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Structure of Glutaraldehyde Cross-Linked Ryanodine Receptor

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Abstract

The ryanodine receptor (RyR) family of calcium release channels plays a vital role in excitation-contraction coupling (ECC). Along with the dihydropyridine receptor (DHPR), calsequestrin, and several other smaller regulatory and adaptor proteins, RyRs form a large dynamic complex referred to as ECC machinery. Here we describe a simple cross-linking procedure that can be used to stabilize fragile components of the ECC machinery, for the purpose of structural elucidation by single particle cryo-electron microscopy (cryo-EM). As a model system, the complex of the FK506-binding protein (FKBP12) and RyR1 was used to test the cross-linking protocol. Glutaraldehyde fixation led to complete cross-linking of receptor-bound FKBP12 to RyR1, and also to extensive cross-linking of the four subunits comprising RyR to one another without compromising the RyR1 ultrastructure. FKBP12 cross-linked with RyR1 was visualized in 2D averages by single particle cryo-EM. Comparison of control RyR1 and cross-linked RyR1 3D reconstructions revealed minor conformational changes at the transmembrane assembly and at the cytoplasmic region. Intersubunit cross-linking enhanced [³H]ryanodine binding to RyR1. Based on our findings we propose that intersubunit cross-linking of RyR1 by glutaraldehyde induced RyR1 to adopt an open like conformation.

Keywords

Ryanodine receptor; single-particle cryo-electron microscopy; glutaraldehyde

Introduction

Ryanodine receptors (RyRs) are gigantic homotetrameric calcium release channels located in sarco(endo)plasmic reticulum (SR/ER) (Inui et al., 1986; Lia et al., 1989; Lin et al., 1988). Each RyR subunit consists of a single peptide consisting of ~5,000 amino acids, and four of these subunits assemble to form the calcium release channel, which is ~ 2.3 MDa in mass. Mammals express three different RyR isoforms; RyR1, RyR2 and RyR3, which share ~65% amino acid sequence homology (Hakamata et al., 1992). RyRs play an important role in excitation-contraction coupling (ECC) (Dulhunty, 2006), the process by which an electrical stimulus generated by a neuron triggers muscle contraction (Sandow, 1952). Changes in the plasma membrane electrical potential are detected by the voltage gated L-

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type calcium channel/ dihydropyridine receptor (DHPR) (Rios et al., 1987; Schneider et al., 1973; and Tanabe et al., 1988), which activates RyRs by two distinct mechanisms: by physical coupling of the two proteins as in skeletal muscle or by calcium induced calcium release as in the heart (Klein et al., 1996; Nabauer et al., 1989). RyR opening enables calcium ions that are actively sequestered in SER to diffuse into the cytoplasm. Once in the cytoplasm calcium ions bind to troponin and activate the actin-myosin contraction apparatus, thus triggering muscle contraction.

Although located in separate membranes (SER and plasma membrane) RyR1 and DHPR exist in close proximity and physically interact with each other (Block et al., 1998; Paolini et al., 2004). The RyR and DHPR are the main components of a large and dynamic complex which is sometimes referred to as excitation-contraction machinery, or calcium release unit (Dulhunty, 2006). Many additional proteins that are thought to contribute to the mass of ECC machinery, some by interacting directly with RyR: calmodulin, calsequestrin, FK506 binding proteins (FKBP12 and FKBP12.6), triadin, junctin, homer, sorcin, mitsugumin-29, mitsugumin-33 and others. (Capes et al., 2011; Treves et al., 2009). In addition RyRs are modified posttranslationally by protein kinases and phosphatases including: cAMP-specific phosphodiesterase and Ca^{2+} /calmodulin-dependent protein kinase (Capes et al., 2011). Structural information on the ECC machinery has come from X-ray crystallography and single particle cryo-EM studies of ECC components (Wagenknecht et al., 2010) and electron microscopy of intact muscle (Franzini-Armstrong and Protasi, 1997). Structures of RyR bound to various protein ligands have been solved by single particle cryo-EM. These include: FKBP12, FKBP12.6, calmodulin, chloride intracellular channel 2, natrin toxin, and imperatoxin A (and high affinity anti-RyR polyclonal antibodies (Benacquist et al., 2000; Hung et al., 2012; Meng et al., 2007; Meng et al., 2009; Peralarez-Marin et al., 2012; Samso et al., 1999, Samso et al., 2005; Samso and Wagenknecht, 2002; Sharma et al., 2006; Wagenknecht et al., 1996; Wagenknecht et al., 1997; Zhou et al., 2008). These studies were feasible, in part, because these protein ligands bind tightly to purified RyR. Weakly binding RyR ligands generally do not form sufficiently stable complexes with RyR *in vitro* for study by single particle cryo-EM. Glutaraldehyde has been used to stabilize large fragile cytoplasmic complexes for single particle cryo-EM (Carlo et al., 2010; Lee et al., 2003; Richter et al., 2010; Southworth and Agar, 2011; Yu et al., 2008), and could similarly be used to stabilize RyR or components of ECC. However this approach has not been tried with RyR before.

In this study we describe a cross-linking protocol that can be used to stabilize fragile RyR complexes that is compatible with single particle cryo-EM. The effect of glutaraldehyde on RyR1 structure was characterized biochemically and by 3D cryo-EM. Cross-linking RyR1 with glutaraldehyde preserved the overall morphology of RyR1, but, surprisingly, induced minor conformational changes at the cytoplasmic and transmembrane region that are suggestive of an “open” state for RyR.

2. Materials and methods

2.1. RyR1 Purification

R4 terminal cisternae vesicles (R4TCV) were isolated from flash-frozen New Zealand white rabbit muscle (Pel-Freez Biologicals) using a published protocol (Saito et al., 1984). RyR1 was purified from R4TCV as described previously (Inui et al., 1986; Wagenknecht et al., 1996), with a few minor alterations to the procedure. Briefly, R4TCV (50–40 mg) were diluted to 3.0 mg/ml and solubilized by addition of 10% CHAPS/5% soybean lecithin. The post-solubilization buffer consisted of; 1 M NaCl, 135 mM sucrose, 20 mM Na-MOPS (pH 7.4), 4 % CHAPS, 2 % soybean lecithin, 2 mM DTT and 2 $\mu\text{g}/\text{ml}$ leupeptin. After incubating for 15 min on ice the sample was centrifuged for 30 minutes at 27,300 rpm with a

SW60Ti rotor (Beckman Coulter, Inc) to pellet insoluble material. The supernatants were loaded on 10–20% (w/v) sucrose gradients and centrifuged for 24 hours at 20,000 rpm in a SW28Ti rotor (Beckman Coulter, Inc). Sucrose gradients were fractionated and the RyR1 peak was detected colorimetrically using Bio-Rad protein assay dye (Bio-Rad). Fractions containing RyR1 were pooled and passed through a Sephadex G25 fine (GE HealthCare Life Sciences) column, equilibrated with desalting column buffer (100 mM NaCl, 20 mM Na-MOPS (pH 7.4), 1.0 % CHAPS/0.5 % soybean lecithin, 2 mM DTT and 2 µg/ml leupeptin). The RyR sample was concentrated on a heparin agarose (Bio-Rad) column, (and eluted in the following buffer; 1 M KCl, 20 mM Na-MOPS (pH 7.4), 0.5% CHAPS, 2 mM DTT and 2 µg/ml leupeptin). Aliquots (10–50 µl) of RyR1 were flash frozen in liquid N₂ stored at –80°C. Total protein concentration of purified RyR1 was determined by Bradford assay (Bradford, 1976). Purity of the RyR1 was assessed by SDS-PAGE (Fig S1) and [³H]ryanodine binding was performed as an indicator of functional RyRs.

2.2. [³H]Ryanodine Binding Assay

[³H]Ryanodine was purchased from PerkinElmer, and the binding assay was carried out using a previously published protocol (Hawkes et al., 1989) with minor modifications. Purified RyR1 (4–5 µg) was incubated in 300 µl of total binding solution (1M NaCl, 50 mM Na-MOPS (pH 7.4), 0.5 % CHAPS/0.25% soy bean lecithin, 6 mM ATP, 0.52 mM CaCl₂, 0.1 mg/ml BSA, 20 nM [³H]-ryanodine) for 2 hours at 25 °C. A 1000 fold molar excess of cold ryanodine was added to determine nonspecific binding. Samples were filtered through Whatman GF/B filters presoaked with 0.5 % polyethylenimine, then dried overnight on a hot plate. Filters were submerged in 5 ml of EcoLume scintillation cocktail and counted in a Beckman scintillation counter.

2.3 Chemical Cross-Linking

EM grade glutaraldehyde (Polysciences, Inc) was diluted in ddH₂O immediately before cross-linking samples, solutions of glutaraldehyde was stored at 4°C or kept on ice during an experiment. Unless otherwise specified, RyR1 (~0.1 mg/ml final concentration) was chemically cross-linked with 0.04 % glutaraldehyde for 20 minutes on ice. The cross-linking reaction was quenched by addition of a 20-fold molar excess of glycine (pH 7.4), and then [³H]ryanodine binding was determined.

2.4 SDS-PAGE

RyR1 was treated with Laemmli buffer (final concentration; 75 mM Tris-HCl (pH 6.8), 2 % SDS, 10 % glycerol, 5 % β-mercaptoethanol and 0.1 % bromophenol blue) for 1 hour at room temperature before SDS-PAGE using 4–20 % precast polyacrylamide gels (Bio-Rad). Gels were stained overnight with Coomassie brilliant blue R-250, destained (with 40% methanol and 10% acetic acid solution), and washed with dH₂O. Recombinant human His-FKBP12 (BPS Bioscience) was visualized by His-tag in-gel staining (Invitrogen), according to manufacturer's instructions. Optimal in-gel His-tag staining was obtained after overnight fixation (in 40% methanol and 10% acetic acid), and after washing the gels 5–8 times with 20 mM phosphate buffer (pH 7.8), before imaging with a Typhoon 9400 LASER based scanner (GE Healthcare).

2.4 Cryo-Specimen Preparation

RyR1 was diluted in buffer A (350 mM KCl, 23 mM Na-MOPS (pH 7.4), 4 mM EGTA, 0.6% CHAPS, 12 mM DTT and 2 µg leupeptin), and cross-linked with glutaraldehyde. Final concentration of sample just before cryo-fixation was: 0.09 mg/ml RyR1, 400 mM KCl, 20 mM Na-MOPS (pH 7.4), 3.0 mM EGTA, 0.50 % CHAPS, 10 mM DTT, 0.04 %

glutaraldehyde and 2 μ g leupeptin. Non-cross-linked control RyR1 cryo-grids were prepared with similar buffer but omitting glutaraldehyde.

Recombinant human His-tagged FKBP12 (His-FKBP12) (BPS Bioscience) was dialyzed against 200 ml of dialysis buffer (150 mM NaCl, 20 mM Na-MOPS (pH 7.4), 10 % glycerol, 1 mM EGTA, 1 mM EDTA, 2 mM DTT and 2 μ g/ml leupeptin) first for 2 hours at 4°C then again with fresh dialysis buffer for 16 hours using a Slide-A-Lyzer MINI dialysis kit (Thermo Scientific). Dialyzed His-FKBP12 sample was divided into 10 μ l aliquots, flash frozen in liquid N₂ and stored at -80 °C. We found that dialyzing His-FKBP12 sample reduced clumping and formation of irregular shaped His-FKBP12-RyR1 particles, based on comparison of electron micrographs of cross-linked and non-cross-linked sample (data not shown). To promote formation of His-FKBP12:RyR1 complex a 22-fold molar excess of His-FKBP12 was added to RyR1 in buffer A and allowed to incubate at room temperature for 20 minutes. Tubes containing His-FKBP12:RyR1 or RyR1 were transferred onto ice and cross-linked with glutaraldehyde prior to cryo-grid preparation.

Cryo-grids were prepared in an environmentally controlled cold room (the temperature and humidity were maintained at 4°C and 85%) according to a published protocol (Grassucci et al., 2007). Thin carbon films were evaporated onto freshly cleaved mica (SPI) using a Baltec Med 020 evaporator, then floated onto ddH₂O and transferred onto homemade holey grids, which were allowed to air dry for ~16–24 hours. Cryo-grids were prepared using a custom made plunger apparatus (Grassucci et al. 2007) by applying 4 μ l of sample to the thin carbon-bearing surface of a glow discharged grid using a micropipette. Excess buffer was blotted away from the grids with Whatman #1 filter paper for 5–6 seconds, and then the grids were quickly plunged into liquid ethane and stored in liquid N₂.

2.5 Cryo-Electron Microscopy

Cryo-grids were transferred into a Oxford CT 3500 cryo-transfer holder (Gatan, Inc) and imaged with an FEI Tecnai F20 transmission electron microscope (TEM) operated at 200 kV as described (Meng et al., 2007). Underfocused images (between 2 and 5 μ m) were taken at 50,000 X magnification with a low-dose setup (15–20 e⁻/Å²) using the Serial EM software package (Mastronarde et al., 2005). Since RyR1 particles show a strongly preferred orientation when adsorbed to a thin carbon support film (Radermacher et al., 1994), the specimen grid was tilted to 10, 20, 30 and 40 degrees to obtain different views.

2.6. Image processing

TEM Images recorded with a 4K × 4K CCD camera were processed using SPIDER and WEB software packages (Frank et al., 1996). The projection matching method was used to generate 3D reconstructions as described previously (Liu et al., 2001; Penczek et al., 1994). A power spectrum of each CCD image was examined in WEB, and poor quality images that showed signs of drift and or astigmatism were deleted. The contrast transfer function of the TEM was corrected by phase flipping as described (Zhou et al., 2008). Windowed particles were aligned to a set of 48 reference projections generated by backprojecting a previously published structure of RyR1 (accession number EMD 5014), prepared in absence of calcium (closed conformational state) and suspended in vitreous ice over holes (Samso et al., 2005). Aligned particles were screened manually in WEB, before running iterative refinement. Untilted images taken at a similar defocus range (+/- 100 nm) were used to generate 2D averages of “face view” particles (those particles that show a distinct square shape due to being oriented with their 4-fold axes perpendicular to the carbon substrate). Control RyR1 and glutaraldehyde cross-linked RyR1 3D reconstructions were made by averaging 4,048 and 4,567 particles. Nominal resolution was estimated at 32 and 31 Å according to Fourier Shell Correlation (FSC) = 0.5 criteria (Malhotra et al., 1998). Four-fold symmetry was

imposed on final 3D reconstructions, which were low-passed filtered to 32 Å before generating difference maps. Volumes and difference maps were visualized with the UCSF Chimera package (Pettersen et al., 2004). The contour level of isosurface renderings was chosen according to the profile of the density histogram of the reconstructions, the threshold being set midway between the peak corresponding to vitreous ice and that of protein, as discussed previously (Frank, 2006).

3. Results

3.1. Cross-Linking His-FKBP12 to RyR1

We sought to develop a gentle cross-linking procedure that can be used to cross-link components of ECC machinery to RyR1 for structural analysis by cryo-EM. Two important criteria to be fulfilled are: (1) high degree of cross-linking between RyR1 and target peptide, and (2) cross-linking must preserve ultrastructure of RyR1.

We used the FKBP12:RyR1 complex as a model system to test the cross-linking procedure, since the structure of FKBP12:RyR1 complex has been solved by single particle cryo-EM and the interaction between FKBP12 (Samso et al., 2005; Samso et al., 2009; Sharma et al., 2006; Wagenknecht et al., 1996; Wagenknecht et al., 1997) and RyR1 has been characterized *in vitro* (50–52). To find optimal cross-linking conditions we performed cross-linking time series experiments on RyR1:His-FKBP12 complexes (Fig. 1A). According to SDS PAGE, samples treated with glutaraldehyde for two minutes or longer contain a high molecular weight species at the top of the gel (Fig. 1A). This band contains His-FKBP12 as assessed by its fluorescence intensity following staining with a poly-His-specific dye, and the position of this band is comparable to that obtained for RyR1 cross-linked alone (Fig. 1C). The fluorescence intensity of this band reaches its peak after 20 minutes of fixation (Fig. 1A and 1B), indicating that 20–60 minutes are sufficient for efficient cross-linking between His-FKBP12 and RyR1. In addition, extensive cross-linking between monomeric RyR1 subunits occurred after exposure to 0.04 % glutaraldehyde (Fig. 1C), and RyR1 subunits (~565 kDa in mass) were not detected by SDS-PAGE. Although the exact molecular weights of the bands located at the tops of the gels (above the 209-kDa myosin molecular weight standard) cannot be determined, they most likely correspond to tetrameric RyR1 (four RyR1 monomers covalently cross-linked together) or tetrameric RyR1 cross-linked to His-FKBP12 (Fig. 1A and 1C). However, based on results of SDS-PAGE experiments alone, the possibility that glutaraldehyde fixation led to formation of some cross-linked dimers (~1.1 MDa) or trimers (~1.7 MDa) of RyR1 subunits or oligomers of RyR1 tetramers (>2.3 MDa) cannot be excluded. Electron microscopy, discussed below, indicates that the high molecular weight band represents primarily tetramers of RyR1 with or without His-FKBP12.

[³H]Ryanodine binding assays were carried out to investigate the effect that glutaraldehyde chemical cross-linking has on the function of RyR1. Glutaraldehyde chemical cross-linking enhanced [³H]ryanodine binding to purified RyR1 by ~30% (glutaraldehyde treated RyR bound 29.0 ± 3.03 pmol/mg, the control RyR1 sample bound 21.8 ± 1.58 pmol/mg, P-value = 0.035) which indicates that RyR1 retains some function following chemical cross-linking, and perhaps that glutaraldehyde promotes an open state for RyR1, since it is generally accepted that ryanodine binds to RyR1 in the open, but not the closed state.

3.2. Effect of Glutaraldehyde on RyR1 Structure

Single particle cryo-EM was used to visualize the 3D structure of glutaraldehyde cross-linked RyR1. Typical cryo-EM images of untilted specimen (Fig. 2) showed mainly square shaped RyR1 particles, and the gross morphology of the particles seemed unaffected by the glutaraldehyde treatment. Little difference in the degree of particle aggregation was seen in

cross-linked versus control samples, which indicates that glutaraldehyde cross-linking does not damage ultrastructure of RyR1 or promote cross-linking between multiple RyR1 tetramers. Cross-linked and non-cross-linked RyR1 face-view averages were compared to each other by computing a difference map (Fig. 3A–D). Although the averaged images appeared nearly identical, the difference map revealed subtle positive and negative densities at positions within the cytoplasmic and transmembrane assembly. This suggests that glutaraldehyde chemical cross-linking changes the conformation of RyR1, but the overall architecture remains the same. Strong features in the 2D difference map correlate well with the 3D difference map discussed below.

Single-particle cryo-EM was also used to determine the location of His-FKBP12 cross-linked to RyR1. Averages of cross-linked RyR1 face-view particles, with and without added His-FKBP12, were generated and compared to each other by difference mapping (Fig 3E–H). The difference map made by subtracting the glutaraldehyde cross-linked His-FKBP12-RyR1 average from the glutaraldehyde cross-linked RyR1 average contained a region of positive density between cytoplasmic domains 9, 5 and 3 (Fig 3G and 3H). This extra mass, attributed to His-FKBP12, is located at the known FKBP12 binding site (Samso et al., 2005; Samso et al., 2009; Sharma et al., 2006; Wagenknecht et al., 1996; Wagenknecht et al., 1997).

Three-dimensional reconstructions of glutaraldehyde cross-linked RyR1 and non-cross-linked control RyR1 were generated (Fig 4) by single particle cryo-EM. The morphology of the control RyR1 structure is similar to that of the cross-linked reconstruction. Careful comparison of both volumes (Fig 4 and movie S2) revealed the following structural differences: (1) a “plug” is present in the transmembrane region of the control but not in glutaraldehyde cross-linked RyR1, (2) the cross-linked structure has a bridge connecting domains 7 and 11, and (3) cytoplasmic domain 6 becomes more extended and appears closer to domain 5 in the glutaraldehyde cross-linked 3D reconstruction. These structural differences between RyR1 and glutaraldehyde cross-linked RyR1 are likely genuine because the difference volumes were not overtly sensitive to moderate changes in contour levels, and the bridge between domains 7–11 and movement of domain 6 correspond to statistically significant regions of the 2D difference map (Fig. 3A). The bridge connecting domains 7 and 11 has not been reported previously in published structures of RyR which include all three isoforms of RyR, RyR bound to various regulatory proteins, RyR trapped in open or closed functional states and RyR determined at different resolutions (Hung et al., 2012; Liu et al., 2001; Meng et al., 2007; Meng et al., 2009; Orlova et al., 1996; Peralarez-Marín et al., 2012; Radermacher et al., 1994; Samso et al., 1999; Samso et al., 2005; Samso et al., 2006; Samso et al., 2009; Samso and Wagenknecht, 2002; Serysheva et al., 1999; Sharma et al., 2000; Sharma et al., 2006; Wagenknecht et al., 1996; Wagenknecht et al., 1997; Zhou et al., 2008).

4. Discussion

4.1. Effect of glutaraldehyde on RyR1 Structure

For the purpose of stabilizing labile RyR complexes we sought to attain (1) high cross-linking efficiency between target peptide and RyR and (2) preservation of RyR1 structure. We have found that cross-linking with 0.04% glutaraldehyde for 20 minutes on ice led to extensive inter-subunit crosslinking of RyR1 as well as quantitative cross-linking of the RyR1 ligand His-FKBP12 to RyR1 (Fig 1). Further, cross-linking did not appear to foster aggregation of RyR1 particles, as judged by examination of cryo-TEM images (Fig 2). Glutaraldehyde is among the most general of protein cross-linking agents, and the conditions that we have used here will be applicable in future studies of other RyR:ligand complexes.

Previous cross-linking studies which used different cross-linking procedures (pH, temperature, concentration of detergent, ionic strength, and omission of DTT) reported that RyR1 was completely (as assessed by the absence of monomeric RyR1 subunits by SDS-PAGE) cross-linked after treating purified RyR1 with either 0.1 % glutaraldehyde for 60 minutes at 23 °C or with 0.005% glutaraldehyde for 15 minutes at 37 °C. The buffer conditions described previously (Lia et al., 1989; Shoshan-Barmatz et al., 1995) are not optimal for structural studies by cryo-EM, and hence we sought to develop a cross-linking protocol *de novo*.

We found that glutaraldehyde moderately enhanced [³H]ryanodine binding to purified RyR1, which suggests that covalent cross-linking of RyR1 promotes channel activation. A previous study reported that chemically cross-linking RyR1 using glutaraldehyde did not inhibit ryanodine binding to a high affinity binding site, but did inhibit ryanodine binding to the low affinity binding site (Shoshan-Barmatz et al., 1995). These authors used a much lower concentration of glutaraldehyde and different buffer conditions than we used, which may explain why no significant increase in ryanodine binding was reported. However in both cases glutaraldehyde was found to not greatly affect ryanodine binding to RyR1.

Glutaraldehyde chemical cross-linking did not appear to damage the ultrastructure of RyR1 or His-FKBP12-RyR1 complex. The overall morphology of the cross-linked RyR1 was similar to that of the non-cross-linked control (Fig. 2A–2D). Comparison between the 3D reconstruction of glutaraldehyde cross-linked RyR1 and the control RyR1 reconstruction revealed structural differences at the cytoplasmic and transmembrane regions. The conformational changes (Fig. 4) included: (1) reduced density along the ion channel at the center of the TM region, (2) formation of a bridge between domains 7 and 11 and (3) an upward extension and radial movement of cytoplasmic domain 6 toward the center of the channel and to domain 5.

4.2. Are the glutaraldehyde induced structural changes related to natural gating mechanism of RyR?

Except for the bridge between domains 7 and 11, similar conformational changes from the closed state have been reported for 3D reconstructions of putatively open states of RyR1 (Orlova et al., 1996; Samso et al., 2009; Serysheva et al., 1999) as well as RyR2 (Sharma et al., 2006) and RyR3 (Sharma et al., 2000); as discussed above, an open state is consistent with the enhanced ryanodine binding observed for cross-linked RyR1. We speculate that tethering of domain 7 to 11 by glutaraldehyde forces the transmembrane assembly to adopt an open-like conformation, thereby activating the ion channel. In the native structure of RyR1, in the absence of glutaraldehyde, these domains are well separated from each other. Domain 11 is situated on top of the transmembrane assembly, and so tethering of domain 11 to domain 7 by glutaraldehyde could plausibly be coupled to conformational changes affecting the channel gate located in the transmembrane assembly and vice-versa.

RyR is sensitive to the redox state of its environment: reactive oxygen and nitrogen compounds have been shown to activate and inhibit RyR (Lanner et al., 2010; Turan and Vassort, 2011). Previous studies (Aghdasi et al., 1997; Wu et al., 1997) reported that diamide treatment induced cross-linking of RyR1 subunits (both intra and inter-molecularly) and increased [³H]ryanodine binding to SER vesicles and the open probability of RyR1 in lipid bilayers. It has been proposed that oxidation of reactive cysteines activates RyR1 by formation of disulfide bonds between RyR1 subunits, which alters the structure of RyR1 (Han et al., 2006).

However, electron microscopy by negative staining showed that purified RyR1 (but presumably not RyR1 in a membrane bilayer environment) underwent profound

morphological changes, probably not reflective of a native state (e.g., fourfold symmetry was disrupted) if the purification was performed in the absence of DTT and under oxidizing conditions; interestingly, addition of DTT to such receptors restored their characteristic “normal” morphology (Han et al., 2006). For this study we found that glutaraldehyde cross-linking appears to promote activation of RyR1 and altered the structure of cytoplasmic and transmembrane assemblies, but the symmetry remained intact after glutaraldehyde cross-linking, unlike the result when sulfhydryls are oxidized. The observation that covalent cross-linking between RyR subunits (mediated by either glutaraldehyde or by sulfhydryl oxidizing reagents) leads to activation may reflect an underlying mechanism inherent in RyR gating, not merely an effect of chemical cross-linking reagents.

4.3. Visualizing core components of ECC machinery stabilized by glutaraldehyde by single particle cryo-EM

Typically specimens are prepared for single particle cryo-EM without any chemical fixatives (Wang et al., 2006). However, within in the last few years several groups have used chemical fixatives (mainly glutaraldehyde) to stabilize large macromolecular complexes and determine their structures by single particle cryo-EM (Carlo et al., 2010; Lee et al., 2003; Richter et al., 2010; Southworth and Agar, 2011). In particular the “GraFix” (Kastner et al., 2008) procedure has proved useful for preparing various cytoplasmic complexes for cryo-EM including the ribosome, spliceosomes and anaphase promoting complex. Combining chemical cross-linking and cryo-EM may prove a useful method for solving structures of novel RyR-ECC protein complexes.

To determine if such an approach is practical we used the His-FKBP12:RyR1 complex as a model system. Efficient glutaraldehyde cross-linking was shown biochemically, and cryo-EM showed that the location of His-FKBP12 cross-linked to RyR1 was identical to the known FKBP12 binding site. Thus by proof of principle we demonstrate the utility of using single particle cryo-EM to study the structure of RyR1 cross-linked to components of the ECC machinery.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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References

- Aghdasi B, Zhang J, Wu Y, Reid MB, Hamilton SL. Multiple classes of sulfhydryls modulate the skeletal muscle Ca^{2+} release channel. *J Biol Chem.* 1997; 272:3739–3748. [PubMed: 9013631]
- Benacquista BL, Sharma MR, Samso M, Zorzato F, Treves S, et al. Amino acid residues 4425–4621 localized on the three-dimensional structure of the skeletal muscle ryanodine receptor. *Biophys J.* 2000; 78:1349–1358. [PubMed: 10692321]
- Block BA, Imagawa T, Campbell KP, Franzini-Armstrong C. Structural evidence for direct interaction between the molecular components of the transverse tubule/ sarcoplasmic reticulum junction in skeletal muscle. *J Cell Biol.* 1988; 107:2587–2600. [PubMed: 2849609]
- Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem.* 1976; 72:248–54. [PubMed: 942051]

- Capes ME, Loziza R, Valdivia HH. Ryanodine receptors. *Skeletal Muscle*. 2011; 1:18–31. [PubMed: 21798098]
- Carlo SD, Lin S, Taatjes DJ, Hoenger A. Molecular basis of transcription initiation in Archaea. *Transcription*. 2010; 1:103–111. [PubMed: 21326901]
- Dulhunty AF. Excitation-contraction coupling from the 1950s into the new millennium. *Clin Exp Pharmacol Physiol*. 2006; 33:763–772. [PubMed: 16922804]
- Frank, J. Three-dimensional electron microscopy of macromolecular assemblies. 2. Oxford University Press; 2006.
- Frank J, Radermacher M, Penczek P, Zhu J, Li Yanghong, et al. SPIDER and WEB: processing and visualization of images in 3D electron microscopy and related fields. *J Struct Biol*. 1996; 116:190–199. [PubMed: 8742743]
- Franzini-Armstrong C, Protasi F. Ryanodine receptors of striated muscles: a complex channel capable of multiple interactions. *Physiol Rev*. 1997; 77:699–720. [PubMed: 9234963]
- Grassucci RA, Taylor DJ, Frank J. Preparation of macromolecular complexes for cryo-electron microscopy. *Nat Protoc*. 2007; 2:3239–3246. [PubMed: 18079724]
- Hakamata Y, Nakai J, Takeshima H, Imoto K. Primary structure and distribution of a novel ryanodine receptor/calcium release channel from rabbit brain. *FEBS Lett*. 1992; 312:229–235. [PubMed: 1330694]
- Han H, Wei R, Lai AF, Yin C. Molecular nature of sulfhydryl modification by hydrogen peroxide on type 1 ryanodine receptor. *Acta Pharmacol Sin*. 2006; 27:888–894. [PubMed: 16787573]
- Hawkes JM, Diaz-Munoz M, Hamilton SL. A procedure for purification of the ryanodine receptor from skeletal muscle. *Membr Biochem*. 1989; 8:133–145. [PubMed: 2641949]
- Hung X, Fruen B, Farrington DT, Wagenknecht T, Liu Z. Calmodulin binding locations on the skeletal and cardiac ryanodine receptors. *J Biol Chem*. 2012; 287:30328–30335. [PubMed: 22773841]
- Inui M, Saito A, Fleischer S. Purification of the ryanodine receptor and identity with feet structures of junctional terminal cisternae of sarcoplasmic reticulum from fast skeletal muscle. *J Biol Chem*. 1986; 262:1740–1747. [PubMed: 3805051]
- Jones J, Reynolds DF, Lia AF, Blaney LM. Ryanodine receptor binding to FKBP12 is modulated by channel activation state. *J Cell Sci*. 2005; 118:4613–4619. [PubMed: 16176935]
- Kastner B, Fischer N, Golas MM, Sander B, Dube P, et al. GraFix: sample preparation for single-particle electron cryomicroscopy. *Nat Methods*. 2008; 5:53–55. [PubMed: 18157137]
- Klein MG, Cheng H, Santana LF, Jiang YH, Lederer WJ, et al. Two mechanisms of quantized calcium release in skeletal muscle. *Nature*. 1996; 379:455–458. [PubMed: 8559251]
- Lanner JT, Georgiou DK, Joshi AD, Hamilton LS. Ryanodine receptors: structure, expression, molecular details, and function in calcium release. *Cold Spring Harb Perspect Biol*. 2010; 2:003996.
- Lai AF, Misra M, Xu L, Smith AH, Meissner G. The ryanodine receptor-Ca²⁺ release channel complex of skeletal muscle sarcoplasmic reticulum. *J Biol Chem*. 1989; 264:16776–16785. [PubMed: 2550460]
- Lee S, Sowa ME, Watanabe Y, Sigler PB, Chui W, et al. The structure of ClpB: A molecular chaperone that rescues proteins from an aggregated state. *Cell*. 2003; 115:229–240. [PubMed: 14567920]
- Lin H, Inui M, Fleischer S, Schindler H. Purified ryanodine receptor of skeletal muscle sarcoplasmic reticulum form Ca²⁺-activated oligomeric Ca²⁺ channels in planar bilayers. *Proc Natl Acad Sci U S A*. 1988; 85:441–445. [PubMed: 2448775]
- Liu Z, Zhang J, Sharma MR, Li P, Chen SRW, et al. Three-dimensional reconstruction of the recombinant type 3 ryanodine receptor and localization of its amino terminus. *Proc Natl Acad Sci*. 2001; 98:6104–6109. [PubMed: 11353864]
- Macrill JJ, O'Driscoll S, Lai AF, McCarthy TV. Analysis of type 1 ryanodine receptor-12 kDa FK506-binding protein interaction. *Biochem Biophys Res Commun*. 2001; 285:52–57. [PubMed: 11437371]
- Malhotra A, Penczek P, Agrawal RK, Gabashvili IS, Grassucci RA, et al. Escherichia coli 70 S ribosome at 15 Å. *J Mol Biol*. 1998; 286:1285–1291.

- Mastronarde DN. Automated electron microscope tomography using robust prediction of specimen movements. *J Struct Biol.* 2005; 152:36–51. [PubMed: 16182563]
- Meng X, Wang G, Viero C, Wang Q, Mi W, et al. CLIC2-RyR1 interaction and structural characterization by cryo-electron microscopy. *J Mol Biol.* 2009; 387:320–334. [PubMed: 19356589]
- Meng X, Xiao B, Cai S, Huang X, Li F, et al. Three-dimensional localization of serine 2808, a phosphorylation site in cardiac ryanodine receptor. *J Biol Chem.* 2007; 282:25929–25939. [PubMed: 17606610]
- Nabauer M, Callewaert G, Cleemann L, Morad M. Regulation of calcium release is gated by calcium current, not gating charge, in cardiac myocytes. *Science.* 1989; 244:800–803. [PubMed: 2543067]
- Orlova EV, Serysheva II, van Heel M, Hamilton SL, Chiu W. Two structural configurations of the skeletal muscle calcium release channel. *Nat Struct Biol.* 1996; 3:547–552. [PubMed: 8646541]
- Paolini C, Fessenden JD, Pessah IN, Franzini-Armstrong. Evidence for conformational coupling between two calcium channels. *Proc Natl Acad Sci U S A.* 2004; 101:12748–12752. [PubMed: 15310845]
- Penczek PA, Grassucci RA, Frank J. The ribosome at improved resolution: new techniques for merging and orientation refinement in 3D cryo-electron microscopy of biological particles. *Ultramicroscopy.* 1994; 53:251–270. [PubMed: 8160308]
- Peralarez-Marin A, Tae H, Board PG, Casarotto MG, Dulhunty AF, et al. 3D mapping of the SPRY2 domain of ryanodine receptor 1 by single-particle cryo-EM. *PLoS One.* 2012; 6:e25813.
- Pettersen EF, Goddard TD, Huang CC, Couch GS, Greenblatt DM, et al. UCSF Chimera--a visualization system for exploratory research analysis. *J Comput Chem.* 2004; 25:1605–1612. [PubMed: 15264254]
- Radermacher M, Rao V, Grassucci R, Frank J, Timmerman AP, et al. Cryo-electron microscopy and three-dimensional reconstruction of the calcium release channel/ryanodine receptor from skeletal muscle. *J Cell Biol.* 1994; 127:411–423. [PubMed: 7929585]
- Richter FM, Sander B, Golas MM, Stark H, Urlaub H. Merging molecular electron microscopy and mass spectrometry by carbon film-assisted endoprotease digestion. *Mol Cell Proteomics.* 2010; 9:1729–1741. [PubMed: 20530635]
- Rios E, Brum G. Involvement of dihydropyridine receptors in excitation-contraction coupling in skeletal muscle. *Nature.* 1987; 325:717–720. [PubMed: 2434854]
- Saito A, Seiler S, Chu A, Fleischer S. Preparation and morphology of sarcoplasmic reticulum terminal cisternae from rabbit skeletal muscle. *J Cell Biol.* 1984; 99:875–885. [PubMed: 6147356]
- Samso M, Feng W, Pessah IN, Allen PD. Coordinated movement of cytoplasmic and transmembrane domains of RyR1 upon gating. *PLoS Biol.* 2009; 7:e85. [PubMed: 19402748]
- Samso M, Shen X, Allen PD. Structural characterization of the RyR1- FKBP12 interaction. *J Mol Biol.* 2006; 356:917–927. [PubMed: 16405911]
- Samso M, Trujillo R, Gurrola GB, Valdivia HH, Wagenknecht T. Three-dimensional location of the imperatoxin A binding site on the ryanodine receptor. *J Cell Biol.* 1999; 146:493–499. [PubMed: 10427100]
- Samso M, Wagenknecht T. Apocalmodulin and Ca^{2+} calmodulin bind to neighboring locations on the ryanodine receptor. *J Biol Chem.* 2002; 277:1349–1353. [PubMed: 11694536]
- Samso M, Wagenknecht T, Allen PD. Internal structure and visualization of transmembrane domains of the RyR1 calcium release channel by cryo-EM. *Nat Struct Mol Biol.* 2005; 12:539–544. [PubMed: 15908964]
- Sandow A. Excitation-contraction coupling in muscular response. *Yale J Biol Med.* 1952; 25:176–201. [PubMed: 13015950]
- Schneider MF, Chandler WK. Voltage dependent charge movement in skeletal muscle: a possible step in excitation-contraction coupling. *Nature.* 1973; 242:244–246. [PubMed: 4540479]
- Serysheva II, Schatz M, van Heel M, Chu W, Hamilton SL. Structure of the Skeletal Muscle Calcium Release Channel Activated with Ca^{2+} and AMP-PCP. *Biophysical J.* 1999; 77:1936–1944.
- Sharma MR, Jeyakumar LH, Fleischer S, Wagenknecht T. Three-dimensional structure of ryanodine receptor isoforms three in two conformational states as visualized by cryo-electron microscopy. *J Biol Chem.* 2000; 275:9485–9491. [PubMed: 10734096]

- Sharma MR, Jeyakumar LH, Fleischer S, Wagenknecht T. Three-dimensional visualization of FKBP12.6 binding to an open conformation of cardiac ryanodine receptor. *Biophys J*. 2006; 90:164–172. [PubMed: 16214874]
- Shoshan-Barmatz V, Hadad-Halfon N, Ostersehter O. Cross-linking of the ryanodine receptor/ Ca^{2+} release channel from skeletal muscle. *Biochim Biophys Acta*. 1995; 1237:151–161. [PubMed: 7632708]
- Southworth DR, Agard DA. Client-loading conformation of the Hsp90 molecular chaperone revealed in the cryo-EM structure of human Hsp90: Hop complex. *Mol Cell*. 2011; 42:771–781. [PubMed: 21700222]
- Tanabe T, Beam KG, Powell JA, Numa S. Restoration of excitation-contraction coupling and slow calcium current in dysgenic muscle by dihydropyridine receptor complementary DNA. *Nature*. 1988; 336:134–139. [PubMed: 2903448]
- Treves S, Vukcevic M, Maj M, Thurnheer R, Mosca B, et al. Minor sarcoplasmic reticulum membrane components that modulate excitation-contraction coupling in striated muscle. *J Physiol*. 2009; 587:3071–3079. [PubMed: 19403606]
- Turan B, Vassort G. Ryanodine receptor: a new therapeutic target to control diabetic cardiomyopathy. *Antioxid Redox Signal*. 2011; 15:1847–1861. [PubMed: 21091075]
- Wagenknecht T, Grassucci R, Berkowitz J, Wiederrecht GJ, Xin H, et al. Cryoelectron microscopy resolves FK506-binding protein sties on the skeletal muscle ryanodine receptor. *Biophys J*. 1996; 70:1709–1715. [PubMed: 8785329]
- Wagenknecht T, Liu Z. Electron microscopy of ryanodine receptors. *Curr Top Membr*. 2010; 66:27–47. [PubMed: 22353475]
- Wagenknecht T, Radermacher M, Grassucci R, Berkowitz J, Xin H, et al. Locations of calmodulin and fk506-binding protein on the three-dimensional architecture of the skeletal muscle ryanodine receptor. *J Biol Chem*. 1997; 272:32463–32471. [PubMed: 9405457]
- Wang L, Sigworth FJ. Cryo-EM and single particles. *Physiology*. 2006; 21:13–18. [PubMed: 16443818]
- Wu Y, Aghdasi B, Dou SJ, Zhang J, Liu SQ, et al. Functional interactions between cytoplasmic domains of the skeletal muscle Ca^{2+} release channel. *J Biol Chem*. 1997; 272:25051–25061. [PubMed: 9312113]
- Xin H, Timerman AP, Onoue H, Wiederrecht GJ, Fleischer S. Affinity purification of the ryanodine receptor/calcium release channel from fast twitch skeletal muscle based on its tight association with FKBP12. *Biochem Biophys Res Commun*. 1995; 214:263–270. [PubMed: 7669046]
- Yu Z, Gonciarz MD, Sundquist WI, Hill CP, Jensen GJ. Cryo-EM structure of dodecameric Vps4p and its 2:1 complex with Vta1p. *J Mol Biol*. 2008; 377:364–377. [PubMed: 18280501]
- Zhou Q, Wang QL, Meng X, Shu Y, Jiang T, et al. Structural and functional characterization of ryanodine receptor-natratin toxin interaction. *Biophys J*. 2008; 95:4289–4299. [PubMed: 18658224]

Fig 1. SDS-PAGE of glutaraldehyde cross-linked RyR1 and RyR1:His-FKBP12. (A) Time course of His-FKBP12 cross-linking to RyR1. Reaction solutions were prepared as described in Experimental Procedures and cross-linked with 0.4 % glutaraldehyde on ice for the indicated times. Cross-linking was terminated by addition of Laemmli buffer, and the gel was stained with a His-tag specific dye to reveal the presence of the His-FKBP12. His-tagged molecular weight protein standards indicated on gel. (B) Data were pooled from 3 independent cross-linking experiments. Error bars represent standard deviation, P-value of Students unpaired T-test calculated relative to zero time point * P < 0.05, ** P < 0.01. C, SDS-PAGE of RyR1 alone cross-linked for 20 min as in (A) and stained with Coomassie Blue. Subunits (565

kDa) of RyR1 migrate as a single band just above the 210 kDa myosin prestained molecular standard, whereas as the cross-linked RyR1 barely enters the top of the gel (as indicated by arrow).

Fig 2.
Cryo-electron microscopy of RyR1 and Glutaraldehyde Cross-Linked RyR1. Typical image of glutaraldehyde cross-linked RyR1 and control RyR1. Circles placed over selected RyR1 particles. Scale bar 500 Å

Fig 3.

2D Cryo-EM Analysis of Glutaraldehyde Cross-Linked RyR1 and His-FKBP12:RyR1 complex. (A and B) Two-dimensional averages of control RyR1 and glutaraldehyde cross-linked RyR1, respectively (N=369 and N=404). Fourfold symmetry was imposed on both reconstructions, averages were low-passed filtered to 29 Å resolution. (C) Difference map made by subtracting glutaraldehyde cross-linked RyR1 from control RyR1 2D average. (D) The T-test corresponding to difference map displayed at 99.99 % confidence. (E and F) Two-dimensional averages of cross-linked RyR1:His-FKBP12 complex, respectively, and cross-linked RyR1 (N=419 and N=400). Fourfold symmetry was imposed and averages were low-passed filtered to 31 Å resolution. (G) Difference map obtained by subtracting cross-linked His-FKBP12:RyR1 2D average from cross-linked RyR1 2D average. (H) T-test corresponding to difference map displayed at 99.99 % confidence. The location of His-FKBP12 revealed by 2D analysis is identical to that of FKBP12 described previously (Wagenknecht et al., 1996). Arrow points to His-FKBP12. Scale bars 100 Å.

Fig 4.

3D Reconstruction of RyR1 and Glutaraldehyde Cross-Linked RyR1. (A and B) Single-particle cryo-EM 3D reconstructions of control RyR1 in red and glutaraldehyde cross-linked RyR1 in yellow, shown in top, bottom and side views. Reconstructions made by combining 4,048 and 4,567 images, respectively. Both volumes were low-pass filtered to 32 Å for comparison purposes. (C) Difference maps derived by comparing control RyR1 and glutaraldehyde cross-linked RyR1 reconstructions. The yellow differences correspond to major regions of excess mass density present in cross-linked RyR1 relative to control, and red differences correspond to excess mass present in control relative to cross-linked RyR1. The difference maps are shown superimposed on the control RyR1 volume in grey. RyR1 domains labeled according to (Radermacher et al., 1994). Density connecting domains 7 and 11 is marked by arrow and the plug in transmembrane region is marked by “p” Scale Bars 100 Å.