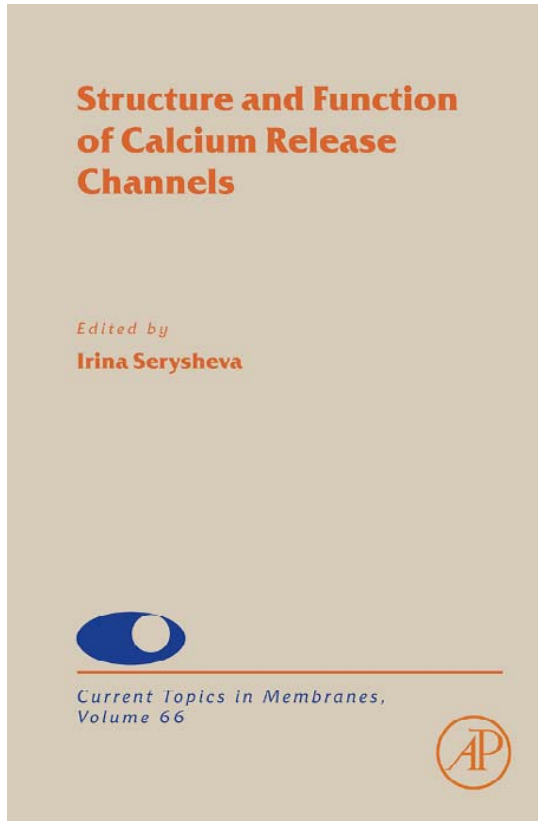


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CHAPTER 3

The Ryanodine Receptor Pore: Is There a Consensus View?

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I. OVERVIEW

The intracellular Ca²⁺-release channel referred to as the ryanodine receptor (RyR) is a key factor in a plethora of biological and pathophysiological processes and is therefore the focus of interest of many scientists and

clinicians. In recent years, an ever-growing body of evidence has emerged detailing the ligands and mechanisms involved in the regulation of RyR activity, and how this might be altered in disease. This information is reviewed in other articles in this publication. Here we review a fundamental aspect of RyR function, namely the structures and mechanisms that control which, and how many, ions can flow through the open channel and underpin the unusual ion handling characteristics of RyR and its great efficiency as a Ca^{2+} -release channel. Therefore, we will limit our discussion to a relatively small region of the channel that provides the pathway for ion movement across the membrane: the pore-forming region (PFR). Our present understanding of these processes has been informed by a wide range of approaches. Information on the likely structure of the PFR has been provided by high-resolution cryo-electron microscopy (cryo-EM) and by molecular modeling, following the identification of structural analogies between RyR and other ion channels. The mechanisms involved in discrimination and translocation have emerged from detailed characterization of channel function, theoretical models, and the interpretation of the consequences of residue mutation in the PFR. Together, these approaches have been used to identify regions of RyR that are critical for ion movement and may contribute to the binding site for ryanodine.

II. INTRODUCTION

Endo/sarcoplasmic reticulum (ER/SR) intracellular membrane systems contain two species of Ca^{2+} -release channel, the inositol trisphosphate receptor (InsP_3R) and the RyR, so named because this channel possesses a high-affinity binding site for the plant alkaloid ryanodine (Sutko, Airey, Welch, & Ruest, 1997). Three mammalian isoforms of RyR have been identified: RyR1 found predominantly in skeletal muscle, RyR2 found in both cardiac muscle and the brain, and RyR3 which is expressed at low levels in a wide range of tissues (Fleischer, 2008). Functional RyR channels are homotetramers with each monomer containing approximately 5000 amino acids. Detailed information on the functional properties of RyR channels has been obtained following the incorporation of individual channels into artificial phospholipid bilayers which permits the measurement of single channel gating and ion handling (Williams, West, & Sitsapesan, 2001). RyR channels are extremely efficient Ca^{2+} -release channels and this efficiency is underpinned by the structure and mechanisms of ion handling of the PFR of the molecule. Our present understanding of these parameters will be reviewed here.

III. ION HANDLING IN RYR

A. Basic Properties of Ion Handling in the RyR Isoforms

The first characterization of ion handling in RyR was carried out by Meissner and colleagues using purified rabbit RyR1 (Smith et al., 1988). The studies demonstrated that RyR1 (a) is cation selective, (b) is divalent selective, (c) has a higher unitary conductance for monovalent than divalent cations, and (d) is essentially nonselective between group 1a monovalent cations. These basic features were confirmed in detailed characterizations of monovalent (Lindsay, Manning, & Williams, 1991) and divalent ion handling (Tinker & Williams, 1992) in purified sheep RyR2 (see Table I).

In addition to the above, RyR2 was found to be relatively nonselective between individual mono- or divalent cations, somewhat selective for divalents ($P_{X^{2+}}/P_{K^+} \sim 6$), while exhibiting a high affinity (μM) for divalents (Tinker, Lindsay, & Williams, 1992b) compared to a relatively low affinity (mM) for monovalent cations. The different experimental conditions that have been used to examine ion handling in the RyR isoforms makes a direct comparison of properties difficult; however, basic features are compared in Table II.

TABLE I
Ion Handling in RyR2

X^+	pX^+/pK^+	γ (pS)	K_D (mM)	γ_{\max} (pS)
K^+	1.00	723	19.9	900
Na^+	1.15	446	17.8	516
Cs^+	0.61	440	34.0	588
Rb^+	0.87	621	n.d.	n.d.
Li^+	0.99	215	9.1	248
X^{2+}	pX^{2+}/pK^+	γ (pS)	* K_D (mM)	pX^{2+}/pBa^{2+}
Ba^{2+}	5.8	202 ± 5	0.165	1.0
Sr^{2+}	6.7	166 ± 4	0.123	1.1
Ca^{2+}	6.5	135 ± 5	0.116	1.1
Mg^{2+}	5.9	89 ± 4	0.116	1.1

pX^+/pK^+ : bi-ionic 210 mM.

γ (pS): determined at symmetrical 210 mM $[X^+]$ or bi-ionic with $[K^+/X^{2+}]$.

K_D and γ_{\max} from Michaelis–Menten conductance–activity plots. $[X^+]$ or *predicted from RyR Eyring rate theory model (Tinker et al., 1992b).

pX^{2+}/pK^+ : bi-ionic 210 mM.

pX^{2+}/pBa^{2+} : bi-ionic with 210 mM $[Ba^{2+}/X^{2+}]$.

n.d.: not determined.

TABLE II
Comparison of Ion Handling in RyR Subtypes

	RyR1	RyR2	RyR3
[K ⁺] γ (pS)	772	723	777
[Ca ²⁺] γ (pS)	123	135	137
pCa ²⁺ /pK ⁺	6.6	6.5	6.3
Permeability sequence	Li ⁺ >Na ⁺ >K ⁺ >Rb ⁺ >Cs ⁺	Na ⁺ >K ⁺ >Li ⁺ >Rb ⁺ >Cs ⁺	n.d.
Conductance sequence	K ⁺ >Cs ⁺ >Rb ⁺ >Na ⁺ >Li ⁺	K ⁺ >Rb ⁺ >Na ⁺ >Cs ⁺ >Li ⁺	n.d.

RyR1, RyR2 and RyR3 K⁺ slope conductances were examined in symmetrical [K⁺] 250 mM/210 mM/250 mM, respectively. Divalent permeability ratios were examined in bi-ionic [K⁺/Ca²⁺] at 50/50 mM, 210/210 mM and 250/250 mM, respectively. Monovalent permeability ratios were examined in bi-ionic [K⁺/X⁺] 210/200–210 mM, 210/210 mM and 250/250 mM, respectively. Conductance sequences were compared in symmetrical [X⁺] 200–215 mM (RyR1) and in symmetrical 210 mM (RyR2). n.d. : not determined. Readers may refer to the following publications for further information: RyR1 (Shomer et al., 1994; Smith et al., 1988; Xu et al., 1993), RyR2 (Lindsay et al., 1991; Tinker & Williams, 1992) and RyR3 (Chen et al., 1997b).

Differences between the RyR isoforms are minimal; however, there are some limited differences in the relative conductance of monovalent cations and permeability sequences of RyR1 and RyR2 that may suggest some minor differences in the mechanisms governing ion handling in these channels. The sequence for monovalent and divalent cation binding in RyR2 correspond to Eisenman (1962) and Sherry (1969) sequences XI and VII, respectively, indicating the involvement of high field strength sites in the process of discrimination (Tinker et al., 1992b). Based on the structure of Ca²⁺ binding proteins, it was predicted that cation binding at such sites could be coordinated by carboxyl oxygens. To account for the higher relative permeability of divalents over monovalents, it was proposed that the RyR PFR was likely to contain a high density of such sites (Tinker et al., 1992b).

B. What is Responsible for the High Rate of Ion Translocation in RyR?

The maximum rate of cation translocation in RyR is phenomenal, with saturating unitary conductance for monovalent cations approaching 1 nS (approximately twice the theoretical limit for a selective channel based on the laws of diffusion; Hille, 1991). Clearly an enormous unitary conductance is a useful property for a Ca²⁺-release channel, but how is this achieved? A range of potential contributing mechanisms have been proposed, for example, rates of delivery of cations could be maximized by a high density of acidic residues at the entrance of the PFR (Tinker et al., 1992b) or the RyR PFR could be occupied

by more than one ion at a time leading to increased rates of ion exit by ion–ion repulsion (Smith et al., 1988). Another suggestion, based on earlier predictions for large conductance K^+ channels (Latorre & Miller, 1983), was that the PFR of RyR, in comparison with other ion channels, was both wide and short so maximizing both rates of ion entry and exit (Williams, 1992).

The dimensions of some aspects of the RyR PFR have been investigated. The minimum radius of the RyR PFR has been estimated as 3.5 Å based on the relative permeability of organic monovalent cations of known dimensions (Tinker & Williams, 1993). However, subsequent experiments have shown that this region of the PFR is likely to be flexible. With a high driving force neomycin (minimum radius 5 Å), a normally impermeant blocking polycation, can move through the PFR (Mead & Williams, 2002a). The binding of ryanodine to RyR prevents this translocation presumably by decreasing the flexibility of the PFR (Mead & Williams, 2002b). Information on aspects of the length of the PFR is also available.

The length of the voltage drop across the channel has been measured at approximately 10 Å by monitoring blocking parameters of bis-quaternary ammonium ions of varying length (Tinker & Williams, 1995) and the region of RyR in which single-file diffusion occurs has been estimated as 9 Å from measurements of streaming potentials (Tu, Velez, Brodwick, & Fill, 1994). Taken together these parameters are consistent with the proposal that the PFR of RyR is, in comparison with more selective, lower conductance, channels such as the bacterial K^+ channel KcsA, relatively wide and short (Williams et al., 2001).

IV. WHERE IS THE PFR IN THE RYR CHANNEL?

A. Evidence from Cryo-Electron Microscopy

Three-dimensional structures of RyR tetramers have been obtained by reconstruction of images gathered from electron micrographs of frozen-hydrated single isolated channels. Of the three RyR isoforms, the most extensive structure–function analysis has been carried out with RyR1 due to its high abundance in skeletal muscle and the relative ease with which it can be purified. Until recently, the most detailed structures available were at resolutions of only 25–30 Å (for a review see Serysheva, Chiu, & Ludtke, 2007). Although no secondary structure elements can be identified at this resolution, it is clear that RyRs have an overall mushroom shape with a large cytoplasmic foot assembly connected to a transmembrane (TM) stalk-like structure (Radermacher et al., 1994; Serysheva et al., 1995). This technique has been used to identify domains in the cytoplasmic foot which

are binding sites for various effectors (Liu, Zhang, Wang, Wayne Chen, & Wagenknecht, 2004; Samso & Wagenknecht, 2002; Wagenknecht et al., 1997). By analogy with other ion channels, the PFR of RyR is likely to be located in the TM portion of the structure and an apparent opening running axially into the TM domain has been identified in what is believed to be an open conformation of RyR1 (Orlova, Serysheva, van Heel, Hamilton, & Chiu, 1996). This opening is not visible in the closed conformation of the channel and these studies have also highlighted other structural alterations in RyR that may occur during the transition from a closed state to an open state of the channel. Recent technical advances have yielded structural information on the TM domain of RyR1 at considerably improved resolution and this will be discussed in Section V.B.

B. Analogies with K^+ Channels and Evidence from Functional Studies of Mutant RyR Channels

In 1999, Balshaw, Gao, and Meissner (1999) identified a motif located in a luminal loop between the last two membrane spanning helices in the primary structure of RyR (GGGIGD) (see Fig. 1) as being analogous to the signature selectivity sequence of K^+ channels and suggested that this region was likely to be a component of a PFR in RyR. Consistent with this proposal, mutation of various residues within this motif, and of residues flanking this motif, were shown to alter ion handling properties of both RyR1 and RyR2 (Fig. 1).

1. RyR1

Meissner's laboratory expressed rabbit RyR1 in HEK cells and screened amino acids at the end of the predicted "loop 2" of the PFR (see Fig. 1). R4913E RyR1 channels released Ca^{2+} after caffeine stimulation *in situ*, but [3H]-ryanodine binding was lost and unitary K^+ conductance was decreased by 60% while Ca^{2+} current was also reduced. In contrast, D4917A mutants showed no caffeine-induced Ca^{2+} transients, no [3H]-ryanodine binding, no detectable Ca^{2+} current, but retained 60% of the unitary K^+ conductance (Gao et al., 2000). Of particular interest are mutations of two residues conserved in all RyR isoforms: D4903A and D4907A. Both mutants retained Ca^{2+} -dependent [3H]-ryanodine binding and had unitary conductance indistinguishable from wild-type (WT) channels. Caffeine-evoked Ca^{2+} transients were seen in cells expressing both mutants, but were increased by the D4907A substitution (Gao et al., 2000). The mutant G4894A induced dramatic changes in ion conductance. Maximum unitary K^+ conductance was severely decreased and Ca^{2+} current was abolished, while responses to caffeine and [3H]-ryanodine binding were preserved (Gao et al., 2000).

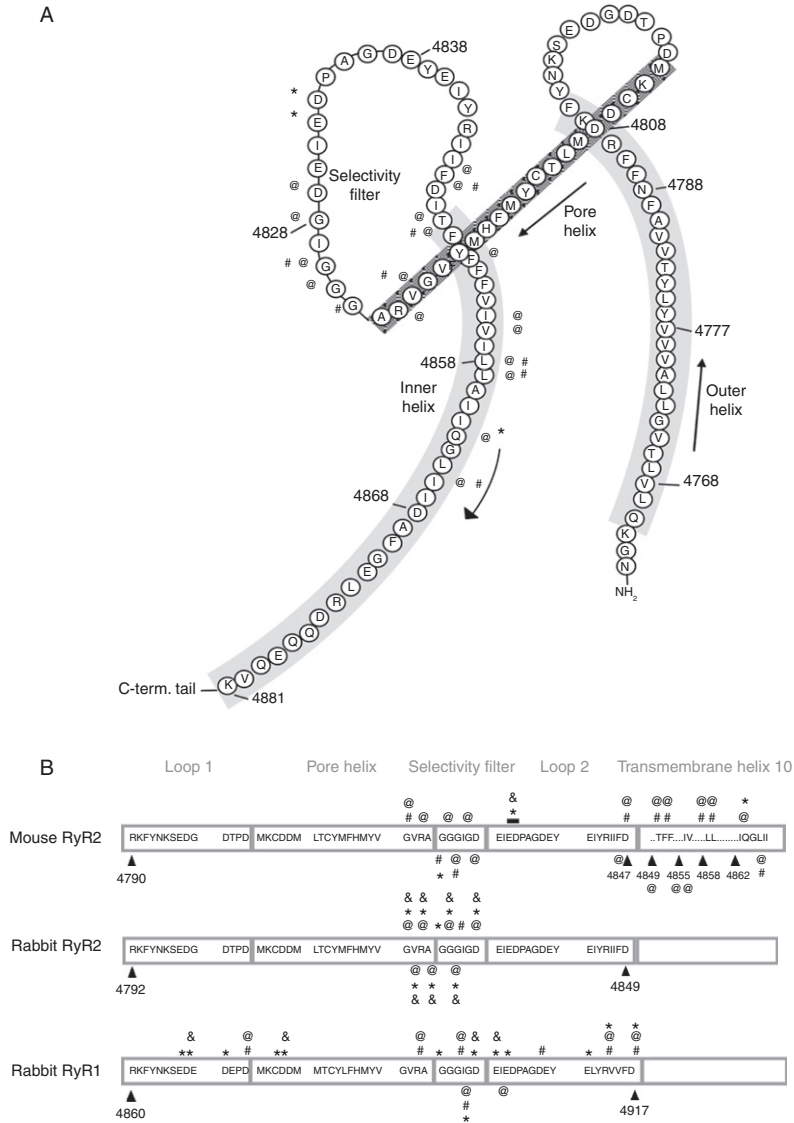


FIGURE 1 Mutagenesis characterization and sequence alignments of the putative pore-forming regions of RyR Ca^{2+} -release channels. (A) Tube map of our model of the mouse RyR2 pore. Meaning of the symbols can be found below. (B) This schematic representation depicts the localization of various residues of the different predicted pore regions (rabbit RyR1, rabbit and mouse RyR2, according to available published data) that underwent substitution into another residue or a series of diverse amino acids. The arrowheads specify the residue numbering according to each isoform of each species. The consequences of the mutations on channel function are indicated as follows: *, ion translocation impairment (permeation, conductance); #, Ca^{2+} release is compromised (in microsomes or cells; stimulation by caffeine); @, decrease or abolition of [^3H]-ryanodine binding; &, disruption of the normal ion discrimination (selectivity).

Recently, MacLennan's laboratory characterized the I4898T substitution in the human RyR1 sequence (corresponding to I4897T in rabbits and I4895T in mice), a mutation in the putative selectivity filter which occurs in central-core disease (CCD) patients (Zvaritch et al., 2007). Transient expression of the rabbit ortholog in HEK-293 cells led to the identification of leaky channels (Lynch et al., 1999). However, homozygous I4895T mice displayed altered developmental features with a suppressed RyR1-mediated Ca^{2+} release while the morphology of *in situ* RyR1 clusters was preserved (Zvaritch et al., 2007).

In addition, previous investigations from the same group provided evidence that R4892W, I4897T, and G4898E (three CCD mutations) resulted in reduced Ca^{2+} sensitivity and amplitude of Ca^{2+} -dependent Ca^{2+} release, together with a decrease of [^3H]-ryanodine binding, when rabbit channels were coexpressed with SERCA1a in HEK-293 cells (Du, Khanna, Guo, & MacLennan, 2004).

2. RyR2

The involvement of residues in and around the putative selectivity filter in RyR2 has also been investigated. Residue substitutions in the rabbit motif, GVRAGGGIGD (Fig. 1), were studied by MacLennan and colleagues (Du, Guo, Khanna, & MacLennan, 2001). With the exception of I4829A and I4829T (corresponding to the CCD mutant I4897T in RyR1), *in situ* caffeine-evoked Ca^{2+} transients were observed in all mutants (either Ala substitution or Ala into Val). Moreover, there was a complete loss of [^3H]-ryanodine binding in all mutants except G4826A, I4829V, and G4830A. A high concentration of ryanodine (10 μM) induced *in situ* Ca^{2+} release in all Ala mutants from 4823 to 4827. Ryanodine also raised the amplitude of caffeine-evoked Ca^{2+} transients in G4828A and restored caffeine sensitivity/responsiveness in I4829A and I4829T. Single-channel recording was possible for all mutants except G4822A and A4825V; however, the unitary K^+ conductance of all mutants within this group except I4829V and G4830A was reduced; in G4824A (corresponding to G4894 in RyR1) conductance was reduced by 97%. The ability to discriminate between Ca^{2+} and K^+ was lost in all of these mutants, except G4826A, I4829V, and G4830A (Du et al., 2001). These findings confirmed the results of earlier investigations reported by Chen and colleagues (Zhao et al., 1999) who characterized residues in the equivalent motif (GVRAGGGIGD) in mouse RyR2 channels. These studies established that residue substitutions at R4822, G4825, G4828, and D4829 reduced or abolished high-affinity [^3H]-ryanodine binding, while *in situ* caffeine-induced Ca^{2+} release in cells expressing these mutant RyR2s was comparable with that of WT channels. In contrast, substitution at G4824 yielded RyR2 channels that retained [^3H]-ryanodine binding, but displayed

decreased caffeine-induced Ca^{2+} release. The K^+ unitary conductance of these channels was reduced drastically (22 pS as compared with 798 pS for the WT). Mutations at G4820 and G4826 reduced caffeine-induced Ca^{2+} release and abolished [^3H]-ryanodine binding.

C. Further Analogies Between the PFR of K^+ Channels and RyR

A breakthrough in our understanding of the mechanisms involved in the function of ion channels came about when the crystal structure of the bacterial K^+ channel, KcsA, was solved by MacKinnon and coworkers (Doyle et al., 1998). This prototypical ion channel structure revolutionized our understanding of the structures involved in ion discrimination, ion translocation, and the gating process of K^+ channels, allowing direct comparisons with other cation channels. The pore of KcsA is formed by four identical subunits arranged around a fourfold symmetry axis to form a functional tetramer, each consisting of two TM helices (Fig. 2). An extracellular loop, which folds back into the membrane, connects the helices and is composed of a short pore helix and a sequence of amino acids that contain a selectivity filter incorporating the K^+ channel signature selectivity sequence. The pore forms an inverted tepee with a gate at the cytosolic entrance. The transition from a closed to an open state occurs by the flexing of a

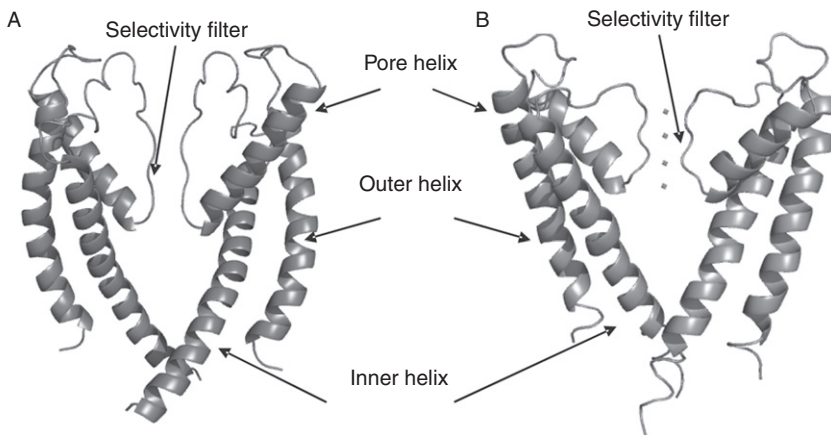


FIGURE 2 (A) RyR pore (analogy model from Welch et al., 2004) and (B) KcsA with four potassium ions shown in the four binding sites of the selectivity filter (1BL8.pdb, Doyle et al., 1998). Two opposing monomers of each channel are shown for clarity.

glycine hinge located approximately halfway along the inner helix (Jiang et al., 2002b). The relatively rigid structure of the selectivity filter of KcsA allows precise coordination of K^+ ions with backbone carbonyl atoms of residues. The hydration state of the permeating ion is also a crucial factor in selecting K^+ over Na^+ (Dudev & Lim, 2009).

V. ATTEMPTS TO IDENTIFY THE STRUCTURE OF THE RYR PFR

A. A Model of the RyR Pore Using KcsA as a Template

Following the identification of a presumed selectivity filter, secondary structure predictions for the loop linking the last two TM helices in RyR indicated that this region was likely to contain structural elements equivalent to those found in the PFR of K^+ channels (Williams et al., 2001). To further test possible structural similarities between the PFRs of RyR and K^+ channels Welch, Rheault, West, and Williams (2004) constructed a model of the putative RyR PFR using the recently solved crystal structure of KcsA as a template (see Fig. 2).

The model comprises just 2.4% of the total RyR2 monomer incorporating the last two TM helices and the luminal loop of each monomer. By combining information from secondary structure prediction programs and comparing sequence analogies with KcsA, corresponding structural elements in RyR were identified and, using molecular modeling software, a stable tetrameric model for RyR was constructed.

By making quantitative comparisons of the physicochemical properties of KcsA and the RyR homology model, Welch et al. (2004) were able to demonstrate that their model represents a highly plausible model for the PFR region of RyR. While the mechanisms governing ion handling must be vastly different in KcsA and RyR the actual structural elements of the PFRs and their organization are likely to be very similar with both structures having a cytosolic cavity lined by the inner helices that taper inward to form a gate. The most obvious differences between the two structures are the arrangements of the loop between the outer helix and the pore helix and the width and shape of the selectivity filter, RyR being much wider than that of KcsA. There are also marked differences in the solvent accessibility in this region with water penetrating all the way across the selectivity filter in the RyR2 model. Rings of negative charge are located at both cytosolic and luminal ends of both structures although the charge density is much higher in RyR2, a factor that could be important in concentrating cations around the entrance to the pore (see above). The much greater degree of flexibility of the selectivity filter observed in the RyR model, compared to

KcsA, and the higher density of negative charge around the mouths are likely to be key determinants in the observed differences in ion handling between RyRs and KcsA. Molecular dynamics (MD) simulations performed in the RyR model correlate extremely well with previous experimental data with regards to selective ion permeation (Williams et al., 2001) and block of K^+ translocation by tetraethyl ammonium ions (Tinker, Lindsay, & Williams, 1992a). The reader is referred to the original paper for more detailed and quantitative comparisons and assessments of structural components, physicochemical comparisons, and ion handling capabilities of the model (Welch et al., 2004). The RyR pore analogy model contains a glycine residue in the putative inner helix (GLIIDA) (Fig. 1) that could act as a hinge in a gating motif (GXXXXA), as originally identified by Jiang et al. (2002b) in K^+ channels. This raises the possibility that the conformational changes underlying gating in RyRs could be similar to those of K^+ channels.

B. High-Resolution Images of the PFR of RyR

Two reports in 2005 detailed the structures of presumed closed states of RyR1 obtained using cryo-EM at resolutions of around 10 Å. The first structure was compared directly to the atomic model of KcsA, docking this structure into the 3D map of RyR1 (Samso, Wagenknecht, & Allen, 2005).

Apart from the outer helices, the overall orientation and arrangement of structural elements display a high degree of architectural compatibility, with the selectivity filter orientated toward the lumen and the constriction of the inner helices forming a gate at the cytoplasmic face of the structure. The second structure, published by Ludtke, Serysheva, Hamilton, and Chiu (2005) is modeled independently of any known structures and identifies five α -helices in the RyR1 TM domain of which two are identified as the inner helix and pore helix. In contrast to the cryo-EM structure obtained by Samso et al. (2005) the inner helix is modeled with a kink at the putative glycine gating hinge and is therefore compared to the open structure of the K^+ channel MthK (Jiang et al., 2002a; Samso et al., 2005).

The different conformations of the inner helices in these two structures mean that disagreement exists regarding the closed structure of RyR and the theory that kinking of the inner helices is required to open the channel. This may be due to the relatively low level of resolution and the ambiguity as to which conformational state the protein is in during the imaging process (Hamilton & Serysheva, 2009). What is clear from these cryo-EM structures is that the RyR PFR orientates around a central fourfold axis of symmetry and that a funnel like cavity is formed with a wide entrance at the luminal

side tapering in towards the cytoplasmic side. These observations strongly correlate with the earlier analogy model (Welch et al., 2004) and indicate that the PFR of RyRs are indeed similar in architecture to K^+ channels and could gate using similar elements.

C. A RyR PFR Model Based on a High-Resolution Cryo-EM Structure

In an attempt to bridge the gap between the low resolution limits of cryo-EM and the previously published RyR PFR model, Ramachandran, Serohijos, Xu, Meissner, and Dokholyan (2009) recently built a molecular model of the putative PFR of RyR1 based on the cryo-EM structure of Ludtke et al. (2005). The unambiguous assignment of several helix-like densities gave a model with remarkable similarities to the MthK K^+ channel, crystallized in the open state. The two main helices, inner helix and pore-forming helix, were found to face each other and line the channel pore, as evidenced in all previous cryo-EM structures. As in previous assignments of the pore structure, acidic residues are located at the mouth of the pore with residues D4899 and E4900, postulated to be important for channel function in RyR1, positioned within the selectivity filter (Wang, Xu, Pasek, Gillespie, & Meissner, 2005; Xu, Wang, Gillespie, & Meissner, 2006).

The low resolution limits of cryo-EM do not allow for modeling of amino acid side chains. However, by replacing the missing side chains and adding the luminal loop, Ramachandran et al. (2009) were able to perform MD simulations and, by taking into consideration data from previously published single channel experiments, were able to examine interactions of the PFR with mono- and divalent ions known to permeate the channel (Lindsay et al., 1991; Tinker & Williams, 1992). MD simulations of selectivity filter mutants known to alter ion handling correlate well with experimental data with ion occupancies observed in the model agreeing well with the charge space competition (CSC) theory, as discussed below. Specific residues located near the selectivity filter show preferential affinity for Ca^{2+} over K^+ , supporting the accuracy of the model as a Ca^{2+} -selective channel. The proposed glycine gating hinge occurs at residue 4934 in RyR1, analogous to G4864 modeled in RyR2 by Welch et al. (2004). That recent structures resemble the open state of MthK even when solved in conditions apparently favoring the closed state of the channel indicate that mechanisms other than flexing at the inner helix glycine hinge may be responsible for the transition between closed and open states in RyR. In order to ascertain the correct conformation of the inner helix in the closed state, an improvement in cryo-specimen presentation and image processing is needed.

VI. THEORETICAL APPROACHES TO UNDERSTANDING THE MECHANISMS UNDERLYING ION TRANSLOCATION AND DISCRIMINATION IN RYR

In the absence of detailed information on the structure of the RyR PFR, potential mechanisms underlying ion handling have been explored using theoretical models. A major area of debate that has arisen from these studies is whether the high unitary conductance of RyR is achieved as the result of ion–ion repulsion in a multi-ion pore. It is frequently acknowledged within the field that the interpretation of experimental ion channel permeation phenomena and ion channel occupancy are model dependent. For example, the saturation of conductance in RyR suggests that ions do not move independently of one another (as in solution) with a fit consistent with a single occupancy pore (Lindsay et al., 1991; Tinker & Williams, 1992); however, multi-ion models can show saturation (Gillespie & Boda, 2008; Gillespie & Eisenberg, 2002).

For this reason the ion occupancy state of RyR has been a matter of strong debate with no clear conclusion made due to the presence of two opposing theories; rate theories such as that of Eyring and continuum models such as Poisson–Nernst–Planck (PNP) (Levitt, 1986; Miller, 1999; Nonner, Chen & Eisenberg, 1999) which both describe the patterns of ion permeation in RyR (Chen, Xu, Tripathy, Meissner, & Eisenberg, 1997, 1999; Tinker et al., 1992b). Although there are multiple lines of “model-dependent” experimental evidence supporting multi- (Smith et al., 1988) or single-ion occupancy (Lindsay et al., 1991; Tinker & Williams, 1992; Tinker et al., 1992b) one phenomenon has become central to the argument of ion occupancy in RyR, the anomalous mole fraction effect (AMFE). AMFE is observed as a minima in current, conductance or Erev when the concentration of ions within a bi-ionic mixture varies to produce different ratios of ions (mole fractions). AMFE can be interpreted in terms of Eyring rate theory with a single filing multi-ion pore (Lindsay et al., 1991; Tinker & Williams, 1992) or in terms of resistors in series (PNP—continuum model) which can be shown when (on average) < 1 ion is in the pore (Nonner, Chen, & Eisenberg, 1998). It has long been established that multi-ion channels may not necessarily display AMFE (Hille & Schwarz, 1978) and that single filing is not necessary to observe this phenomenon (Gillespie, Boda, He, Apel, & Siwy, 2008). In RyR, the presence of AMFE is highly dependent on the choice of cation, ion activities, and the method of detecting AMFE (Tomaskova & Gaburjakova, 2008). Therefore, advocates of rate theory models of RyR will be satisfied that single occupancy exists in some ion mixtures (Lindsay et al., 1991; Tinker & Williams, 1992) whereas in others it does not (Gillespie, Giri, & Fill, 2009;

Tomaskova & Gaburjakova, 2008). Recent PNP theories adopt density function theories (DFT) to improve on the previous flawed models which do not describe ions of a finite size and do not consider interactions between ions and channel structure. New models describe selectivity in RyR by CSC where ions can interact with flexible, time-averaged, charged residues where “charge space competition created by ions in a confined geometry results in significant selectivity” (Gillespie & Eisenberg, 2002). In this model, RyR, on average, is multiply occupied in various mole fraction mixtures of monovalent and divalent cations while one, approaching two, divalent cation(s) are present in the pore in pure divalent solutions (Gillespie, Xu, Wang, & Meissner, 2005). However, this model assumes that ions are dehydrated once reaching the pore, whereas an alternative model of RyR demonstrates that ions are partially hydrated within the pore (Welch et al., 2004). In summary, there is agreement within the literature that there is multi-ion occupancy in RyR and therefore this could serve as a mechanism, along with pore dimensions (Section III.B) and negative charge (Sections V.A and VII.B) to maximize ion conduction in the channel.

VII. TESTING PHYSICAL AND THEORETICAL MODELS OF THE RYR PFR BY RESIDUE SUBSTITUTION

A. *RyR1*

Meissner's group identified potentially important residues by using mutagenesis in conjunction with a theoretical PNP–DFT model and MD simulations in their model of the PFR of RyR1. D4899 and E4900 were shown to be critical for both conductance (ion permeation) and selectivity (high ion binding) (Wang et al., 2005). Simulations and electrophysiological experiments revealed that the mutant D4899Q exhibited a loss of Ca^{2+} preference in the selectivity filter (Ramachandran et al., 2009). Furthermore, analysis of G4898R, a CCD mutant showed a decrease of Ca^{2+} conductance experimentally, whereas K^+ conductance was constitutively high. These data were confirmed by simulations with a decrease in the preference of Ca^{2+} over K^+ in the selectivity filter (Ramachandran et al., 2009). Noteworthy is that both CCD mutations G4898E and G4898R can form homotetramers and heterotetramers in combination with WT channels when expressed in HEK-293 cells. In terms of function, while heterotetrameric mutants can maintain their Ca^{2+} -dependent activity and K^+ conductance, but display decreased Ca^{2+} selectivity (depending on the type of mutants and the combination of subunits), homotetrameric mutants have negligible Ca^{2+} -dependent activity and Ca^{2+} permeation and reduced K^+ conductance (Xu, Wang, Yamaguchi, Pasek, & Meissner, 2008).

B. RyR2

In agreement with earlier experimental data (Mead, Sullivan & Williams, 1998), the RyR PFR analogy model based on KcsA demonstrates the existence of a high density of negatively charged residues at both the luminal and cytosolic mouths of RyR2 pore (Welch et al., 2004). To investigate the significance of this charge, substitutions of acidic residues at the luminal side of the channel were performed. Earlier studies in RyR1 had demonstrated that the neutralization of either the glutamic acid residue equivalent to E4832 or the aspartic acid residue equivalent to D4833 in RyR2 produced no significant change in K^+ conductance (Wang et al., 2005). However, introducing the double substitution ED4832AA in mouse RyR2 channels led to very significant changes in function (Mead-Savery et al., 2009). In comparison with WT RyR2, the open probability of ED4832AA RyR2 is greatly increased and a wide variety of subconductance states are observed. ED4832AA discriminates ideally between cations and anions but discrimination between divalent and monovalent cations is lost.

At low ionic activities unitary conductance of ED4832AA is reduced but conductance increases to values above those seen in WT channels at high activities. MD simulations provide some insights into the consequences of this double substitution suggesting that (a) interactions of E4832 and D4833 with other residues in the PFR stabilize the selectivity filter, (b) E4832 and D4833 neutralization reduces the electric field at the luminal face that in turn could reduce cation delivery to the pore at low activities, and (c) neutralization lowers the electric field in the selectivity filter which, together with destabilization of the filter could underlie enhanced conductance at high ionic activity. In addition to its role as a Ca^{2+} -release channel, RyR also contains a high-affinity binding site for the plant alkaloid ryanodine. Interaction of ryanodine or its derivatives and congeners (ryanoids) results in alterations in both channel gating and ion handling (Sutko et al., 1997; Tinker et al., 1996). As outlined above, residue substitution in and around the proposed selectivity filter of the RyR channels can reduce or abolish the high-affinity interaction of [3H]-ryanodine with its receptor. Another region of the channel that may contribute to the site of ryanoid interaction was uncovered by the investigation of residue substitution in the predicted pore-lining TM helix (TM 10). Mutations D4847A, F4850A, F4851A, L4858A, L4859A, and I4866A severely reduced the release of Ca^{2+} from the ER in HEK-293 cells in response to caffeine and diminished [3H]-ryanodine binding to cell lysates. Mutations F4846A, T4849A, I4855A, V4856A, and Q4863A eliminated or markedly reduced [3H]-ryanodine binding, but cells expressing these mutants responded to caffeine by releasing Ca^{2+} from intracellular stores (Wang et al., 2003). Investigations of single RyR2

channels containing one of these mutants; Q4863A, revealed that ryanodine and other ryanoids bind to the channel as readily as to the WT RyR2. The absence of measureable equilibrium binding of [³H]-ryanodine to Q4863A RyR2 reflects an almost 600-fold increase in the rate of dissociation of the bound ryanoid (Ranatunga, Wayne Chen, Ruest, Welch, & Williams, 2007).

VIII. CONCLUDING REMARKS

In this contribution we have attempted to demonstrate how recent developments in structural biology, electrophysiology and molecular modeling have contributed to an improved understanding of the architecture of the PFR and the mechanisms governing the way in which the RyR channel translocates and discriminates between ions. Together these properties allow SR Ca²⁺ to be released at phenomenal rates through the open RyR channel during excitation–contraction coupling.

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