Cryo-Electron Microscopy and 3D Reconstruction of Ryanodine Receptors and Their Interactions with E-C coupling Proteins

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Abstract

Cryo-electron microscopy (EM) and image processing have proven to be powerful tools for determining the three-dimensional (3D) architecture of ryanodine receptors (RyRs). Over the past few years, 3D models of RyRs have become available at resolutions in the range 25-35 Å for all the three isoforms that have been identified in mammals. The information available from 3D reconstruction reveals the four-fold symmetric architecture of RyRs: an overall mushroom-shaped structure with a large cytoplasmic portion containing domain-like features, and a distinctive transmembrane assembly. Binding sites of various protein ligands (e.g., calmodulin, FK506-binding protein) that interact in vivo with RyR have been mapped onto the surface of RyRs using cryo-EM. Conformational changes in RyRs associated with calcium transport have also been characterized, by employing buffer conditions that favor the two different functional states, 'open' and 'closed'. This review will discuss these results, and will demonstrate the utility of cryo-EM and 3D image processing reconstruction in striving towards our ultimate goal of obtaining sufficient structural detail to elucidate the mechanism of excitation-contraction coupling in muscle.

Key words: 3D: three-dimensional reconstruction; EM: electron microscopy; RyR ryanodine receptor.

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Ryanodine receptors (RyRs) form a class of intracellular calcium release channels that are widely expressed but are particularly prevalent in muscle. In muscle, the neuron-induced depolarization of transverse (t)-tubules is translated into a signal for calcium release from the sarcoplasmic reticulum (SR) at structures known as triad junctions, where RyRs respond to this signal. RyRs are homotetramers, composed of four identical subunits, and have a molecular mass in the range from 2.2 - 2.3 MDa. Three known isoforms are responsible for calcium release from ER/SR: RyR1, the isoform found in skeletal muscle, RyR2, the form in heart muscle, and RyR3, often referred to as the brain isoform. However, we now know that these isoforms are expressed more widely then their nomenclature would indicate [6, 16, 18, 27, 37]. Calcium release from the SR is a finely regulated process that involves not only RyRs but also several other accessory proteins that modulate RyR activity. Among these proteins are members of the FK506- binding protein (FKBP) family and calmodulin (CaM), both of which produce physiological effects when associated with RyRs [49].

It has not been possible so far to obtain ordered crystals of RyRs. This is perhaps not surprising; RyRs are not only structurally complex but they are also integral membrane proteins, which tend to be poor candidates for characterization by X-ray crystallography. The first detailed visualizations of these calcium release channels became available more than a decade ago by the implementation of image processing technique (using the SPIDER software) in our laboratory of micrographs of negatively stained purified receptors [42]. Since then, there have been significant developments in the application of cryo-electron microscopy (EM) to isolated (non-crystalline) macromolecules embedded in vitreous ice. In this technique, small amounts (< 1µg protein) of aqueous sample are placed on the specimen grid, which is then extensively blotted and rapidly plunged into liquid ethane, thereby vitrifying the thin water layer. The advantage of frozen-hydrated specimen preparation is that specimen collapse ("flattening") is avoided, along with the other artifacts associated with dehydration, contrast enhancement using heavy metal, and chemical fixation. Cryo-EM, in combination with image

processing using software packages such as SPIDER [12, 13], has allowed a quantum leap in the field of biological EM as it has now become feasible to obtain images of fully hydrated macromolecules, enabling direct comparisons and integration with high-resolution structural results from X-ray crystallography and NMR [3, 7, 9, 12, 13]. Already, this technique has been applied to obtain 3D structures of all three RyR isoforms (Fig. 1) albeit at moderate levels of resolution (typically 25-30 Å resolution) [29, 30, 33, 34, 35].

When the overall surface maps for the three isoforms are compared, they appear quite similar. This is not surprising, due to the isoforms' high degree of sequence identity. The differences among the receptor isoforms occur mainly in three regions that are known as divergent regions DR1, DR2, and DR3, and these have been recently mapped in 3D reconstructions by a GFP insertion method [23, 24]. In RyR1, the DR1 region comprises residues 4254-4631; RyR2 comprises residues 4210-4562. The DR2 region, which is absent in RyR3 [34], includes residues 1342-1403 in RyR1, and residues 1353-1397 in RyR2. DR3 contains residues 1872-1923 in RyR1 and residues 1852-1890 in RyR2.

This review focuses on the results obtained by cryo-EM and image processing to reveal the 3D architecture of RyRs themselves and also the structure of their complexes with associated proteins that underlie and modulate excitation-contraction (E-C) coupling in muscle.

3D structure of RyRs visualized by cryo-EM

Over the past decade, cryo-EM has progressed since negative stain EM provided the first two-dimensional (2D) projection density maps of native RyR, to the more recently available three-dimensional reconstructions (3D) of all three RyR isoforms. Two research groups, namely, our laboratory (Albany group), which was the first to report 3D structures of RyRs, and another at the Baylor College of Medicine (Baylor group) have made progress in determining the 3D structures of RyRs [12, 29, 30, 33, 34, 35, 42]. The 3D structures determined for RyR1 by both the groups are currently at resolutions of ~20-30 Å. The two structures appear to be in good agreement; despite differences in the methods of data collection and image analysis applied to obtain the 3D reconstructions [30, 35]. In their initial studies the Baylor group used electron micrographs collected from untilted specimen cryo-grids and applied an angular reconstitution algorithm implemented in the IMAGIC [50] software suite for image processing, while the Albany group obtained data from tilted and untilted cryo-grids and used a random conical algorithm implemented in the SPIDER software package [30, 35].

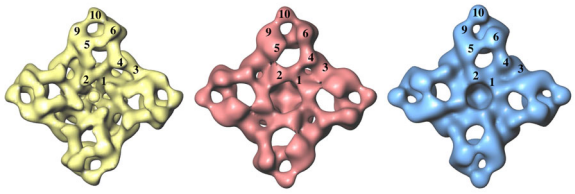

Figure 1. Solid body representations of the three isoforms of ryanodine receptor, RyR1 (yellow), RyR2 (red), RyR3 (blue). The three receptors are shown as viewed from the cytoplasmic face. Although the surfaces may look very similar, pair wise difference maps computed for the 3D reconstructions show that there are several significant structural differences among the isoforms. Such structural differences may be related to functional differences in the mechanisms of E-C coupling in heart and skeletal muscle, and to differing modes of interaction of the isoforms with various ligands. The domains are numbered (1-10) in one quarter of the tetramer molecule.

Even with the current limitation of the resolution to 20-30 Å, the available reconstructions of the receptors provide useful information and serve as a framework for functional interpretations (discussed below). The characteristic square appearance of most RyRs in cryo-images arises from the receptor's massive cytoplasmic region, a structure that we term the cytoplasmic assembly (CA). A smaller "baseplate" or transmembrane assembly (TA) projects from one of the faces of the CA and it contains the transmembrane regions of the receptor (Fig. 2). The CA has dimensions of 290 x 290 x120 Å and the TA protrudes 70 Å from the mushroom-shaped cap, resulting in an overall height (thickness) for the receptor of 190 Å (Fig. 2).

The Cytoplasmic Assembly

The CA was first recognized as the "foot" structure in electron micrographs of sectioned muscle (reviewed in [14]), before RyRs were characterized and analyzed by cryo-EM and image processing; thus the molecular identity of the foot was unknown for almost 20 years. About half of the volume of CA is occupied by solvent and its characteristic square form is made up of

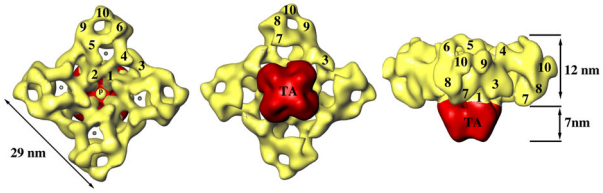

Figure 2. Surface representation of skeletal muscle RyR1 structure shown in three different orientations. From left to right are seen the cytoplasmic face, SR view, and a side view. These views show the cytoplasmic assembly structure in yellow, and the transmembrane assembly in red; domains 1-10 are numbered. Dots indicate the peripheral cavities inside the receptor (see text for details). (Adapted from [30]).

reproducible, interconnected globular folds or domains, which were assigned arbitrary numerical designations (numbers 1-10) by the Albany Group [30]. These domain-like structures form each subunit of the tetramer (Fig. 2). Domains 2, 4, 5, 6, 9, and 10 form much of the face that interacts with the t-tubule in myofibers, and some of these domains may interact with the dihydropyridine receptors (DHPR) (Fig. 3). Domains 7, 8, 9, and 10 face the SR *in situ*, and domain 10, the most distal domain, forms the tip of each corner of the CA. RyR has four symmetrically situated peripheral cavities running through the CA which are ~ 4.5 nm in diameter and are surrounded by domains 2, 4, 5, and 6 present in each subunit (marked by dots in Figure 2). The clusters of domains 5-10 are also referred to as "clamps" while the four copies of domain 3, which form the sides of the CA, have been named the "handles" by the Baylor group. Domain 1 joins the CA to the TA, and is flanked by domains 2 and 3. Although the 3D density maps derived from cryo-EM by image processing method reveal the molecular boundary of the mushroom CA in the tetrameric structure of the receptor, the maps are not of sufficient resolution to depict precisely how the domains form the individual subunits, or to define the folding of the polypeptide chain or the contours of the ion channel within the TA.

The 3D structure discussed so far that appears to be common for all three RyR isoforms is shown in Figure 1 and the detailed features of RyR1 in Figure 2. These 3D maps of RyR relate to the state of channel that was determined under buffer conditions favoring the functional state referred to as "closed". The assumption that this is the non-conducting state was derived from conductance measurements of RyRs reconstituted into lipid bilayers. The structural features of the receptor's

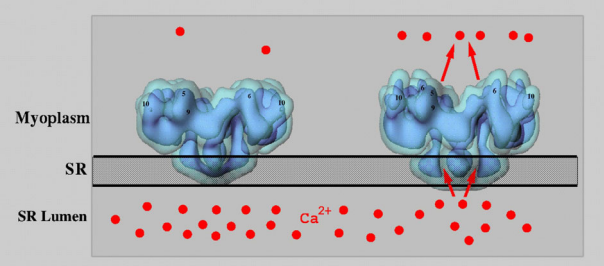

Figure 3. Open (right) and closed (left) states of RyR3. The 3D reconstruction of RyR3 is shown at two density levels (darker blue is the higher level), to better illustrate shifts in density between the two states. Note expansion of the high-density regions in the transmembrane region in going from closed to open, and also the upward movement of domain 6 (for details refer Sharma et al., 1998). Red dots represent calcium ions. In the open state, Ca^{2+} might also emanate from the sides of the receptor near the myoplasmic surface of the SR, in addition to the top of the receptor (indicated by the arrows).

"open" state, that is, the ion-conducting state of RyR, have also been studied by cryo-EM. Visual comparison of the 3D maps reported for open state RyR1 (Baylor group) and open state RyR3 (Albany) shows similar structural conformational changes when compared with the closed state maps for the corresponding receptor isoform. Notable changes in the RyR structure are evident in domains 3, 5, and 6. Domain 6 in open RyR1 and RyR3 was seen to be elongated by about 15 Å as compared its dimensions in the closed state RyR1 and RyR3 (Fig. 3). Density maps of open RyR1 show a weak, almost broken, connection of domains 9 and 10 relative to the closed state appearance, but this difference is less pronounced for RyR3 [29, 34]. Isoform-specific differences such as this might be related to functional differences among the isoforms, such as the involvement of RyR1 (but not RyR3) in skeletal-type E-C coupling (refer to the **Movie** for better appreciation of the conformational changes depicted in the 3D maps of closed and open RyR3).

Transmembrane Assembly

Attempts have been made to elucidate the structure and topology of the TA based on the 3D structure information and on hydropathy profiles. The topology of the transmembrane segments of the receptor has still not been fully elucidated. Whereas there is agreement that most, probably all, of the transmembrane helices occur in the amino-terminal 1/10 – 1/5 of the RyR subunit's sequence, the number (4-12 have been proposed) and locations of the helical segments within the sequence are still debated [54]. In principle, the volume of the TA in the 3D reconstructions should permit an estimation of the number of transmembrane helices that can be packed into it, but volume determinations from cryo-EM reconstructions are prone to error. Uncertainty in volume estimation is partly due to such factors as unknown amounts of receptor-associated detergent or lipid [12,13]. Nevertheless, both the Albany and Baylor reconstructions appear inconsistent with the presence of only four transmembrane helices; rather, they appear capable of accommodating up to 10 such helices per subunit. The mass of the TA, as estimated from cryo-EM 3D maps, is estimated to be 360 kDa (90 kDa per subunit) [30], a figure consistent with that predicted by the most recent experimentally supported topology model [10], which is illustrated in Figure 4 (right-hand panel). Although perhaps this model does not represent the final word, certain aspects of it, such as the even number of transmembrane helices and the fact that these helices include M5, M6, M8, and M10, are strongly supported by work from several laboratories [19, 54]. Sequence homology and site-directed mutagenesis studies implicate helices M8 and M10 together with the intervening membrane-associated loop, as contributing to the formation of the ion-conducting transmembrane pore, similar to the structure found for tetrameric

bacterial K^+ channels [1, 8, 26, 41, 47, 54]. Thus, helices M8 and M10 should be located near the four-fold symmetry axis of the TA (Fig. 4).

Cryo-EM studies from both the Baylor and Albany groups have shown rather extensive conformational differences in the TA region between putatively open and closed states of the RyR [29, 34]. Experimentally, the change in conformation was induced by simply exposing the receptors in buffer, either to 0.1 mM Ca^{2+} and 1mM millimolar ATP or AMP, to favor the open state, or submicromolar Ca^{2+} and absence of nucleotide to favor the closed state. The changes in density shown in Figure 3 provide a speculative model about the structural changes that occur during functioning of the TA (see Figure 3 legend for details). Associated changes in the CA are also seen, particularly in domain 6, which increases its height (by 15 Å) in the open state and perhaps reflecting an alteration of its interaction with dihydropyridine receptor (DHPR). At present, information on such structural details of RyR can only be obtained by cryo-EM.

Mapping of other E-C coupling proteins onto the 3D structure of RyR

The functioning of RyR is subject to exquisite modulation by several allosteric factors, including calcium, magnesium, ATP, and calmodulin (CaM), and FKBP. Some of the binding sites for these ligands, which interact with RyR during E-C coupling, have been localized on the region of CA of RyR's 3D structure [43, 44]. The Albany group has successfully used the 3D cryo-EM technique to map the following ligand binding sites: Imperatoxin A (IpTxA; a putative analogue representing a portion of DHPR that interacts with RyR [22]), CaM, and FKBP (refer to Fig. 4). Knowledge of such specific interactions visualized on 3D density maps should aid in understanding the molecular mechanism of E-C coupling [31, 32, 43, 44].

FKBP12 and FKBP12.6

Two FKBPs, a 12-kDa protein (FKBP12) and one that is slightly larger and 85% identical in sequence (FKBP12.6), have been found to tightly bind RyRs and to modulate their activity [21, 25, 40]. RyR tetramers bind four copies of FKBP12/12.6, one per subunit. The FKBPs can be dissociated quantitatively from the receptors by treatment with immuno-suppressant drugs such as rapamycin or FK506, either in vitro or in intact muscle, with concomitant effects on muscle contraction. RyR1 and RyR3 are capable of binding both FKBP12 and FKBP12.6, whereas RyR2, the cardiac RyR, shows a preference for FKBP12.6 in some species [5, 21, 27]. In one study involving site-directed mutagenesis, three amino acids, Gln31, Asn32, and Phe59, were identified as being involved in the selective binding of FKBP12.6 to RyR2 [51]. For RyR1, and possibly for RyR2, FKBP12/12.6 stabilizes the receptor, preventing aberrant activation of the channel during the resting

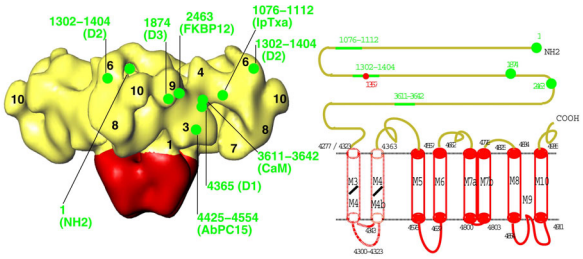

Figure 4. Correlation of RyR1's amino acid sequence with its cryo-EM 3D structure. In both panels, the red color depicts the transmembrane region, while yellow depicts the cytoplasmic assembly, and green dots or sequence segments represent specific regions identified by cryo-EM in the 3D map. Shown on left side is the side view of the 3D map of RyR1 labeled with the position of mapped sequence regions (NH_2 or amino terminus, and divergent regions D1, D2, and D3) and binding sites if ligands (FKBP, CaM, AbPC15, and IpTxa). On the right is a schematic representation of the RyR1 sequence (relevant residues from 1-5037 are labeled in the figure). Shown with the cytosolic N and C terminus are the proposed boundaries for the eight transmembrane helices (shown as red cylinders) organized as four hairpin loops; M3-M4, M5-M6, M7a-M7b, and M8-M10. The trans-membrane topology illustrated is reproduced from Du et al. [10].

phase of contraction. Thus, depletion of FKBP12.6 results in a defective channel. This effect may play a role in one of the molecular mechanisms underlying the aberrant calcium handling associated with heart diseases, including some arrhythmias [46].

Localization of FKBP12 by cryo-EM (Fig. 4) showed its binding site on RyR1 3D map to be adjacent to domain 9, near the joining point to domain 3 [43, 44]. This result was obtained by subtracting a 3D reconstruction of RyR1 treated with FK506 from a 3D map of a RyR1-FKBP12 complex. The difference map so obtained revealed a mass of density that could be attributed unambiguously to FKBP12. A surprising and intriguing finding was that the location of this binding site is approximately 120 Å from the postulated location of the ion-conducting pore at the center of TA (Fig. 4).

The domain 9 region has been shown to contain DR3 [53], and also a region (1815-1855) upstream of DR3 in RyR2, that has been identified as being essential for binding of FKBP12.6 to RyR2. Considering the similarity that is apparent among the 3D structures of all three RyR isoforms, it seems reasonable at this point to assume that homologous regions will map essentially at same locations on all three isoform maps. Thus we may conclude that domain 9 (containing DR3), which also

binds FKBP, is likely to be responsible for stabilizing the conformational changes for RyR [53].

Calmodulin

CaM acts as a Ca^{2+} sensor and signaling protein in virtually all cell types, regulating many cellular proteins such as kinases and ion pumps and channels, including RyRs. CaM partially inhibits all three isoforms of RyR at calcium concentrations above 1 μM but differential effects among the isoforms have been found at submicromolar concentration of calcium [2, 11, 28, 52]. Nevertheless, the significance of RyR-CaM interactions in vivo remains to be determined. For example, a recent mutation study of RyR1 residues 3614-3643, which comprise part of the CaM binding domain, indicated that CaM binding to RyR1 is not essential for voltage-dependent Ca^{2+} release by RyR1 *in situ* [28].

The binding of CaM, a 16-kDa protein, has been mapped onto the surface of RyR1 by applying the difference map technique described above for FKBP12. Both forms of CaM, apo-CaM and Ca^{2+}-CaM, bind to RyR1, and their binding sites have been mapped with slightly different results (Fig. 4). Ca^{2+}-CaM binds to each domain 3 of RyR1, near domains 4, whereas apo-CaM has been localized to the external surface of domain 3 but at a distance of about 35 Å from Ca^{2+}-CaM [32, 44]. Intriguingly, CaM, like FKBP12/12.6, binds far (~100 Å) from the presumed location of the ion-conducting channel.

Dihydropyridine Receptor

During E-C coupling, depolarization of the cell membrane is detected by DHPRs, which are voltage-regulated $Ca2^{+}$ channels that are most abundant in the transverse (T)-tubules, tubular invaginations of the plasma membrane [17]. Direct or indirect interactions between DHPRs and RyRs, in skeletal and cardiac muscle respectively, result in activation of RyR. EM by the freeze-fracture technique has shown that ordered arrays of RyRs and DHPRs are present in skeletal muscle. These junctional arrays have been referred to as calcium release units (CRUs) [4, 15, 17]. DHPR is a heteromer consisting of five different subunits (α1, α2, β, δ, γ) of which the α1 subunit, which is present in heart and skeletal muscle in distinct isoforms, forms the voltage-regulated ion-conducting channel [39]. Recently, the first 3D reconstructions from EM were reported for DHPRs, and there was speculation about how the RyR and DHPR reconstructions could be docked to one another at CRUs [36, 45, 48]. Unfortunately, due to the low affinity between DHPR and RyR1, it has not been possible to apply 3D cryo-EM to this complex and characterize their interaction directly. Efforts to understand the molecular basis for the interaction of RyR with DHPR have employed IpTxa, a 33-residue toxin peptide from scorpion venom that is thought to mimic a region of the DHPR (the II-III loop) that interacts with RyR [20]. IpTxA interacts at nanomolar affinity with RyR1, and activates it. Recent structural and mutagenesis studies show that IpTxA exhibits a functional surface that is based on a short double-stranded antiparallel beta-sheet composed of six essential residues [22].

The location of IpTxA on the 3D structure of RyR1, and hence possibly the location of the II-III loop of DHPR, is shown in Figure 4. The toxin is seen to bind on the surface of RyR1 just below domain 4, near the junction of domain 3 with the clamp assembly domains 7/8 [31]. This location is ~110 Å from the putative ion channel, indicating that IpTxA must act via a mechanism involving long-range conformational changes.

Conclusion

Efforts are ongoing to attain high-resolution structures for the RyRs. Since, however, this goal is proving difficult, we must rely in future on cryo-EM to obtain 3D structural information. It should be appreciated that 3D cryo-EM itself has the potential to achieve levels of resolution well below 10 Å, but due to several characteristics of the specimen, like lack of large variety of views of RyRs due to its behavior on grid, structures of RyR or its isoforms have thus far not been solved to this level of resolution. A reasonable goal that should be achievable at current resolutions is to determine the amino acids that comprise the various domains that have been resolved in RyRs by cryo-EM [32, 42-44]. Already a rough, albeit tentative, model of the positions of the >5,000 amino acid-residues of the primary sequence to the receptor's 3D structure is emerging. Residues 1-1,400 probably contribute to the formation of the clamp regions (domains 5-10). While the sequence from ~3,600-4,400 lies within domain 3 (or the handle). The TA region is composed of the remaining ~ 1000 carboxy-terminal amino acid residues.

Recent studies in our laboratory provide a framework for a methodic, comprehensive approach to relating RyR's sequence and its 3D architecture. Divergent regions were mapped by cryo-EM using a technique that should be generally applicable to the localization of other surface-exposed regions of RyR's sequence [23-24]. This approach involves inserting the sequence of green fluorescent protein into the sequence of RyR and then isolating the chimeric proteins from cell lines that have been programmed to express them. DR2 (region 1342-1403 in RyR1) was identified by this method (unpublished results from Albany group), and the result was verified through the comparison of 3D reconstructions of RyR3, which lacks the DR2 region, and RyR1, which contains it [34].

It is interesting that the locations of DR2 and DR3 and the sites of binding ligands (such as FKBP12, IpTxa, and CaM) identified on the 3D maps are largely confined to the clamp regions (refer to Figure 4). We are also intrigued that conformational changes between the open and closed states of the receptor show major

involvement of domains 5, 6, 9, and 10. It seems clear that the clamps, which form the corners of the square-shaped cytoplasmic region, play an essential role in the macromolecular apparatus that carries out E-C coupling.

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Supplemental material:

Movie of open and closed state of RyR3. The transition between the two states and the concomitant conformation changes can be better appreciated in this movie that provide a glimpse of the rate for one of the several structural changes that occur during E-C coupling mechanism. In BAM On-Line: http://www.bio.unipd.it/bam/bam.html