content exceeds a threshold level, which can be caused by either an increase in SR Ca^{2+} concentration higher than the threshold or a reduction in the threshold to a level less than the SR Ca^{2+} concentration (Venetucci et al., 2007). Although SOICR has been well characterized as an underlying mechanism of multiple pathologies (Jiang et al., 2004, 2005, 2008; Lehnart et al., 2008; Tang et al., 2012; Zhabyeyev et al., 2013), exactly how RyR2 detects and responds to SR luminal Ca^{2+} has yet to be defined. Here we argue that SR luminal Ca^{2+} is able to activate RyR2 via a luminal rather than a cytosolic site, and we discuss the functional evidence for this in light of recent structural information.

Activation of RyR2 by Ca^{2+}

RyR2 is a massive, "mushroom-like" protein with a very large cytosolic domain and a much smaller luminal domain within the SR lumen (Efremov et al., 2015; Yan et al., 2015; Zalk et al., 2015; Peng et al., 2016; Fig. 1). It is formed by four identical 565-kD monomers with fourfold symmetry. RyR2 is one of three isoforms, the others being the skeletal muscle isoform, RyR1, and the more ubiquitously expressed RyR3 (Fill and Copello, 2002).

It has been proposed that calsequestrin 2 (CASQ2), an SR Ca^{2+}-buffering protein, is responsible for the luminal Ca^{2+} activation of RyR2 (Terentyev et al., 2003; Györke et al., 2009). This notion is supported by the fact that several mutations within CASQ2 lead to catecholaminergic polymorphic ventricular tachycardia (CPVT), a life-threatening, stress-induced arrhythmogenic disease that is known to be linked to inappropriate activation of RyR2 (di Barletta et al., 2006). However, subsequent studies have shown that CASQ2-null mice can still respond to changes in SR Ca^{2+}, albeit with some changes in RyR2 function and SR volume (Knollmann et al., 2006; Zhang et al., 2014a). Moreover, purified RyR2 channels (devoid of CASQ2) remain sensitive to SR Ca^{2+} (Sitsapesan and Williams, 1997; Xu et al., 1998; Kong et al., 2008; Chen et al., 2014). Combined, these observations suggest that although CASQ2 is important for regulating SR Ca^{2+} content and the response of RyR2 to SR Ca^{2+}, it is unlikely to be the only mechanism of RyR2 activation by SR luminal Ca^{2+} (Knollmann et al., 2006). An alternative proposal is that RyR2 itself responds to SR luminal Ca^{2+}, which is consistent with the fact that purified RyR2 channels are activated by SR luminal Ca^{2+} (Sitsapesan and Williams, 1997; Xu et al., 1998; Kong et al., 2008; Chen et al., 2014).

One theory to explain the response of single RyR2 channels to luminal Ca^{2+} comes from observations showing that the activity of RyR is dependent on Ca^{2+} flux from the lumen to the cytosol (Tripathy and Meissner, 1996; Xu and Meissner, 1998). In this model, termed "feed-through," luminal Ca^{2+} passing through an open channel results in the activation of that channel because of Ca^{2+} interacting with the channel's own cytosolic Ca^{2+} activation site.

An alternative theory to explain the response of RyR2 to SR luminal Ca^{2+} has been proposed by Laver (2007), who hypothesized that RyR2 contains a discrete luminal Ca^{2+} activation site that promotes the initial opening of the channel in response to increases in SR luminal

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Abbreviations used: CASQ, calsequestrin; CTD, C-terminal domain; FKBP, FK506 binding protein; SOICR, store overload–induced Ca^{2+} release.

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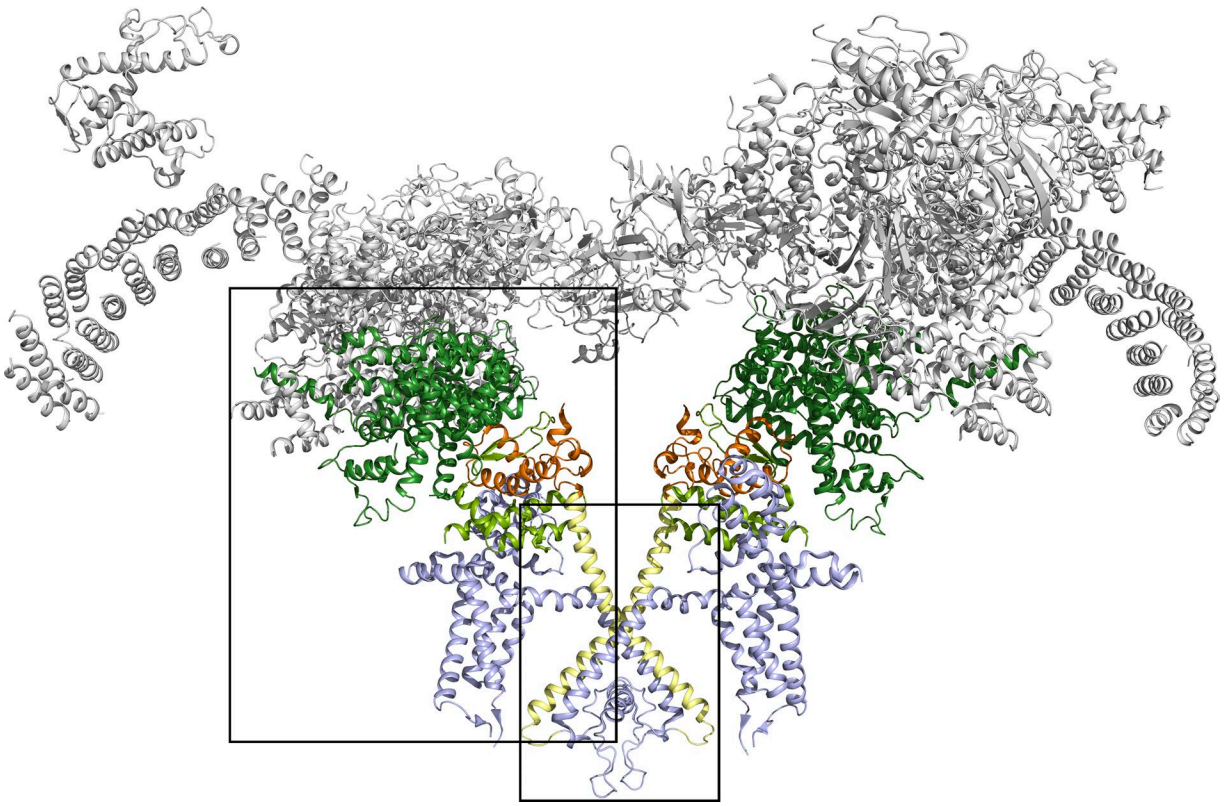

Figure 1. **Three-dimensional structure of RyR2.** Three-dimensional structure of RyR2 showing two opposing subunits (Peng et al., 2016). Boxes highlight the structures shown in more detail in Figs. 2 A and 3.

Ca^{2+}. Once the channel is open, luminal Ca^{2+} can pass through and interact with the cytosolic Ca^{2+} activation site, further enhancing activation. Interestingly, this model also predicts that Ca^{2+} passing through the channel can inactivate the channel via a cytosolic Ca^{2+} inactivation site (Xu and Meissner, 1998; Laver, 2007).

Our proposal for luminal Ca^{2+} activation of RyR2 is that SR luminal Ca^{2+} is able to activate RyR2 through a luminal Ca^{2+} sensor rather than the cytosolic Ca^{2+} activation site. Evidence for this model comes from the RyR2 mutation A4860G, which causes CPVT (Jiang et al., 2007; Zhao et al., 2015). The A4860G mutation abolishes luminal but not cytosolic Ca^{2+} activation of the channel. Despite the presence of luminal-to-cytosolic Ca^{2+} flux (feed-through), 50 mM luminal Ca^{2+} was unable to activate single A4860G mutant channels (Jiang et al., 2007). This finding is consistent with the work of Liu et al. (2010), who demonstrated that single RyR2 channels are immune to feed-through luminal Ca^{2+} activation.

Following on from this study (Jiang et al., 2007), in 2014 we identified an amino acid residue that is critical for the luminal Ca^{2+} activation of RyR2. We found that mutation of E4872 to alanine (E4872A) abolished luminal, but not cytosolic, Ca^{2+} activation of RyR2 (Chen et al., 2014). This selective action on luminal Ca^{2+} activation suggests that the E4872A mutation does not simply cause gross perturbations in protein folding. Furthermore, introducing metal binding histidines at this site transforms RyR2 from a luminal Ca^{2+}-gated channel to a luminal Ni^{2+}-gated channel. These data strongly suggest that the region encompassing E4872 contains one or more cation-binding pockets that contribute to RyR2 activation by luminal Ca^{2+}, and that residue E4872 is an essential component of these binding pockets (Chen et al., 2014). The stark changes in luminal and cytosolic Ca^{2+} activation as a result of mutating this residue illustrate that RyR2 has distinct Ca^{2+} sensors that, although they may act in concert physiologically, can differentially respond to cytosolic and luminal Ca^{2+}.

With the recent breakthroughs in solving the 3-D structure of RyR2 (and RyR1), the mechanisms of luminal Ca^{2+} activation of RyR2 can be further explored. In the next sections, we provide our interpretation of the functional data on Ca^{2+} activation of RyR2 in light of the new structures. However, when interpreting these structures, it is important to acknowledge that they represent averaged snapshots in which the nuances of transitions between open and closed cannot be observed. Moreover, the structures are devoid of many of their binding partners, including proteins and lipids, which will undoubtedly modify the structure.

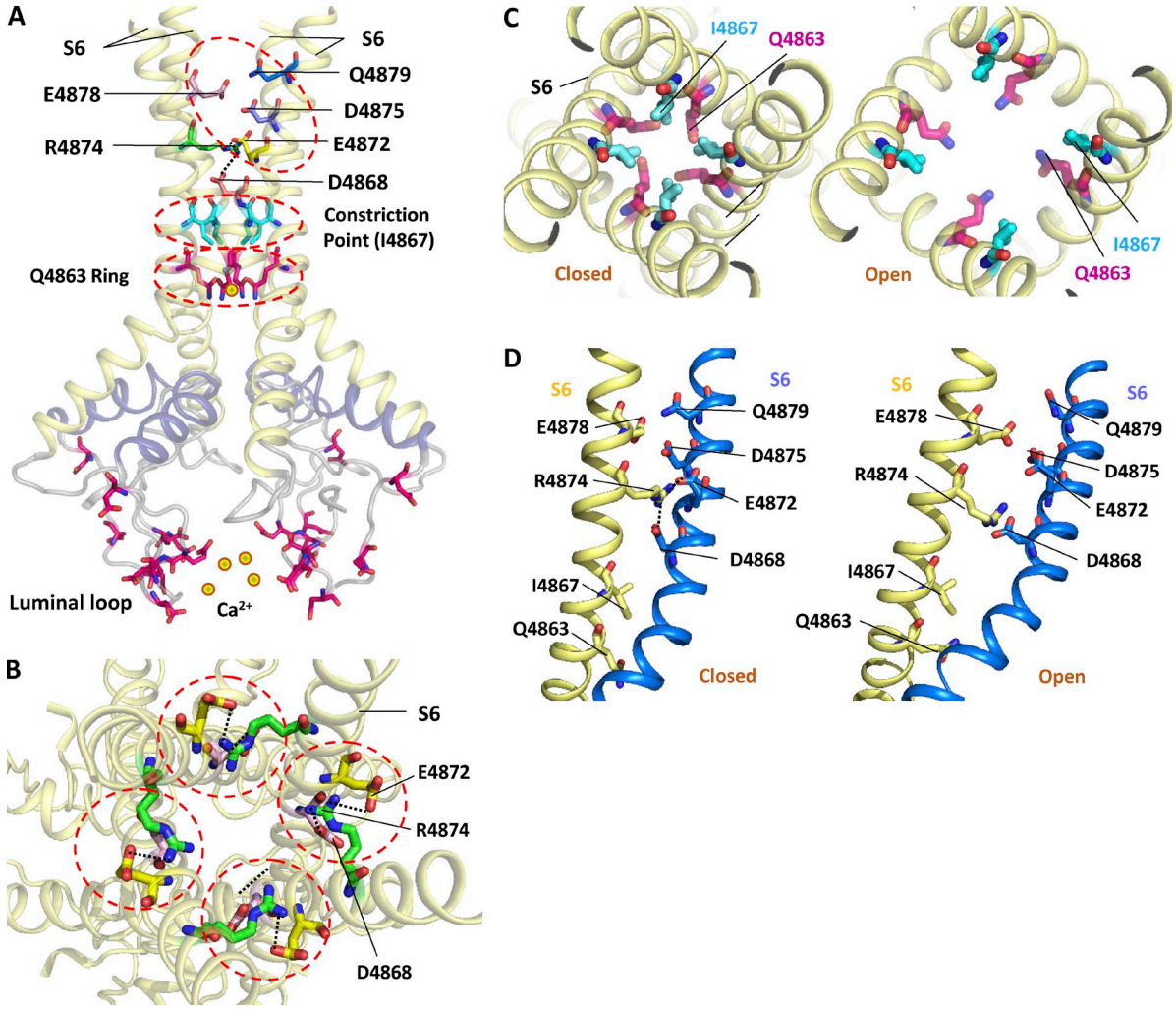

Figure 2. **Structure of the RyR2 channel pore.** (A) Detailed structure of the channel pore of RyR2, illustrating the major structural elements potentially involved in Ca^{2+} activation. (B) Top view of the channel pore, showing salt bridges potentially formed between D4868, E4872, and R4874. (C) Top view of the channel pore in a closed (left) and open (right) conformation. In the closed state, the constriction point (the narrowest part of the pore) is formed by I4867; when the channel opens, the constriction point shifts to Q4863. (D) Side chain orientation of critical residues in the S6 helices from neighboring subunits. The salt bridge between R4874 and E4872 is disrupted when the channel opens.

Structural evidence for activation of RyR2 by luminal Ca^{2+}

In 2015, three landmark studies presented the first high-resolution structures of RyR1 (Efremov et al., 2015; Yan et al., 2015; Zalk et al., 2015). Using cryo-electron microscopy (cryo-EM), these groups solved the structure of RyR1 to a resolution as high as 3.8 Å. More recently, the cryo-EM structure of RyR2 was solved to a similar, near-atomic resolution (4.2 Å; Peng et al., 2016). For the first time, the 3-D location of individual amino acids, especially in the channel pore region, could be determined.

As predicted from functional studies, the negatively charged residue E4872 was located at a key position within the channel pore. The structure suggests that formation of a salt bridge between D4868 and E4872 in one subunit and the positively charged R4874 in a neighboring subunit is possible (Fig. 2, A and B; Peng et al., 2016). These salt bridges are likely to be involved in channel activation, since mutating D4868, E4872, or R4874 has a dramatic impact on channel activation. The D4868, E4872, and R4874 residues are located immediately adjacent to the constriction point (residue I4867), the likely "gate" of the channel (Fig. 2 A). Interestingly, E4872 lies on the cytosolic side of the channel gate, indicating that the E4872 residue is not accessible to luminal Ca^{2+} when the channel is in a closed state (Fig. 2 A; Peng et al., 2016).

Fascinatingly, the high-resolution 3-D structures of RyRs also reveal a cluster of oxygen-containing residues surrounding E4872, including D4875 and Q4879 in one subunit and E4878 in the neighboring subunit (Fig. 2,

A and D). Mutations of some of these residues markedly reduce the activation of RyR2 by luminal Ca^{2+} or caffeine, suggesting that they are important for channel activation. The cytosolic location of all of these residues hints that they may not be directly involved in luminal Ca^{2+} activation when the channel is in the closed state. The arrangement of these residues undergoes substantial changes during channel activation. In the closed state, residue E4872 likely forms an intersubunit salt bridge with R4874, and E4878 may interact with Q4879 via hydrogen bonding. In the open state, R4874 moves away from E4872, whereas residues E4872, E4878, and Q4879 move closer to one another to form a putative Ca^{2+}-binding pocket (Fig. 2 D). In other words, the putative luminal Ca^{2+}-binding pocket is formed only in the open state. These state-dependent conformational changes suggest that E4872, previously shown to be critical for luminal Ca^{2+} activation (Chen et al., 2014), may be involved in stabilizing the open state of the channel by forming a Ca^{2+}-binding pocket immediately abutting the gate when the channel is open. However, given that these residues are all located on the cytosolic side of the gate, an important unresolved question is how they are involved in the luminal Ca^{2+} activation of RyR2.

The 3-D structure of RyR2 shows that there is a ring of glutamines (Q4863) directly to the luminal side of the channel gate (formed by I4867; Fig. 2 A). The Q4863 residue is the only amino acid inside the pore cavity with its oxygen-containing side chain pointing toward the core of the cavity (Fig. 2, A and C). Importantly, the Q4863 ring abuts the constriction point (I4867) on the luminal side of the pore. Mutations of Q4863 markedly reduce the activation of RyR2 by caffeine (Peng et al., 2016), suggesting that Q4863 is important for channel activation. Although the exact role of Q4863 in luminal Ca^{2+} activation is unknown, we speculate that, given its location, it may provide one or more interaction sites for luminal Ca^{2+} inside the hydrophobic internal pore close to the channel gate, and that binding of luminal Ca^{2+} to these sites may affect the gate (I4867). Interestingly, comparison of the open and closed structures of RyR2 reveals that the narrowest part of the channel pore moves from I4867 in the closed state to Q4863 in the open state (Peng et al., 2016; Fig. 2, A and C). Further evidence for the importance of the Q4863 ring in luminal Ca^{2+} activation of RyR2 comes from a homologous channel, the inositol 1,4,5-trisphosphate receptors (ITPRs). ITPRs share a similar pore-forming region with RyR channels but lack the oxygen-containing glutamine residue in an equivalent location to Q4863 in RyR2. Importantly, unlike single RyR2 channels that can be activated by luminal Ca^{2+} in the absence of cytosolic Ca^{2+} (Chen et al., 2014), single ITPR channels cannot be activated by luminal Ca^{2+} in the absence of inositol trisphosphate (Vais et al., 2012).

In addition to the glutamine ring formed by Q4863, the luminal side of the channel pore contains clusters of large numbers (>40) of negatively charged amino acids (Fig. 2 A). These negatively charged residues are thought to be important for attracting and concentrating Ca^{2+} ions near to and within the luminal mouth of the pore (Williams et al., 2001; Welch et al., 2004; Mead-Savery et al., 2009).

In summary, structural analysis suggests that multiple elements within the channel pore and the helix bundle crossing region may be involved in the activation of RyR2 by luminal Ca^{2+}. From the cytosolic side of the channel, these elements include (a) a putative Ca^{2+}-binding pocket formed by E4872, D4875, E4878, and Q4879 in the open conformation; (b) a network of intersubunit salt bridges formed by D4868, E4872, and R4874; (c) the constriction point (I4867); (d) a ring of Q4863; and (e) clusters of negatively charged residues located in the luminal mouth of the channel pore (Fig. 2, A and B). The precise mechanism by which each of these structural elements promotes luminal Ca^{2+} activation of RyR2 remains to be determined. However, the close arrangement of these structural elements in relation to the RyR2 channel gate makes it unique and interesting and may represent a novel mechanism of channel activation.

Luminal Ca^{2+} activation hypothesis: store overload–induced Ca^{2+} release

Based on the close arrangement of the unique structural elements in the RyR2 channel pore, we speculate that the closed state of RyR2 is stabilized by a network of intersubunit salt bridges within the helix bundle crossing region (Fig. 2, A and B). When SR luminal Ca^{2+} content exceeds a certain level (threshold), luminal Ca^{2+} enters the internal pore cavity and interacts with the Q4863 ring. This may be aided by the large clusters of negatively charged residues in the luminal mouth of the pore. The coordination of luminal Ca^{2+} by the Q4863 ring immediately adjacent to the constriction point (I4867) may then destabilize the closed state of the channel. This would occur because of the positively charged Ca^{2+} ion, coordinated by Q4863, weakening or disrupting the nearby intersubunit salt bridges (R4874–D4868) via electrostatic effects. This could lead to brief openings of the constriction point (gate; Fig. 2, A and D). These brief openings would allow luminal Ca^{2+} flux through the gate and a potentiation of opening because of luminal Ca^{2+} binding to the E4872 Ca^{2+}-binding pocket, and thus the stabilization of the open state. Fascinatingly, the E4872 Ca^{2+}-binding pocket may have a dual role in channel activation. When SR Ca^{2+} exceeds the store overload–induced Ca^{2+} release (SOICR) threshold, it could open a closed RyR2 channel via interaction with the Q4863 ring, allowing access of Ca^{2+} to the E4872 Ca^{2+}-binding pocket to further activate the channel. Under normal SR Ca^{2+} loads, and with a normal SOICR threshold, binding of Ca^{2+} to the E4872

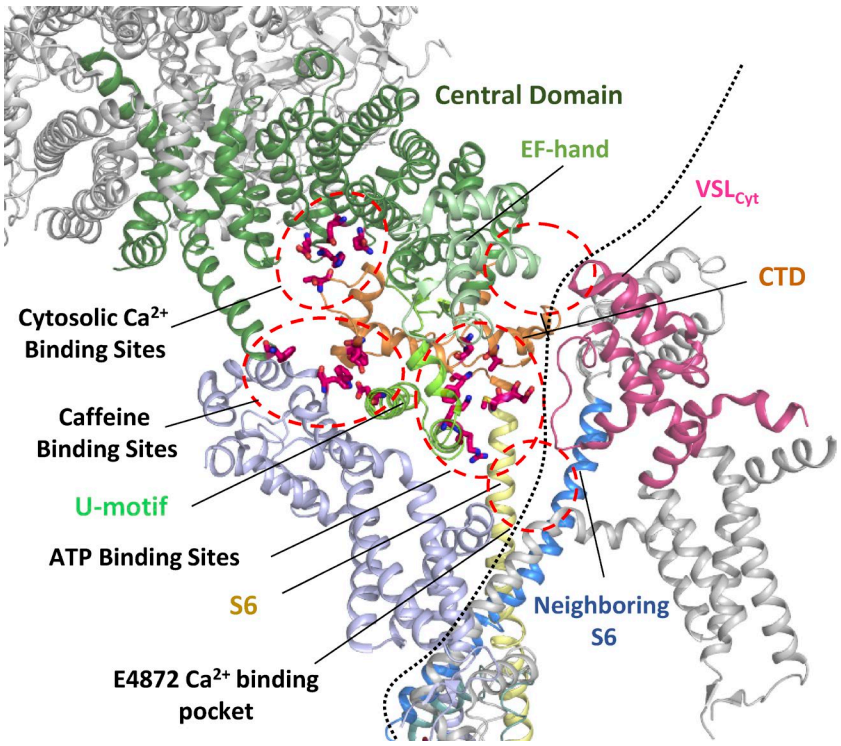

Figure 3. **Structural elements within RyR2 important for channel activation.** The dotted line divides structures from the neighboring subunits. The EF-hand domain of one subunit may interact with the VSL_{cyt} of the neighboring unit. The Ca^{2+}-binding site, caffeine-binding site, ATP-binding site, E4872 Ca^{2+}-binding pocket, and contact site between the EF-hand domain and the VSL_{cyt} domain are highlighted by red dashed ellipses. Locations are based on des Georges et al. (2016).

Ca^{2+}-binding pocket would occur only during cytosolic Ca^{2+} activation of the channel, where it would stabilize and increase the open duration of the channel. This suggests that although the putative E4872 Ca^{2+}-binding pocket plays a critical role in SOICR when the SR Ca^{2+} threshold is surpassed, it also has a crucial role in prolonging the open duration during normal cytosolic Ca^{2+} activation (Ca^{2+}-induced Ca^{2+} release; CICR) of the channel (Chen et al., 2014).

Cytosolic Ca^{2+} activation hypothesis: CICR

In accordance with the relationship of luminal and cytoplasmic Ca^{2+} activation of RyR2, the location of the Ca^{2+}-binding site responsible for cytosolic Ca^{2+} activation places them in an ideal position to influence the E4872-containing luminal Ca^{2+}-binding pocket. As shown in Fig. 3, the cytosolic Ca^{2+}-binding site is located close to the interface of the central domain, S6 helix, C-terminal domain (CTD), and U-motif. Comparing the open and closed states of the channel, this appears to be a region undergoing large intradomain movements within RyR2 (Peng et al., 2016). Therefore, it is likely that Ca^{2+} binding to this region modifies the local interactions between the S6 helix, CTD, and U-motif, leading to a conformational shift that is transduced to the channel gate. A model of this form of activation has previously been proposed by Wei et al. (2016). Although their model is based on channel activation caused by binding of Ca^{2+} to the EF-hand motif, a domain we have shown not to be required for cytosolic Ca^{2+} activation (Guo et al., 2016), it does represent a plausible template mechanism for cytosolic Ca^{2+} activation of RyR2. In this model, Ca^{2+} binding to the Ca^{2+}-binding site in the central domain would reduce the interaction between the CTD and U-motif. This movement results in the upward movement of the S6 helix, reducing its interaction with the outer S5 helix and allowing the S6 helix to flex. A flex in S6 will alter the interactions between S6 helices in neighboring subunits, potentially weakening the salt bridges within the E4872 Ca^{2+}-binding pocket and priming it for Ca^{2+} occupation, thereby effectively promoting an open state. The movement of the S6 helix also results in the rotation and widening of the channel pore, resulting in the opening of the channel. Combining these ideas, we propose that binding of Ca^{2+} at the cytosolic binding site opens the channel through movement of the S6 helix, and once the channel is open, the favorable conformation of the E4872 binding pocket allows luminal Ca^{2+} binding that stabilizes the channel in the open state, prolonging Ca^{2+} flux during CICR. This effect on cytosolic Ca^{2+} activation of the channel is consistent with our previous experimental findings, in which mutation of E4872 resulted in shorter channel open times (Chen et al., 2014).

Relationship between luminal and cytosolic Ca^{2+} activation

Just as cytosolic Ca^{2+} binding to the cytosolic Ca^{2+} activation site sensitizes the channel to luminal Ca^{2+}, changes in the structure around E4872 in response to luminal Ca^{2+} could alter the stability of the S6 helix, making the channel more sensitive to cytosolic Ca^{2+}. It is likely

that these two-way allosteric conformational changes in structure underlie the synergistic response to Ca^{2+} on both sides of the channel, as recognized by Bassani et al. (1995) more than two decades ago. This concept is supported by our recent mutagenesis data, which show that alanine scanning mutagenesis within the S6 helix dramatically alters both the cytosolic and luminal Ca^{2+} activation of the channel (Jiang et al., 2007; Sun et al., 2016). Interestingly, the side of the helix on which each amino acid is located can be used to predict its impact on function. Mutating the residues facing the U-motif generally results in an increase in cytosolic and luminal Ca^{2+} activation, whereas mutating those on the other side appears to depress activity (Jiang et al., 2007; Sun et al., 2016). Considering the proposal that cytosolic Ca^{2+} results in the movement of the U-motif and the CTD, leading to the upward movement of the S6 helix and channel activation (Wei et al., 2016), it is possible that disrupting the interaction of the S6 helix and U-motif through mutations has a similar effect. Alternatively, rather than enhancing activation of the channel, the loss of S6 helix and U-motif interaction may destabilize the closed state of RyR2. Evidence for this comes from the specific mutant V4880A, which leads to decreased closed time (indicative of a less stable closed state) and almost completely abolishes the requirement for cytosolic Ca^{2+} (Sun et al., 2016). The location and ability of V4880A to remove the requirement for cytosolic Ca^{2+} further supports the idea that a long-range signal from the cytosolic Ca^{2+} activation site is normally transduced into the S6 helix to gate the channel.

Modulators of RyR2 activation

In addition to Ca^{2+}, it is likely that other modulators of RyR2 may work through related mechanisms. In the absence of cytosolic Ca^{2+}, RyR2 requires ATP or caffeine for effective luminal Ca^{2+} activation (Chen et al., 2014). Indeed, we have previously shown caffeine to activate RyR2 by preferentially sensitizing the channel to luminal Ca^{2+} (Kong et al., 2008). As shown in Fig. 3, both ATP and caffeine interact with RyR2 in the same region as cytosolic Ca^{2+}, namely where the central domain, CTD, U-motif, and S6 helix interact (des Georges et al., 2016). As described earlier in this article, the structure of this region appears critical in the function of the channel and undergoes substantial movements between the open and closed states (Peng et al., 2016). Therefore, if we assume that RyR1 and RyR2 have a similar structure, it is likely that both ATP and caffeine are able to sensitize the channel to luminal Ca^{2+} by modifying this structure and destabilizing the S6 helix in a manner similar to activation of the channel by cytosolic Ca^{2+}. However, the lack of direct activation of RyR2 by these agents suggests that although they may destabilize the gate and favor the binding of luminal Ca^{2+} to the E4872 binding pocket, unlike cytosolic Ca^{2+}, they are not sufficient to open the channel directly. In essence, it appears that ATP or caffeine binding to either side of the U-motif promotes a preopen state, which favors the open state–dependent formation of the luminal Ca^{2+}-binding pocket, thus enhancing luminal Ca^{2+} activation. Interestingly, caffeine preferentially alters luminal Ca^{2+} activation (Kong et al., 2008). This implies that although binding of Ca^{2+} to the cytosolic activation site can modify the state of the luminal Ca^{2+}-binding pocket via the U-motif, caffeine-mediated changes in the U-motif may not alter the cytosolic Ca^{2+}-binding site in the same way.

The presence of EF-hands within RyR2 led to them being described as the cytosolic Ca^{2+} sensor (Wei et al., 2016); however, we have recently shown that they are not required for cytosolic Ca^{2+} activation (Guo et al., 2016). Nevertheless, our data suggest that EF-hands play an important role in luminal Ca^{2+} regulation of RyR2. Deletion of the EF-hand domain markedly reduces the luminal Ca^{2+} sensitivity of the channel (Guo et al., 2016). In support of its role in luminal Ca^{2+}activation, the EF-hand domain of one subunit sits in close proximity to the voltage sensor–like cytosolic domain (VSL_{cyt}; S2–S3 loop) in the neighboring subunit (Fig. 3). It is possible that the binding of Ca^{2+} to the EF-hand domain alters the conformation of the S2 and S3 helices in the neighboring subunit, in turn altering helix packing and increasing the flexibility of S6 in a manner similar to that described earlier (Wei et al., 2016).

Further modulators of RyR2 function include small interacting proteins. On the luminal side of the channel, these comprise CASQ2, junctin, triadin, and histidine-rich Ca^{2+}-binding protein (HRC; Dulhunty et al., 2012). In addition to the effects of CASQ2 described earlier, both CASQ2 and HRC are Ca^{2+}-binding proteins, therefore their ability to buffer, and consequently reduce, local luminal Ca^{2+} levels could partially explain their action (Zhang et al., 2014a,b). However, this mechanism cannot apply to junctin and triadin. It is more likely that the interaction of these proteins with the luminal/trans-membrane segments of RyR2 alters the constraints on the pore region, something not possible to observe in the current structures. If the outer helices of the pore become more or less mobile, this would change the packing and flexibility of the inner S6 helix, potentially modifying channel activation.

Besides regulation by luminally expressed proteins, RyR2 function is modified by the cytosolic proteins calmodulin and FK506 binding protein (FKBP). FKBP was originally shown to decrease RyR2 opening (Marx et al., 2000; Marks, 2002); however, more recent studies suggest that this is not the case (Hunt et al., 2007; Xiao et al., 2007; Guo et al., 2010). Intriguingly, rather than affecting activation, both FKBP and calmodulin have now been shown to promote earlier termination of Ca^{2+} release, suggesting that they may alter the closing of the

channel (Tian et al., 2013; Zhang et al., 2016). However, given their respective binding sites (both far from the channel pore), the mechanism by which these proteins promote RyR2 inactivation is unclear.

Similarly, posttranslational modifications, such as phosphorylation and oxidation, are also well established as altering RyR2 sensitivity to both cytosolic and luminal Ca^{2+} (Marx et al., 2000; Xiao et al., 2005; Huke and Bers, 2008; Hanna et al., 2014; Oda et al., 2015; Waddell et al., 2016). As with calmodulin and FKBP, these modifications occur on the cytosolic side of the channel; thus the mechanism by which they alter cytosolic Ca^{2+} activation, luminal Ca^{2+} activation, or both has yet to be defined.

Therefore, in summary, although the high-resolution structure of RyR1 and RyR2 have provided unprecedented insights into the activation mechanisms of RyR2 by cytosolic and luminal Ca^{2+}, the finer details of how these are regulated by cell signaling pathways remain to be determined.

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