Ca²⁺-inactivation of the mammalian ryanodine receptor type 1 in a lipidic environment revealed by cryo-EM

Ashok R. Nayak¹, Montserrat Samsó^{1*}.

¹*Department of Physiology and Biophysics, Virginia Commonwealth University, Richmond, VA, USA.*

*Correspondence to: montserrat.samso@vcuhealth.org

Abstract

Activation of the intracellular Ca²⁺ channel ryanodine receptor (RyR) triggers a cytosolic Ca²⁺ surge, while elevated cytosolic Ca²⁺ inhibits the channel in a negative feedback mechanism. Cryo-EM of rabbit RyR1 embedded in nanodiscs under partially inactivating Ca²⁺ conditions revealed an open and a closed-inactivated conformation. Ca²⁺ binding to the high affinity site engages the central and C-terminal domains into a block, which pries the S6 four-helix bundle open. Further rotation of this block pushes S6 toward the central axis, closing (inactivating) the channel. Main characteristics of the Ca²⁺-inactivated conformation are downward conformation of the cytoplasmic assembly and tightly-knit subunit interface contributed by a fully occupied Ca²⁺ activation site, two inter-subunit resolved lipids, and two salt bridges between the EF hand domain and the S2-S3 loop validated by disease-causing mutations. The structural insight illustrates the prior Ca²⁺ activation prerequisite for Ca²⁺ inactivation and provides for seamless transition from inactivated to closed conformations.

Keywords ryanodine receptor, inactivation, calcium, excitation-contraction coupling, cryo-electron microscopy

Introduction

Ryanodine Receptors (RyRs) are complex intracellular, multi-domain Ca²⁺ channels that generate Ca²⁺ transients in cells of higher metazoans. They are pivotal for contraction of skeletal and cardiac muscles (Flucher & Franzini-Armstrong, 1996, Meissner, 2017, Ríos, 2018), supporting transient rise in cytosolic Ca²⁺ from its resting level of ~100 nM, which is driven by a more than 1,000 fold Ca²⁺ concentration gradient across the membrane of the endoplasmic reticulum (sarcoplasmic reticulum in muscle, SR) (Ziman *et al*, 2010). RyRs also play a role in neuron excitability (Albrecht *et al*, 2001, Arias-Cavieres *et al*, 2018, Bouchard *et al*, 2003) and a myriad of other Ca²⁺ dependent pathways such as differentiation, survival and apoptosis (Bagur & Hajnóczky, 2017, Tu *et al*, 2016). Dysregulation of the channel leads to several life-threatening diseases such as malignant hyperthermia, central core disease, sudden cardiac death, and lethal fetal akinesia deformation sequence syndrome (Alkhunaizi *et al*, 2019, McCarthy *et al*, 2000, Priori *et al*, 2001, Tiso *et al*, 2001, Treves *et al*, 2008).

The high-conductance RyR channel is strongly regulated, with cytosolic Ca²⁺ concentration having a biphasic effect on the probability of channel opening. Notably, both intracellular Ca²⁺ channels, RyRs and inositol P₃ receptors (IP₃Rs), display a bell-shaped curve of Ca²⁺ dependence (Bezprozvanny *et al*, 1991, Meissner, 2017, Meissner *et al*, 1986), characteristic of dual regulation by cytosolic Ca²⁺. In RyR, the ascendant branch of Ca²⁺ dependence with half-maximal concentration (*K_a*) of 1-5 μM

(Laver, 2018, Laver & Lamb, 1998) is mediated by a high affinity cytosolic Ca^{2+} site (des Georges *et al*, 2016). Ca^{2+} concentrations above 100 μM , reached in the tight nanodomain surrounding the RyR upon its opening (Langer & Peskoff, 1996), inhibit the channel (Chen *et al*, 1997, Xu & Meissner, 1998, Yamaguchi, 2020) which rapidly enters a refractory period that has been proposed to prevent depletion of the SR (Bers, 2002, Ríos *et al*, 2008). Out of the three isoforms, the “skeletal muscle” RyR1 isoform studied here is the most sensitive to Ca^{2+} inactivation (Meissner, 2017), where inactivation-impairing mutations are known to cause malignant hyperthermia (Gomez *et al*, 2016). While inactivation of RyR by Ca^{2+} has been characterized at the functional level, its underlying mechanism is unknown. Outstanding questions are whether high Ca^{2+} induces an allosteric change in RyR that dampens the affinity of its activation site for Ca^{2+} , and whether the Ca^{2+} inactivated conformation is similar or distinct with respect to the closed conformation obtained in the absence of Ca^{2+} .

Here, cryo-EM and single-particle 3D reconstruction were applied to rabbit RyR1 embedded in nanodiscs under conditions of partial Ca^{2+} inactivation. Classification of two independent cryo-EM datasets revealed, in both cases, coexistence of closed and open conformations, in agreement with functional experiments performed on the same channels using tritiated ryanodine binding. In the closed (inactivated) conformation, the resolution of the cryo-EM maps of nanodisc-embedded RyR1 enabled visualization of two lipids buried in a pocket of the transmembrane domain (TMD). To our knowledge, this is the first time that lipid is visualized in direct contact with the RyR. The open state, obtained by classification of the same dataset, represents the first RyR1 open conformation achieved in the absence of any extra activator other than the physiological activators, Ca^{2+} and ATP. We also carried out a control 3D reconstruction of RyR1 under the same conditions except for the absence of Ca^{2+} , which yielded a closed channel. Comparison of the closed conformations of RyR1 at high Ca^{2+} and of RyR1 in the absence of Ca^{2+} revealed unique features associated to Ca^{2+} -inactivation. Both Ca^{2+} -induced activation and inactivation can be explained by a unifying mechanism that involves conformational rearrangements within the central region of RyR1. Thus, the 3D structures of closed, open and inactivated RyR1 embedded in lipidic nanodisc provide a mechanistic framework to understand the biphasic response of RyR1 to Ca^{2+} . In addition, two inter-subunit salt bridges appear to mediate the Ca^{2+} -inactivated structural rearrangement, a finding supported by disease-causing mutations hindering such interactions and known to impair Ca^{2+} -induced inactivation of the RyR1.

Results

Experimental design and functional validation of RyR1

Experimental conditions were fine-tuned to obtain the inactivated state. The channels were prepared in 2 mM free Ca^{2+} , a concentration that inactivates the channel. As RyR1 is constitutively bound to the sensitizing ATP in the muscle cell (Kushmerick *et al*, 1992, Meissner *et al*, 1986, Xu *et al*, 1996), we included its non-hydrolyzable form, AMP-PCP (ACP) for its structural determination. In order to visualize the direct effect of Ca^{2+} on RyR1's conformation FKBP12, a stabilizer of the cytoplasmic domain, was excluded. To determine the structure of RyR1 in a detergent-free, membrane-embedded natural state, RyR1 was reconstituted into membrane scaffold protein (MSP) 1E3D1 nanodiscs in the presence of phosphatidylcholine, an abundant phospholipid in membrane fraction preparations. As a control, we carried out cryo-EM and 3D reconstruction of RyR1 using identical buffer conditions and

89 saturating ACP, and substituted Ca^{2+} by 1 mM EGTA plus 1 mM EDTA (dataset denominated RyR1-
90 ACP/EGTA). The channels were also reconstituted into nanodiscs in the presence of
91 phosphatidylcholine.

92 The Ca^{2+} -induced activity profile of rabbit RyR1 in SR membranes was determined using the tritiated
93 ryanodine binding assay, which reflects the probability of channel opening. The assay indicated RyR1
94 channel inactivation above 0.1 mM Ca^{2+} , with an IC_{50} of 0.6 mM (Figure 1A). Presence of 2 mM ATP
95 increased the efficacy of Ca^{2+} -induced activation by ~3- fold at peak activation with similar potency
96 for Ca^{2+} -induced inactivation (IC_{50} of 0.7 mM), in agreement with earlier results obtained in lipid
97 bilayer (Laver *et al*, 1995). We replicated the ryanodine binding experiments over the same Ca^{2+}
98 concentration range with 2 mM ACP. Maximal ryanodine binding was ~1.5 fold higher in the presence
99 of ACP compared to ATP, and in this case slightly higher Ca^{2+} concentration was necessary for the
100 same degree of RyR1 inhibition, with an IC_{50} value for Ca^{2+} of 1.5 mM in the presence of ACP. Thus,
101 under our experimental conditions of 2 mM free Ca^{2+} , RyR1 inactivation was partial.

102

103 **Classification of the high Ca^{2+} dataset reveals an *open* conformation and a *closed-inactivated*** 104 **conformation**

105 Cryo-EM of rabbit RyR1 embedded in nanodisc in the presence of 2 mM ACP and 2 mM free Ca^{2+}
106 followed by single-particle analysis and 3D classification revealed two classes of particles, with their
107 pore either fully open or fully closed. The findings, reproduced in two independent datasets (A and B),
108 reflected the partial inhibition observed at 2 mM free Ca^{2+} (pCa 2.7) in the ryanodine binding studies
109 (Figure 1A). The 3D reconstructions of RyR1-ACP/ Ca^{2+} open reported here are the first RyR1 open
110 structures obtained in the presence of natural activators alone, possibly owing to the use of nanodiscs.
111 The RyR1-ACP/ Ca^{2+} reconstructions with a closed pore suggest a distinct conformation that we refer
112 to as RyR1-ACP/ Ca^{2+} -inactivated henceforth, as further supported by our data.

113 A symmetry of C4 was imposed after ascertaining true four-fold symmetry of the protein. The closed-
114 pore (inactivated) channels were resolved to 3.8 Å and 4.1 Å resolution for the A and B datasets,
115 respectively, which improved to 3.5 Å and 3.8 Å respectively using a phase improvement procedure
116 (Terwilliger *et al*, 2020). Despite this improvement, we followed a conservative approach and used the
117 non-density modified maps for subsequent analysis. The open conformation represented a smaller
118 fraction of the data (13% and 19% for the A and B datasets), which limited the resolution to 4.6 Å and
119 5.8 Å, respectively (Figure 1-figure supplement 1, 2, and 3). The RyR1-ACP/ Ca^{2+} _A open subset
120 improved to a resolution of 4.0 Å after a symmetry expansion step. Unless specified, the reported
121 observations correspond to dataset A.

122 Classification of the control RyR1-ACP/EGTA dataset yielded a single class with resolution of 4.3 Å
123 (0.143 FSC gold standard), but further symmetry expansion and focused classification using a
124 quadrant-shaped mask increased resolution to 3.9 Å (Figure 1-figure supplement 4). Despite the
125 presence of the ACP activator, the 3D reconstruction of RyR1-ACP/EGTA yielded a closed state.
126 Tables 1 and 2 summarize the cryo-EM data collection, single-particle image processing, model
127 quality attributes and database IDs for all datasets, and Table 3 compiles the main characteristics of the
128 three conformations analyzed (closed, open, inactivated).

Pore analysis using the program HOLE (Figure 1B and C) of RyR1-ACP/EGTA indicated a closed pore with a radius constricting to 1 Å at the known hydrophobic gate Ile4937. In both RyR1-ACP/Ca²⁺ open A and B datasets the hydrophobic gate as appraised by the pore profile (Figure 1B and Figure 1-figure supplement 5) and as measured at the S6 helix backbone C_α atoms (for more accurate measurement according to the lower resolution of the two datasets) (Figure 1C, Figure 1-figure supplement 5) had diameters of 15.8 Å and 16.0 Å, which indicates an open, Ca²⁺-permeable pore in both cases. Pore dimensions are comparable to the pore diameter of RyR1 open structures obtained in the presence of activators such as Ca²⁺/PCB95 or Ca²⁺/caffeine/ATP (16.7 Å; PDB ID: 5TAL (des Georges *et al.*, 2016)). The slightly narrower pore in our case could be attributed to the millimolar instead of submicromolar Ca²⁺ in our case, and/or extra activators in addition to endogenous physiological activators in earlier open structures.

The reconstructions corresponding to RyR1-ACP/Ca²⁺ inactivated of the A and B datasets displayed a pore diameter at the Ile4937 gate of ~2 Å when considering the side chains, and 10.7 Å and 11.3 Å diameter respectively when measured at the backbone C_α atoms, making it impermeable to Ca²⁺ ions (Figure 1 B and C, Figure 1-figure supplement 5). The position of the S6 backbone at Ile4937 is similar to our closed structure (r=10.4 Å) and to RyR1-FKBP12/EGTA (r=10.3 Å; PDB ID: 5TB0 (des Georges *et al.*, 2016)). Interestingly, the channel pore was narrower than 1 Å at Gln4933 in RyR1-ACP/Ca²⁺ inactivated (Figure 1B and C).

We previously established that the large square-shaped cytoplasmic shell of the RyR undergoes a conformational change upon opening. The periphery of its four quadrants tilts downward (toward the membrane), while their inner corner tilts away from it, rotating around a pivot point (Samsó *et al.*, 2009). Such tilt can be quantified with the flexion angle, whereby negative angle (downward) correlates with opening, positive angle with closing, and absence of FKBP lowers the flexion angle of closed states (Steele & Samsó, 2019). In general, the approximate ranges of flexion angles are +1° to +2° for RyR1-FKBP12/EGTA closed, -1° to -3° for RyR1-EGTA closed, 0° to -3° for RyR1-FKBP12 “primed” (with activating ligands and closed pore), and -1.5° to -5° for RyR1-Ca²⁺ open with or without FKBP12 (Iyer *et al.*, 2020, Steele & Samsó, 2019). Here, RyR1-ACP/Ca²⁺ open channels have flexion angles of -5.1° and -4.8° for the A and B datasets, within the expected range. The consensus 3D reconstruction of RyR1-ACP/EGTA had flexion angle of -2.2°, and its two classes had flexion angles of -1.8° (24%) and -2.9° (76%) (Figure 1-figure supplement 4). But unexpectedly for closed-pore channels, RyR1-ACP/Ca²⁺ inactivated datasets have a negative flexion angle (-4.6° and -4.2° for A and B datasets, respectively) (Figure 1-figure supplement 1, 3). As there was indication of variability in the cytoplasmic shell of RyR1-ACP/Ca²⁺ inactivated dataset, we carried out fourfold symmetry expansion and focused classification for the monomer and obtained three sub-classes, all showing downward motion: class 1 (22 % of particles, 3.7 Å resolution, -3.6° flexion angle), class 2 (44 % of particles, 3.3 Å resolution, -4.4° flexion angle) and class 3 (20 % of particles, 3.8 Å resolution, -5.6° flexion angle) (Figure 1-figure supplement 1). Such pronounced downward flexion angles in a closed channel cannot be explained just by the lack of FKBP12 in our preparations. The distinct conformation of RyR1-ACP/Ca²⁺ inactivated, consisting of a closed pore and extreme-downward cytoplasmic assembly, prompted further analysis of the central region that joins the cytoplasmic and transmembrane domains.

169

170 Different arrangement of the central region in Ca²⁺-inactivated, closed and open conformations

171 In RyR1-ACP/Ca²⁺ open, the high-affinity Ca²⁺ binding site formed by Glu3967, Glu3893 (from the
172 CD; residues 3668-4070), and Thr5001 (from the CTD; residues 4957-5037) as reported earlier (des
173 Georges *et al*, 2016), and additionally Gln3970 in our case, which was visible up to 20 σ . The Ca²⁺-
174 induced reorientation of CTD with respect to CD and subsequent separation of S6 was obvious when
175 compared to the RyR1-ACP/EGTA structure (Figure 2A, Figure 2-figure supplement 1), consistent
176 with previous reports of Ca²⁺-induced activation (Bai *et al*, 2016, des Georges *et al*, 2016).

177 An outstanding question is how higher Ca²⁺ concentration may result in inactivation. Potential
178 mechanisms are an additional allosteric change that dampens the affinity of the Ca²⁺ activation site, or
179 that the high affinity Ca²⁺ site remains occupied while an additional conformational change overcomes
180 activation. Consistent with the second scenario, the high affinity Ca²⁺ binding site in RyR1-ACP/Ca²⁺
181 inactivated remained fully occupied by Ca²⁺ up to 20 σ , although with additional out-of-plane tilting of
182 the CD and CTD around the Ca²⁺ binding site. The Ca²⁺ ion was coordinated by Glu3893, Glu3967
183 (CD), and Thr5001 (CTD) within contact distances of 2.8 Å, consistent with the open structures.
184 However, on the CD-C' side, Gln3970 lost coordination to Ca²⁺, interacting with Ser4029 instead
185 (Figure 2A, Figure 2-figure supplement 1). Mutations Q3970E/K in RyR1 are implicated in central
186 core disease and equivalent RyR2 mutations in cardiac arrhythmia (Chirasani *et al*, 2019), which
187 highlights the important Ca²⁺ sensing role for this residue.

188 To understand how the fully occupied high affinity Ca²⁺ binding site (CD/CTD interface) led to a
189 closed pore in RyR1-ACP/Ca²⁺ inactivated, we looked for differences between RyR1-ACP/Ca²⁺ open
190 and RyR1-ACP/Ca²⁺ inactivated in the domains spanning from the CD/CTD interface to the pore.
191 Specifically, we focused on domains around the high affinity Ca²⁺ binding site (CD, EF hand, U-motif,
192 CTD, and the C' section of S6 or S6C'; domain boundaries indicated in Figure 2), which we term
193 "central block". Comparison was done for dataset A after confirming similarity for the central block of
194 0.95 Å C α root mean square deviation (RMSD) for the encompassed 672 resolved residues, between
195 inactivated A and B datasets (Figure 2-figure supplement 2A). The RMSD (C α backbone) between
196 individual domains from RyR1-ACP/Ca²⁺ inactivated versus open was below 1.5 Å for all comparisons
197 when the domains were pre-aligned pairwise (Figure 2B, Figure 2-figure supplement 2 B and C),
198 indicating little conformational change. The N' section of S6 was also included in the comparisons.
199 Without pre-alignment, RMSD was higher (Figure 2B), indicating domain repositioning, the degree of
200 which was estimated by subtracting the pre-aligned from the unaligned RMSDs. This yielded an
201 average shift of ~2 Å per domain in going from RyR1-ACP/Ca²⁺ open to RyR1-ACP/Ca²⁺ inactivated,
202 except for S6N' where the shift was ~0.7 Å (Figure 2B). Comparing the same domains between RyR1-
203 ACP/Ca²⁺ inactivated and RyR1-ACP/EGTA (closed) yielded smaller repositioning with average shifts
204 of ~1 Å, reflecting similarity in the domain's locations, with the exception of the EF hand domain that
205 undergoes a ~5 Å repositioning (Figure 2B, Figure 2-figure supplement 2C).

206 When the central blocks were pre-aligned as a unit using their respective CD domains, and the
207 individual domains were compared pairwise (CD-aligned; Figure 2-figure supplement 2A), there was
208 good overlap between RyR1-ACP/Ca²⁺ open and RyR1-ACP/Ca²⁺ inactivated (RMSD below 1.5 Å)
209 except for S6N' (3.8 Å), revealing the bend in the middle of S6 upon opening. In contrast, the CD-
210 aligned RMSD of RyR1-ACP/Ca²⁺ inactivated vs RyR1-ACP/EGTA closed was higher, between 3-4
211 Å for the EF hands, U-motif, and CTD, and increases toward the C-terminus, 5 Å for S6C' and almost
212 7 Å for S6N' (Figure 2B, "CD-aligned"). This indicated that domains CD, EF hands, U-motif, S6C',
213 and CTD relocated together, and that the central block became more compact in the presence of Ca²⁺

214 acting at the CD/CTD interface. Taken together, these results imply that conformational changes from
215 CD are transmitted to S6, only in the presence of Ca^{2+} (Figure 2 C and D).

216 We analyzed how the central block may evolve from RyR1-ACP/EGTA (closed), to RyR1-ACP/ Ca^{2+}
217 open, to RyR1-ACP/ Ca^{2+} inactivated. Upon Ca^{2+} -induced opening, “engagement” of CD and CTD by
218 Ca^{2+} tilts the CD out-of-plane (such that helix h tilts 3° inwards), while the CTD tilts up and outwards.
219 This pulls the S6 helices (which are directly connected to the CTD) away from the pore axis, opening
220 the ion pathway (Figure 2 C and D). To reach the inactivated state, the central block tilts further (see
221 rotation axis in Figure 2-figure supplement 2B) in a movement similar to pushing down the levers of a
222 winged corkscrew, which pushes each S6 helix $2.5 \text{ \AA}$ toward the pore axis, closing the channel. Alpha
223 helices within the CTD act as a lever coupling this out-of-plane rotation to S6C’ and as described
224 further below, ATP reinforces the connection between CTD-S6C’ in the presence of Ca^{2+} . These
225 conformational changes involved in activation, inactivation, and transition from the inactivated to the
226 closed state, are shown in the morph among conformations in Figure 2 - Video 1 and illustrated in the
227 schematics in Figure 2D.

228

229 **Formation of salt bridges between EF hand domain and S2-S3 loop of the neighboring subunit in** 230 **the inactivated state**

231 The EF hand domain of RyR1, a candidate Ca^{2+} regulation site (Du *et al*, 1998, Gomez *et al*, 2016,
232 Gomez & Yamaguchi, 2014, Xiong *et al*, 1998), did not show significant changes, with maximum C α
233 pre-aligned RMSD of $1.5 \text{ \AA}$ among the different states analyzed (closed, open, inactivated). The EF
234 hand loops (residues 4081-4090, 4116-4123) were empty in our high Ca^{2+} conditions, which is
235 consistent with the low affinity of Ca^{2+} to this site (K_d 3.7 mM (Xiong *et al*, 1998)). Nonetheless, the
236 entire EF hand domain, which protrudes from the CD-C’, repositions noticeably during activation, with
237 a 3.4° counterclockwise in-plane rotation (as seen from the cytoplasmic side), and 6.8° upward out-of-
238 plane rotation. This movement brings the EF hand domain in closer proximity to the S2-S3 loop
239 (cytoplasmic loop between S2 and S3 TMD helices; residues 4664-4786) (Figure 3). With inactivation,
240 an additional 1° counterclockwise rotation and 2.7° upward out-of-plane rotations define a physical
241 limit to the counterclockwise motion, forming two salt bridges at this inter-subunit interface: Glu4075-
242 Arg4736 and Lys4101-Asp4730, with their side chains within $3.5 \text{ \AA}$ (Figure 3A). This interaction is
243 critical to support inactivation, as demonstrated by the fact that MH/CCD mutations facing this
244 interface F4732D, G4733E and R4736W/Q, with the latter including the Arg directly involved in the
245 salt bridge (Figure 3B), greatly reduced channel inactivation (Gomez *et al*, 2016). On the other hand,
246 MH/CCD mutations T4082M, S4113L, and N4120Y, in regions of the EF hand domain away from the
247 interface (Figure 3B), did not affect RyR1 inactivation (Gomez *et al*, 2016), serving as a negative
248 control for this hypothesis.

249

250 **Ca^{2+} binds to the ATP binding pocket**

251 The ATP binding site showed full occupancy in the three conformations –open, closed and inactivated.
252 Under closed-state conditions, ACP bound to the pocket formed by the U-motif, S6C’ and CTD
253 (Figure 4A “closed”), in the same position as ATP (des Georges *et al*, 2016), with a high map
254 significance (7σ). An additional elongated non-protein density was associated to ACP in the RyR1-

255 ACP/Ca²⁺ open and inactivated maps. This distinct density has high map significance (15σ) (Figure
 256 4A, Figure 4-figure supplement 1), suggestive of a putative Ca²⁺ ion. Well-resolved local density in
 257 RyR1-ACP/Ca²⁺_A inactivated class-1 map allowed tentative modeling of two waters on either side of
 258 the putative Ca²⁺ that connect on either side to ACP's γ-phosphate and Thr4979 (CTD) (Figure 4A
 259 "inactivated"), suggesting potential coordination through Ca²⁺'s first layer of hydration. Analysis of
 260 protein-ligand interactions based on the atomic coordinates (Laskowski & Swindells, 2011) showed an
 261 increased network of electrostatic and hydrophobic interactions, and three predicted hydrogen bonds,
 262 that the inactivated conformation gained with respect to closed conformation (Figure 4A). Thus, the
 263 nucleotide nestled deeper into the cavity in going from closed to open, and then to inactivated,
 264 increasing connectivity between S6C' and CTD and reducing connectivity to the U-motif (Figure 4B).
 265 Interestingly, ACP in RyR1-ACP/Ca²⁺_A inactivated acquired an interaction with the backbone carbonyl
 266 of His4983, a residue that participates in the C2H2 Zinc motif which is central to the CTD (Figure 4).
 267 Based on the higher map significance of the putative Ca²⁺ ion in our cryo-EM maps obtained at higher
 268 Ca²⁺ concentration (Figure 4-figure supplement 1) and the enhanced inactivation when ATP is present
 269 (Sitsapesan & Williams, 2000), the interface between the nucleotide, the CTD and S6 could be thought
 270 of as an atypical low affinity Ca²⁺ inactivation site. The picture is bound to be more complex, as
 271 presumably Mg²⁺ present in the cytoplasm would also bind to ATP. Thus, under physiological
 272 conditions of high local Ca²⁺, competition between the two divalent cations for ATP may take place.

273

274 **Protein-lipid interactions within the nanodisc environment**

275 To provide a more physiological environment to the TMD domain and avoid the presence of
 276 detergents, we embedded the protein in scaffold protein MSP1E3D1 that assembled into nanodiscs.
 277 The reconstituting lipid was phosphatidylcholine. Refinement focused on the CD-TMD region of
 278 RyR1-ACP/Ca²⁺_A inactivated and open, resulting in reconstructions with 3.5 Å and 4.4 Å resolution
 279 respectively, yielded a visible electron density for the nanodisc. Two molecules of the MSP1E3
 280 scaffold protein wrapped closely around the TMD in a double-belt arrangement (Figure 5A). The
 281 larger top belt adopts a quatrefoil shape, with one voltage sensor (S1-S4) in each lobe, while the lower
 282 belt is rounder and smaller, following the tapered TMD. The larger footprint of the top half of RyR1's
 283 TMD domain, reflected in the surrounding nanodisc, appears to correlate with the curvature of the
 284 membrane around RyR1 observed by electron tomography in its native membrane (Chen &
 285 Kudryashev, 2020, Renken *et al*, 2009). Even considering the tight fit between the nanodisc and
 286 RyR1's TMD, conformational changes were unhindered and the channel opened within the nanodisc
 287 environment. The slight expansion of the top belt of the nanodisc at the level of the ion gate upon
 288 opening (Figure 5 – Video 1) reveals a certain degree of plasticity of the scaffold protein.

289 We resolved two lipid densities at the four inter-subunit interfaces in the RyR1-ACP/Ca²⁺_A inactivated
 290 CD-TMD focused map (Figure 5B). The two lipid densities, observed up to 8σ map significance,
 291 spanned ~20 Å across the inner leaflet of the SR membrane, at a hydrophobic pocket in the domain-
 292 swapped inter-subunit space formed by the S1-S4 bundle and core TMD helices (S5 and S6) (Figure 5
 293 B and C). One density (lipid-1), encompassing two fatty acyl tail moieties of 16 and 11 carbons with a
 294 polar head compatible with a phosphatidylcholine molecule was resolved near S5/S6 (Figure 5C). The
 295 lipid likely originates from a longer unsaturated PC (16:0-18:1) used in 50-fold molar excess while
 296 embedding RyR1 into nanodiscs. Lipid-2, with a resolved 16-carbon fatty acid tail, was sandwiched
 297 between lipid-1 and S3/S4. The lipid's fatty-acyl tails interact extensively with 21 amino acids in the

lipophilic pocket formed by S3/S4 and S5/S6 of neighboring subunits, while the density corresponding to the lipid head moiety was positioned within 5 Å from Tyr4629, Asn4857, and Tyr4909 (Figure 5C). Together, the lipids covered ~622 Å² of the 1990 Å² surface area of the S3/S4-S5/S6 subunit interface forming a hydrophobic crevice (Figure 5D). No lipid density was resolved in the RyR1-ACP/Ca²⁺_A open map: besides the lower resolution of this map, its crevice is narrower, as S3 and S4 remodeled the lipid binding pocket by tilting ~4.4° and ~3.9°, respectively. Furthermore, reorientation in Phe4808 (S4) and Tyr4912 (S6) as modeled for the open state would result in steric clash with lipid-2 and lipid-1 respectively (Figure 5-figure supplement 1 A and B; lipids superimposed from the RyR1-ACP/Ca²⁺ inactivated reconstruction –shown in Figure 5-figure supplement 1 C in the same orientation), rearranging or even excluding the lipids in the open channel. A similar observation was reported for the TRPV3 channel, whereby lipids get squeezed out in going from the closed to the open state (Singh *et al*, 2018). We did not resolve lipids within the crevice of RyR1-ACP/EGTA, probably owing to the lower resolution of this reconstruction (4.30 Å), but examination of the crevice indicates no expected hindrance to lipid entry offered by residues Phe4808 and Tyr4912 in the closed state (Figure 5-figure supplement 1 D). Moreover, we resolved lipids in a higher-resolution (3.27 Å) 3D reconstruction of closed RyR reconstituted in lipids, in this case for the RyR2 isoform (Figure 5-figure supplement 1E; PDB ID: 6WOU (Iyer *et al*, 2020)). The analogous appearance of the lipid densities in the two isoforms is remarkable (three tubular densities with similar lengths and orientations; compare Figure 5-figure supplement 1, panels C and E), suggesting that the interaction of lipids with the RyR TMD crevice is conserved among isoforms, and characteristic of closed and inactivated states.

Plasticity of transmembrane helices and stability of the luminal mouth of the channel

The TMD helices were examined in the membrane-like environment provided by the nanodisc. Significant perturbations in the α-helical configuration were observed. S4 displays near-3₁₀ helix in residues 4807-4813 and (only for the open structure) near-π helix in residues 4814-4819. S5 displays π helix in residues 4856-4859. S6 displays π helix in residues 4921-4928 of S6 (Figure 6A, B). In S4, comparison of the three conformations suggests that the degree of over-coiling of the N' half of S4 is balanced by the uncoiling of its C' half to near-π helix in the open structure (Figure 6A), which causes the reorientation of Phe4808 into the hydrophobic crevice. In S6, the π-helix region introduces non canonical i ← i+5 hydrogen bonding which is energetically less stable than a regular α-helix (Fodje & Al-Karadaghi, 2002, Kumar & Bansal, 2015)(Figure 6B), and may facilitate the flexibility of S6 required for its structural transitions.

The three segments departing from α-helical configuration contain Phe clusters (FFF 4807-4809, FF 4858-4859, FFFF 4920-4923) conserved in all RyR isoforms and IP₃R intracellular Ca²⁺ release channels; with some of them lining the hydrophobic crevice and forming contacts with the lipids (Figure 5C). Deletion of FF 4923-4924 (human sequence; equivalent to rabbit FF 4922-4923) in S6 of RyR1, which is associated to fetal akinesia deformation syndrome or FADS, resulted in loss of Ca²⁺ conductance (Xu *et al*, 2020), which further supports a role for the Phe clusters in the stability of the pore.

The SR luminal loop (4860-4878) between S5 and the P-loop helix (also termed pore helix; residues 4879-4893), proposed to act as luminal Ca²⁺ sensor (Sitsapesan & Williams, 1995) and as anchorage point for proteins within the SR lumen (Beard *et al*, 2009), contains six acidic residues (EDEDEPD 4867-4873). This luminal loop is separated from the rest of the TMD, and its acidic residues point to the lumen interior. As our experimental high Ca²⁺ concentrations correspond to these in the SR lumen,

341 this conformation of the luminal loop (Figure 6C) is probably a close representation of the native state.
342 The higher resolution RyR1-ACP/Ca²⁺ inactivated map revealed a disulfide bond (Cys4876-Cys4882)
343 between S5 and the P-loop (Figure 6C), and two inter-subunit hydrogen bonds (Tyr4888-Arg4913 and
344 Tyr4888-Asp4917) between the P-loop and S6. These interactions may stabilize the luminal mouth of
345 the channel, keeping the proper arrangement of the four SR luminal loops, as well as holding S6
346 through its conformational transitions.

347

348 Discussion

349 The goal of this work was to understand the mechanism of Ca²⁺-induced inactivation of RyR1 from a
350 structural point of view. Although maximum inhibition occurred at 10 mM Ca²⁺, we selected a
351 concentration closer to the highest Ca²⁺ concentration in physiological compartments, around 1-2 mM
352 free Ca²⁺ (in the SR lumen, cytoplasmic Ca²⁺ nanodomains, and extracellular medium). At 2 mM free
353 Ca²⁺, we discerned two classes within each of the two datasets, with their ion gate either in an open or
354 a closed conformation on account of direct pore analysis. The concentration of Ca²⁺ used, 2 mM, is
355 near the IC₅₀ of this cation determined experimentally in the [³H] ryanodine binding assays in the
356 presence of ACP (the ATP analogue used in our studies), a concentration at which Ca²⁺-activated and
357 Ca²⁺-inactivated RyR1 conformations should coexist. Accordingly, we propose that they correspond
358 with the open-pore and closed-pore conformations observed by cryo-EM, respectively.

359 Earlier 3D structures of RyR in the open state required synthetic activators such as caffeine (des
360 Georges *et al*, 2016), which by itself suffices to activate the channel (Xu *et al*, 2018), or PCB95 (Bai *et*
361 *al.*, 2016, Samsó *et al*, 2009). Our cryo-EM maps of RyR1 and lipid were obtained in the absence of
362 any non-physiological activator, and in a nanodisc environment. Given the lipidic environment
363 provided by the nanodisc and the lack of exogenous activators, the structure of RyR1-ACP/Ca²⁺ open
364 (reproduced in two independent datasets, A and B) obtained in the presence of Ca²⁺ and ACP provides
365 an accurate account of the native open state. Overall, our open-state structure validates the previous
366 RyR1 open structures obtained using exogenous activators.

367 The RyR1-ATP/caffeine/Ca²⁺ dataset, obtained in the presence of 30 μM Ca²⁺, 2 mM ATP, and 5 mM
368 caffeine, also yielded a class with a closed pore, which was termed “primed” (des Georges *et al*, 2016).
369 An apparently puzzling result is that our structure of RyR1-ACP/Ca²⁺ inactivated is similar to the
370 primed conformation. Both consist of an occupied Ca²⁺ binding site with a closed pore. The flexion
371 angle of the primed structure (e.g., PDB ID: 5TAQ), around -3° degrees, is also more akin to an open
372 than a closed state. In these studies, caffeine was used as a means to attain the open state of the
373 channel, as caffeine sensitizes RyR1 to Ca²⁺ and increases opening probability. However, caffeine also
374 affects inactivation, and shifts the entire bell-shaped curve of RyR1 activation as a function of Ca²⁺
375 concentration to lower Ca²⁺ concentrations: from a maximum at 30 μM Ca²⁺, to well below 10 μM
376 Ca²⁺ in the presence of saturating caffeine (Meissner *et al*, 1997). Thus, 30 μM Ca²⁺ in the presence of
377 caffeine is on the descending branch of the curve, which corresponds to partially inactivating
378 conditions. According to this, the RyR1 channel conformation qualified as “primed” in the earlier
379 study is, very likely, inactivated, and appears to correspond to the RyR1-ACP/Ca²⁺ inactivated
380 conformation characterized here.

381 Activation and inactivation appear to proceed under an integrated mechanism, where inactivation
382 depends on prior activation. Both RyR1 open and inactivated conformations showed Ca^{2+} bound at the
383 high affinity site in the CD/CTD interface, which joined these and their proximal domains (EF, U-
384 motif, and S6C') into a more rigid central block. In activation, CD and CTD rotate toward each other
385 closing around the Ca^{2+} ion; the rotation of the CTD pulls S6C' outwards, opening the pore. In
386 inactivation, the central block rotates further into the arc initiated by the CD alone, which now pushes
387 the S6 helices toward the central axis. The movement is similar to pushing down the levers of a winged
388 corkscrew while pushing toward the central rod (see schematics in Figure 2D), closing the channel in a
389 distinctive conformation different from the EGTA-closed resting state. This state, with a closed pore
390 and Ca^{2+} bound to the activation site, can no longer be activated by Ca^{2+} , which is the hallmark of an
391 inactivated state.

392 Comparison of the open, closed and inactivated conformations uncovers other novel features. A strong
393 electron density connected to ACP under the high Ca^{2+} conditions, that we attribute to hydrated Ca^{2+} ,
394 progressively supports more interactions with the CTD and S6, increasing cohesiveness of the central
395 block (in the order inactivated > open > closed; Figure 4), which could help to explain the more robust
396 Ca^{2+} -induced inactivation reported in the presence of ATP (Sitsapesan & Williams, 2000). In this
397 context, it is important to keep in mind that Mg^{2+} at physiological concentration also binds to the
398 nucleotide binding pocket of RyR1, which we can confirm with our 3D reconstruction of RyR1-
399 ACP/ Mg^{2+} (PDB ID: 7K0S; manuscript in preparation). While in general ATP has higher affinity for
400 Mg^{2+} than for Ca^{2+} , the Ca^{2+} occupancy of this site in the inactivated state is significant (RMSD 15;
401 Figure 4-figure supplement 1). Thus, it is difficult to predict out how both cations may compete for the
402 same site, something that will require further study. Besides the high affinity Ca^{2+} binding site and the
403 nucleotide binding site, there were no other obvious densities in the resolved regions of the RyR1
404 structure that could account for a bound Ca^{2+} ion, or clusters of negatively charged residues that could
405 support further Ca^{2+} -mediated conformational changes.

406 Mg^{2+} is a potent inhibitor of RyR1, and a mechanism of Mg^{2+} competition for the Ca^{2+} binding site
407 was proposed (Lamb & Stephenson, 1991, Meissner *et al*, 1986). We carried out a 3D reconstruction
408 of RyR1-ACP/ Mg^{2+} (PDB ID: 7K0S; manuscript in preparation) and found that, at a physiological
409 concentration of Mg^{2+} , the high affinity Ca^{2+} binding site remains empty. Importantly, the Mg^{2+} -
410 inhibited conformation of RyR1 is clearly distinct from the Ca^{2+} -inactivated RyR1, and bearing more
411 resemblance to the EGTA-closed conformation.

412 The EF hand domain did not have Ca^{2+} bound in the high Ca^{2+} datasets in keeping with the low affinity
413 of Ca^{2+} to this site (Xiong *et al.*, 1998). However, this protruding domain, with its sequence between
414 the CD and the U-motif, appears to play a distinctive role in inactivation which derives from its further
415 anticlockwise and out-of-plane rotations with respect to the open state (Figure 3). This conformation
416 brings the EF hand domain in contact with the cytoplasmic loop between the S2 and S3 transmembrane
417 helices of the neighboring subunit, forming two inter-subunit electrostatic interactions (Figure 3). One
418 possible scenario is that the energy landscape of the open channel allows for overshoot of the opening
419 motion, allowing interaction of the EF hand and S2-S3 loop domains by such salt bridges. These
420 appear to stabilize the inactivated conformation by providing an extra linkage between subunits, and
421 between the cytoplasmic assembly and TMD. Functional studies of MH mutations in the S2-S3 loop
422 and EF hand domains identified a subset of mutations that altered RyR1 function by impairing Ca^{2+}
423 inactivation (Gomez *et al*, 2016). Interestingly, only this subset of residues, when mutated, would

424 impair the inter-domain interactions that we identified, which supports the proposed role of the two salt
425 bridges (Glu4075-Arg4736 and Lys4101-Asp4730) in stabilizing the inactivated state of RyR1.

426 In the TMD, owing to the nanodisc environment, two lipids in a crevice between S3/S4 of one subunit
427 and S5/S6 of the neighboring subunit were resolved in the inactivated state. The hydrophobic nature of
428 this crevice suggests that lipids may help to stabilize the TMD in the inactivated state. Although we
429 could not resolve lipids in the closed state probably owing to the lower resolution of this 3D
430 reconstruction, we observed lipids for the RyR2 isoform reconstituted in nanodiscs under closed state
431 conditions (Iyer *et al*, 2020). The orientation of the two lipids in the two isoforms of RyR is identical.
432 In the open state the hydrophobic crevice is narrower, and Phe4808 of S4 adopts a different orientation
433 that would clash with lipid-2, suggesting rearrangement in the open state. A similar lipid exclusion of
434 bound lipid in the open channel was reported for the TRPV3 channel (Singh *et al*, 2018). Thus, the
435 closed-state dependent occupation of the TMD crevice by lipids could constitute a common feature
436 among 6-transmembrane domain Ca^{2+} channels.

437 Departure from α -helix geometry was also present in segments of S4 (near- 3_{10} helix and near- π helix),
438 and S5 and S6 (π helix). In the case of S4, helical structural transitions correlate with the closed, open
439 and inactivated conformations. The dynamism of the transmembrane helices through the gating
440 transitions appears to be supported by anchoring of Phe motifs in S4, S5 and S6 to lipids in the
441 membrane. In addition, inter-subunit interactions including disulfide bridges inter-connect the 4860-
442 4878 SR luminal loop, the P-loop and S6N' around the luminal mouth of the channel where the four
443 protomers converge.

444 Functional studies employing single channels embedded in lipid bilayers show rapid channel
445 inactivation following an RyR1 Ca^{2+} release event. Therefore, after RyR1 opening, a refractory period
446 is needed to relieve inactivation and recover the ability to activate again (Laver & Lamb, 1998, Ríos *et al*,
447 2008, Schiefer *et al*, 1995, Sitsapesan & Williams, 2000). The 3D reconstructions reported here
448 provide a structural basis for this refractoriness: we hypothesize that the 3D reconstructions reported
449 here, combined with the time course of Ca^{2+} release, provide a mechanism for this refractoriness as
450 follows. When RyR opens, Ca^{2+} concentration in its surrounding nanodomain increases rapidly, and
451 time from Ca^{2+} release onset also increases. Both augment occupancy of the high affinity Ca^{2+} binding
452 site and the probability of a full conformational change of the CD/CTD block induced by Ca^{2+} , which
453 in turn increases the successful formation of the inter-subunit salt bridges, “sealing” the transition of
454 the channel to the inactivated state. At this time, the Ca^{2+} -inactivated state is in a distinctive locked
455 closed conformation while the high affinity Ca^{2+} binding site is still occupied. This renders this closed
456 conformation unable to be activated by Ca^{2+} , as long as Ca^{2+} occupies the high affinity site. In this
457 way, Ca^{2+} permeation through the RyR1 provides negative feedback through the same binding site.
458 Ca^{2+} -dependent inactivation is often observed in Ca^{2+} permeation pathways, probably to limit cytosolic
459 Ca^{2+} overload that could be detrimental and life-threatening (Dick *et al*, 2016, Gomez *et al*, 2016).
460 Together with previous investigations (Gomez *et al*, 2016, Gomez & Yamaguchi, 2014), we propose a
461 structural mechanism for how naturally occurring mutations disturb RyR1 inactivation producing Ca^{2+}
462 dysregulation and muscle disease. Overall, our study provides a structural basis to understand the
463 transitions from the closed, to the open, and then to the Ca^{2+} -inactivated state of the RyR1 at high
464 resolution.

465

Key Resources Table				
Reagent type (species) or resource	Designation	Source or reference	Identifiers	Additional information
strain, strain background (<i>Oryctolagus cuniculus</i> , Mixed gender)	New Zealand White	Charles River	NZW 052	
recombinant DNA reagent	membrane scaffold protein plasmid pMSP1E3D1	Addgene	Cat #20066	
chemical compound, drug	Ryanodine, [9,21- ³ H(N)]-, 250μCi	PerkinELmer	Part #NET950250UC	
chemical compound, drug	Adenosine-5'-[(α,β)-methyleno]diphosphate, Sodium salt	Jena Bioscience	Cat # NU-420-25	
chemical compound, drug	16:0-18:1 PC(POPC)	Avanti Polar lipids	Cat #850457	
chemical compound, drug	3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS)	Sigma Aldrich	Cat #220201	
chemical compound, drug	HiTrap Heparin HP	Cytiva	Cat #17-0406-01	
chemical	L-α-	Sigma Aldrich	Cat #P3644	

compound, drug	phosphatidylcholine			
chemical compound, drug	Sucrose	Sigma Aldrich	Cat #S9378	
software, algorithm	Maxchelator	Bers et al.,2010	https://somapp.ucdmc.ucdavis.edu/pharmacology/bers/maxchelator/CaMgATPEGTA-TS.htm	
software, algorithm	MotionCor2	Zheng et al.,2017		
software, algorithm	Gctf	Zhang., 2016		
software, algorithm	RELION-3.0	Scheres., 2012		
software, algorithm	PHENIX	Afonine et al., 2018	http://www.phenix-online.org/	
software, algorithm	HELANAL	Bansal et al., 2000	http://nucleix.mbu.iisc.ernet.in/helanalplus/index.html	
software, algorithm	Coot	Emsley et al.,2012	https://www2.mrc-lmb.cam.ac.uk/personal/pemsley/coot/	
software, algorithm	UCSF Chimera	Pettersen et al., 2004	https://www.cgl.ucsf.edu/chimera/	
software, algorithm	UCSF ChimeraX	Pettersen et al., 2021	https://www.cgl.ucsf.edu/chimerax/	
software, algorithm	PYMOL	Schrodinger, 2015	https://pymol.org/2/	
software, algorithm	HOLE	Smart et al.,1993	http://www.holeprogram.org/	

software, algorithm	Adobe Creative Cloud	Adobe	https://www.adobe.com/creativecloud/	
Other	UltraAufoil - 1.2/1.3 Holey-Gold 300 mesh grids	Quantifoil, Germany	https://www.quantifoil.com/products/ultrafoil	

Reagents

All chemicals were purchased from ThermoFisher or Sigma-Aldrich except where indicated.

[³H]Ryanodine binding

RyR1 activity was estimated by measuring the extent of bound [³H]ryanodine in microsomes isolated from rabbit skeletal muscle when incubated with free Ca²⁺ alone (10 μM to 2 mM range), or in the presence of 2 mM ATP or 2 mM AMP-PCP (ACP) sodium salts. Concentrations of total Ca²⁺ added to the reaction mixture were estimated in Maxchelator (<https://somapp.ucdmc.ucdavis.edu/pharmacology/bers/maxchelator>). Preincubated membrane vesicles (~40 μg) were allowed to bind 5 nM [³H]ryanodine (PerkinElmer) in a buffer containing 50 mM MOPS (pH 7.4), 0.15 M KCl, 0.3 mM EGTA, protease inhibitors, and 2 mM DTT for 3 h at 37°C. Sample aliquots were diluted seven-fold with an ice-cold wash buffer (0.1 M KCl) before placing onto Whatman GF/B filter papers in a vacuum-operated filtration apparatus. The remaining radioactivity in the filter papers after washing three times with the wash buffer was measured by liquid scintillation counting. Non-specific ryanodine binding was estimated in the presence of 250 μM unlabeled ryanodine (Calbiochem) and subtracted from the total binding. Data represent the mean specific [³H]ryanodine binding from four independent experiments.

Purification of RyR1 from rabbit skeletal muscle and reconstitution into nanodiscs

Microsomes were purified from rabbit back and hind leg muscles through differential centrifugation as previously described (Hu *et al*, 2021, Samsó *et al*, 2009). 100 mg frozen membranes were thawed and solubilized in buffer A containing 20 mM MOPS pH 7.4, 1 M NaCl, 9.2% (w/v) CHAPS, 2.3% (w/v) Phosphatidylcholine (PC; Sigma), 2 mM DTT and protease inhibitor cocktail for 15 mins at 4° C. The solubilized membranes were centrifuged at 100,000 x g for 60 min and the pellet was discarded. Supernatant was layered onto 10-20% (w/v) discontinuous sucrose gradients, prepared in buffer B (buffer A plus 0.5% CHAPS and 0.125% PC). The layered sucrose gradient tubes were

ultracentrifuged at 120,000 x g for ~20 h at 4° C to allow RyR1 separation. Fractions containing >95% pure RyR1 were pooled and further purified with a HiTrap Heparin HP Agarose column (GE Healthcare), after a five-fold dilution in salt-free buffer and filtration steps. RyR1 was eluted with buffer B containing 0.9 M NaCl, after washing with 20 column volumes of buffer B with 200 mM NaCl. Peak fractions were flash-frozen and stored at -80° C until reconstitution into nanodiscs and cryo-EM. 1.5-2 mg of RyR1 was purified from 100 mg of SR membrane vesicles. RyR1 purity was estimated with 12.5% SDS-PAGE and negative staining with 0.75% uranyl formate. Protein concentration in purified microsomes and RyR1 fractions was measured with Quick Start Bradford Protein Assay (Bio-Rad). The plasmid encoding for MSP1E3D1, pMSP1E3D1, was purchased from Addgene and recombinant MSP1E3D1 was purified in *E. coli* using the manufacturer's instructions. RyR1-nanodiscs were obtained by mixing purified RyR1, MSP1E3D1 and POPC (Avanti polar lipids) at a 1:2:50 molar ratio. The mixture was incubated for 1 h 30 min at 4° C before an overnight dialysis in a CHAPS-free buffer (20 mM MOPS pH 7.4, 635 mM KCl, 2 mM DTT), which contained either 1 mM EGTA + 1mM EDTA for the control “RyR1-ACP/EGTA” dataset or 3.7 mM CaCl₂ for the RyR1-ACP/Ca²⁺ dataset. The dialyzed RyR1-nanodisc preparations were incubated with ACP (sodium salt) for 30 min prior to plunge freezing, at concentrations of 5 mM ACP (control RyR1-ACP/EGTA dataset) or 2 mM ACP (RyR1-ACP/Ca²⁺ datasets). Free Ca²⁺ was estimated with Maxchelator. Integrity of the nanodisc-embedded channels was examined by negative staining.

516

517 Cryo-EM grid preparation and data acquisition

Cryo-EM grids were cleaned with a customized protocol (Passmore & Russo, 2016) prior to glow discharge. Aliquots of 1.25 – 1.5 µl RyR1-nanodisc were applied onto each side of glow-discharged 300 mesh UltraAufoil -1.2/1.3 holey-gold (Quantifoil, Germany). The grids were blotted for 1-1.5 s with an ashless Whatman® Grade 540 filter paper in a Vitrobot Mark IV (ThermoFisher Scientific) and rapidly plunged into liquid ethane. Grid quality and RyR1 sample distribution was assessed on a Tecnai F20 (ThermoFisher Scientific) electron microscope. Data acquisition was carried out in a Titan Krios transmission electron microscope (ThermoFisher Scientific) operated at 300 kV and counting mode, with a K2 or K3 detector (Gatan) for the ACP/Ca²⁺_A and ACP/Ca²⁺_B datasets, respectively. A Gatan Quantum Energy Filter (GIF) with a slit width of 20 eV was employed. The ACP/EGTA dataset was collected on a K2 detector and a 20eV GIF. Datasets were collected in automated mode with the program Latitude (Gatan) with cumulative electron dose of 70 e⁻/Å² applied over 50-60 frames. Image acquisition parameters for RyR1-ACP/EGTA, RyR1-ACP/Ca²⁺_A and RyR2-ACP/Ca²⁺_B datasets are summarized in Table 1.

531

532 Single-Particle Image Processing

Gain-reference normalization, movie-frame alignment, dose-weighting, motion correction of the collected movie stacks was carried out with Motioncor2 (Zheng *et al.*, 2017). Contrast transfer function parameters were estimated from non-dose weighted motion corrected images using Gctf (Zhang, 2016). All subsequent image processing operations were carried out using dose-weighted, motion-corrected micrographs in RELION 3.0 (Scheres, 2012). The micrographs were low-pass filtered to 20 Å before automated particle picking. 2D class average templates for autopicking were generated by reference-free 2D classification of 1000 manually picked particles. Autopicked particles with ethane

540 and hexagonal ice-contaminated areas were removed by visual inspection. Particle image sub-stacks
 541 required for the focused reconstructions residues 3668-5037, encompassing the CD, U-motif, TMD
 542 and CTD domains, were generated using a signal subtraction procedure employed in *relion_project*
 543 module (Bai *et al*, 2015). Particle image stacks of quarter sub-volumes of RyR1 corresponding to a
 544 single subunit were generated by particle subtraction following a symmetry expansion step with
 545 *relion_particle_symmetry_expand* tool (Bai *et al*, 2015, Scheres, 2016). Composite tetrameric maps
 546 were generated from the symmetry expanded monomeric maps with *chimera* (Pettersen *et al*, 2004)
 547 *vop* maximum tool. B-factor applied to the reconstructed maps was estimated with *relion_postprocess*.
 548 Analysis of the TMD was carried out on a focused map of the CD-TMD region. Unfiltered half maps
 549 obtained from the final 3D-refinement step in RELION 3.0 were further density modified with
 550 Phenix.Resolve (Terwilliger *et al*, 2020). The reported resolutions of the cryo-EM maps are based on
 551 FSC 0.143 criterion (Scheres & Chen, 2012). Local resolution was estimated with ResMap (Kucukelbir
 552 *et al*, 2014). Pixel size calibration of post-processed maps was carried out using real space correlation
 553 metric of UCSF Chimera based on a published RyR1 cryo-EM map (des Georges *et al*, 2016). Pixel
 554 size maxima of 1.07, 1.105 and 1.07 Å were obtained in RyR1-ACP/EGTA, RyR1-ACP/Ca²⁺_A, RyR1-
 555 ACP/Ca²⁺_B respectively. Image processing schemes of the RyR1-ACP/Ca²⁺_A, RyR1-ACP/Ca²⁺_B, RyR1-
 556 ACP/EGTA datasets are summarized in Figure 1 -figure supplement 1, 3 and 4, respectively.

557

558 **Model Building and Structure refinement**

559 The cryo-EM based atomic models of RyR1 (PDB ID: 5tb3 for RyR1-ACP/EGTA, RyR1-ACP/Ca²⁺
 560 inactivated and 5ta3 for RyR1-ACP/Ca²⁺ open) were used as the initial models for model building. The
 561 best resolved symmetry expanded cryo-EM map for a single subunit in inactivated or open
 562 conformation was docked within a RyR1 monomer model with Chimera Fit in map tool. Local density
 563 fit of the RyR1 sequence was improved over an iterative process of amino acid fitting in *coot* (Emsley
 564 *et al*, 2010) alternated with real space refinement in PHENIX (Afonine *et al*, 2018). Four copies of the
 565 monomers were docked to the whole RyR1 reconstructions. Real space refinement of the tetrameric
 566 models was carried out with secondary structure and Ramachandran restraints. Further manual fitting
 567 of the CD, TMD and CTD (3668-5037) of RyR1 was carried out in *Coot*. Comprehensive model
 568 validation was carried out with PHENIX and PDB validation server at [https://validate-rcsb-](https://validate-rcsb-2.wwpdb.org/)
 569 [2.wwpdb.org/](https://validate-rcsb-2.wwpdb.org/) and are summarized in Table 2. Molecular lipophilicity potential surfaces were drawn in
 570 *chimeraX* (Ghose *et al*, 1998, Laguerre *et al*, 1997, Pettersen *et al*, 2021). Figures were generated with
 571 *PYMOL* (Schrodinger, 2015) and *chimera* programs (Pettersen *et al*, 2004, Pettersen *et al*, 2021).

572

573 **Pore radius and Helical geometry measurements**

574 Pore radii were measured for the refined atomic model coordinates of the RyR1 pore region (residues
 575 4821-5037) with the *HOLE* program (Smart *et al*, 1993). Dot-surfaces representing the channel ion
 576 permeation pathway were generated with *HOLE* implemented in *Coot*, which were reformatted to
 577 enable visualization in UCSF *chimera*. Residues per turn of S4 and S6 transmembrane helices of RyR1
 578 were calculated with *HELANAL* (Bansal *et al*, 2000).

579

580 **Flexion angle measurement**

581 The cytoplasmic shell flexion angles of RyR1 in different conformations were estimated using (Steele
582 & Samsó, 2019) with minor changes. The angle was calculated between a diagonal running from the
583 N-terminal domain (residue 348) to the P1 domain (residue 984) from the same subunit, and the
584 horizontal plane.

585

586 **Data availability**

587 Tetrameric and focused cryo-EM maps of RyR1-ACP/EGTA, RyR1-ACP/Ca²⁺ inactivated and RyR1-
588 ACP/Ca²⁺ open have been deposited in the Electron Microscopy Databank (EMDB) with the following
589 accession codes: 22616, 22597 (RyR1-ACP/EGTA tetrameric and focused maps), 25828 (RyR1-
590 ACP/Ca²⁺_A inactivated; focused map as an additional map), 25839, 25831, 25832 (three sub-classes of
591 RyR1-ACP/Ca²⁺_A inactivated), 25829 (RyR1-ACP/Ca²⁺_A open), 25833 ((RyR1-ACP/Ca²⁺_B
592 inactivated). Atomic models generated from the cryo-EM maps have been deposited in the RCSB PDB
593 database with the following accession codes: 7K0T (RyR1-ACP/EGTA), 7TDG (RyR1-ACP/Ca²⁺_A
594 inactivated), 7TDJ, 7TDI, 7TDK (three sub-classes of RyR1-ACP/Ca²⁺_A inactivated), 7TDH (RyR1-
595 ACP/Ca²⁺_A open).

596

597 **Appendix**

598 Supplementary material for this article is available in a separate file.

599

600 **Acknowledgments**

601 Cryo-grid preparation and screening were carried out at the Cryo-EM Unit at Virginia Commonwealth
602 University (VCU) supported by the VCU School of Medicine and M.S.'s funds. Cryo-EM data
603 collection was carried out at the Frederick National Laboratory for Cancer Research supported by
604 contract HSSN261200800001E, at the Molecular Electron Microscopy Core Facility at the University
605 of Virginia (UVA) (supported by NIH U24 GM116790). We thank Drs. Thomas Edwards, Ulrich Baxa
606 and Adam Wier for cryo-EM data collection at the Frederick National Laboratory. Supported by NIH
607 R01 AR068431 and the Muscular Dystrophy Association (MDA 352845) (to M.S.).

608

609

610 **Conflict of interest**

611 Authors declare that they have no conflict of interest.

613 **References**

- 614 Afonine PV, Klaholz BP, Moriarty NW, Poon BK, Sobolev OV, Terwilliger TC, Adams PD, Urzhumtsev A
 615 (2018) New tools for the analysis and validation of cryo-EM maps and atomic models. *Acta Crystallogr D*
 616 *Struct Biol* 74: 814-840
- 617 Albrecht MA, Colegrove SL, Hongpaisan J, Pivovarov NB, Andrews SB, Friel DD (2001) Multiple modes of
 618 calcium-induced calcium release in sympathetic neurons I: attenuation of endoplasmic reticulum Ca²⁺
 619 accumulation at low [Ca²⁺]_i during weak depolarization. *The Journal of general physiology* 118: 83-100
- 620 Alkhunaizi E, Shuster S, Shannon P, Siu VM, Darilek S, Mohila CA, Boissel S, Ellezam B, Fallet-Bianco C,
 621 Laberge AM (2019) Homozygous/compound heterozygote RYR1 gene variants: expanding the clinical
 622 spectrum. *American Journal of Medical Genetics Part A* 179: 386-396
- 623 Arias-Cavieres A, Barrientos GC, Sánchez G, Elgueta C, Muñoz P, Hidalgo C (2018) Ryanodine Receptor-
 624 Mediated Calcium Release Has a Key Role in Hippocampal LTD Induction. *Frontiers in Cellular Neuroscience*
 625 12
- 626 Bagur R, Hajnóczky G (2017) Intracellular Ca(2+) Sensing: Its Role in Calcium Homeostasis and Signaling.
 627 *Molecular cell* 66: 780-788
- 628 Bai X-c, Rajendra E, Yang G, Shi Y, Scheres SHW (2015) Sampling the conformational space of the catalytic
 629 subunit of human γ -secretase. *eLife* 4: e11182
- 630 Bai X-C, Yan Z, Wu J, Li Z, Yan N (2016) The Central domain of RyR1 is the transducer for long-range
 631 allosteric gating of channel opening. *Cell Research* 26: 995-1006
- 632 Bansal M, Kumart S, Velavan R (2000) HELANAL: A Program to Characterize Helix Geometry in Proteins.
 633 *Journal of Biomolecular Structure and Dynamics* 17: 811-819
- 634 Beard NA, Wei L, Dulhunty AF (2009) Control of muscle ryanodine receptor calcium release channels by
 635 proteins in the sarcoplasmic reticulum lumen. *Clin Exp Pharmacol Physiol* 36: 340-5
- 636 Bers DM (2002) Cardiac excitation–contraction coupling. *Nature* 415: 198-205
- 637 Bezprozvanny I, Watras J, Ehrlich BE (1991) Bell-shaped calcium-response curves of Ins(1,4,5)P₃- and calcium-
 638 gated channels from endoplasmic reticulum of cerebellum. *Nature* 351: 751-754
- 639 Bouchard R, Pattarini R, Geiger JD (2003) Presence and functional significance of presynaptic ryanodine
 640 receptors. *Prog Neurobiol* 69: 391-418
- 641 Chen SR, Li X, Ebisawa K, Zhang L (1997) Functional characterization of the recombinant type 3 Ca²⁺ release
 642 channel (ryanodine receptor) expressed in HEK293 cells. *The Journal of biological chemistry* 272: 24234-46
- 643 Chen W, Kudryashev M (2020) Structure of RyR1 in native membranes. *EMBO Rep* 21: e49891
- 644 Chirasani VR, Xu L, Addis HG, Pasek DA, Dokholyan NV, Meissner G, Yamaguchi N (2019) A central core
 645 disease mutation in the Ca(2+)-binding site of skeletal muscle ryanodine receptor impairs single-channel
 646 regulation. *American journal of physiology Cell physiology* 317: C358-c365
- 647 des Georges A, Clarke OB, Zalk R, Yuan Q, Condon KJ, Grassucci RA, Hendrickson WA, Marks AR, Frank J
 648 (2016) Structural Basis for Gating and Activation of RyR1. *Cell* 167: 145-157.e17
- 649 Dick IE, Joshi-Mukherjee R, Yang W, Yue DT (2016) Arrhythmogenesis in Timothy Syndrome is associated
 650 with defects in Ca(2+)-dependent inactivation. *Nat Commun* 7: 10370
- 651 Du GG, Imredy JP, MacLennan DH (1998) Characterization of recombinant rabbit cardiac and skeletal muscle
 652 Ca²⁺ release channels (ryanodine receptors) with a novel [3H]ryanodine binding assay. *The Journal of*

653 *biological chemistry* 273: 33259-66

654 Emsley P, Lohkamp B, Scott WG, Cowtan K (2010) Features and development of Coot. *Acta Crystallogr D Biol*
655 *Crystallogr* 66: 486-501

656 Flucher BE, Franzini-Armstrong C (1996) Formation of junctions involved in excitation-contraction coupling in
657 skeletal and cardiac muscle. *Proc Natl Acad Sci U S A* 93: 8101-6

658 Fodje MN, Al-Karadaghi S (2002) Occurrence, conformational features and amino acid propensities for the π -
659 helix. *Protein Engineering, Design and Selection* 15: 353-358

660 Ghose AK, Viswanadhan VN, Wendoloski JJ (1998) Prediction of Hydrophobic (Lipophilic) Properties of
661 Small Organic Molecules Using Fragmental Methods: An Analysis of ALOGP and CLOGP Methods. *The*
662 *Journal of Physical Chemistry A* 102: 3762-3772

663 Gomez AC, Holford TW, Yamaguchi N (2016) Malignant hyperthermia-associated mutations in the S2-S3
664 cytoplasmic loop of type 1 ryanodine receptor calcium channel impair calcium-dependent inactivation.
665 *American journal of physiology Cell physiology* 311: C749-c757

666 Gomez AC, Yamaguchi N (2014) Two regions of the ryanodine receptor calcium channel are involved in
667 $\text{Ca}(2+)$ -dependent inactivation. *Biochemistry* 53: 1373-9

668 Hu Y, Iyer KA, Nayak AR, Kurebayashi N, Murayama T, Samsó M (2021) Purification of Recombinant Wild
669 Type and Mutant Ryanodine Receptor Expressed in HEK293 Cell Culture. *Bio-protocol* 11: e4112

670 Iyer KA, Hu Y, Nayak AR, Kurebayashi N, Murayama T, Samsó M (2020) Structural mechanism of two gain-
671 of-function cardiac and skeletal RyR mutations at an equivalent site by cryo-EM. *Science Advances* 6: eabb2964

672 Kucukelbir A, Sigworth FJ, Tagare HD (2014) Quantifying the local resolution of cryo-EM density maps. *Nat*
673 *Methods* 11: 63-5

674 Kumar P, Bansal M (2015) Dissecting π -helices: sequence, structure and function. *Febs j* 282: 4415-32

675 Kushmerick MJ, Moerland TS, Wiseman RW (1992) Mammalian skeletal muscle fibers distinguished by
676 contents of phosphocreatine, ATP, and Pi. *Proc Natl Acad Sci U S A* 89: 7521-5

677 Laguerre M, Saux M, Dubost JP, Carpy A (1997) MLPP: A Program for the Calculation of Molecular
678 Lipophilicity Potential in Proteins. *Pharmacy and Pharmacology Communications* 3: 217-222

679 Lamb GD, Stephenson, DG (1991) Effects of Mg^{2+} on the control of Ca^{2+} release in skeletal muscle fibers of the
680 toad. *J. Physiol.* 434: 507-528.

681 Langer GA, Peskoff A (1996) Calcium concentration and movement in the diadic cleft space of the cardiac
682 ventricular cell. *Biophysical journal* 70: 1169-82

683 Laskowski RA, Swindells MB (2011) LigPlot+: multiple ligand-protein interaction diagrams for drug discovery.
684 *J Chem Inf Model* 51: 2778-86

685 Laver DR (2018) Regulation of the RyR channel gating by $\text{Ca}(2+)$ and $\text{Mg}(2)$. *Biophys Rev* 10: 1087-1095

686 Laver DR, Lamb GD (1998) Inactivation of Ca^{2+} release channels (ryanodine receptors RyR1 and RyR2) with
687 rapid steps in $[\text{Ca}^{2+}]$ and voltage. *Biophysical journal* 74: 2352-64

688 Laver DR, Roden LD, Ahern GP, Eager KR, Junankar PR, Dulhunty AF (1995) Cytoplasmic Ca^{2+} inhibits the
689 ryanodine receptor from cardiac muscle. *J Membr Biol* 147: 7-22

690 McCarthy TV, Quane KA, Lynch PJ (2000) Ryanodine receptor mutations in malignant hyperthermia and
691 central core disease. *Hum Mutat* 15: 410-7

692 Meissner G (2017) The structural basis of ryanodine receptor ion channel function. *The Journal of general*
693 *physiology* 149: 1065-1089

694 Meissner G, Darling E, Eveleth J (1986) Kinetics of rapid Ca^{2+} release by sarcoplasmic reticulum. Effects of
695 Ca^{2+} , Mg^{2+} , and adenine nucleotides. *Biochemistry* 25: 236-44

696 Meissner G, Rios E, Tripathy A, Pasek DA (1997) Regulation of skeletal muscle Ca^{2+} release channel
697 (ryanodine receptor) by Ca^{2+} and monovalent cations and anions. *The Journal of biological chemistry* 272:
698 1628-38

699 Passmore LA, Russo CJ (2016) Specimen Preparation for High-Resolution Cryo-EM. *Methods Enzymol* 579:
700 51-86

701 Pettersen EF, Goddard TD, Huang CC, Couch GS, Greenblatt DM, Meng EC, Ferrin TE (2004) UCSF Chimera-
702 -a visualization system for exploratory research and analysis. *J Comput Chem* 25: 1605-12

703 Pettersen EF, Goddard TD, Huang CC, Meng EC, Couch GS, Croll TI, Morris JH, Ferrin TE (2021) UCSF
704 ChimeraX: Structure visualization for researchers, educators, and developers. *Protein Sci* 30: 70-82

705 Priori SG, Napolitano C, Tiso N, Memmi M, Vignati G, Bloise R, Sorrentino V, Danieli GA (2001) Mutations
706 in the cardiac ryanodine receptor gene (hRyR2) underlie catecholaminergic polymorphic ventricular
707 tachycardia. *Circulation* 103: 196-200

708 Renken C, Hsieh CE, Marko M, Rath B, Leith A, Wagenknecht T, Frank J, Mannella CA (2009) Structure of
709 frozen-hydrated triad junctions: a case study in motif searching inside tomograms. *Journal of structural biology*
710 165: 53-63

711 Ríos E (2018) Calcium-induced release of calcium in muscle: 50 years of work and the emerging consensus. *The*
712 *Journal of general physiology* 150: 521-537

713 Ríos E, Zhou J, Brum G, Launikonis BS, Stern MD (2008) Calcium-dependent inactivation terminates calcium
714 release in skeletal muscle of amphibians. *The Journal of general physiology* 131: 335-48

715 Samsó M, Feng W, Pessah IN, Allen PD (2009) Coordinated Movement of Cytoplasmic and Transmembrane
716 Domains of RyR1 upon Gating. *PLOS Biology* 7: e1000085

717 Scheres SH (2012) RELION: implementation of a Bayesian approach to cryo-EM structure determination.
718 *Journal of structural biology* 180: 519-30

719 Scheres SH (2016) Processing of Structurally Heterogeneous Cryo-EM Data in RELION. *Methods Enzymol*
720 579: 125-57

721 Scheres SHW, Chen S (2012) Prevention of overfitting in cryo-EM structure determination. *Nature Methods* 9:
722 853-854

723 Schiefer A, Meissner G, Isenberg G (1995) Ca^{2+} activation and Ca^{2+} inactivation of canine reconstituted
724 cardiac sarcoplasmic reticulum Ca^{2+} -release channels. *J Physiol* 489 (Pt 2): 337-48

725 Schrodinger, LLC (2015) The PyMOL Molecular Graphics System, Version 1.8.

726 Singh AK, McGoldrick LL, Sobolevsky AI (2018) Structure and gating mechanism of the transient receptor
727 potential channel TRPV3. *Nat Struct Mol Biol* 25: 805-813

728 Sitsapesan R, Williams AJ (1995) The gating of the sheep skeletal sarcoplasmic reticulum Ca^{2+} -release
729 channel is regulated by luminal Ca^{2+} . *J Membr Biol* 146: 133-44

730 Sitsapesan R, Williams AJ (2000) Do Inactivation Mechanisms Rather than Adaptation Hold the Key to
731 Understanding Ryanodine Receptor Channel Gating? *Journal of General Physiology* 116: 867-872

732 Smart OS, Goodfellow JM, Wallace BA (1993) The pore dimensions of gramicidin A. *Biophysical journal* 65:
733 2455-60

734 Steele TWE, Samsó M (2019) The FKBP12 subunit modifies the long-range allostereism of the ryanodine
735 receptor. *Journal of structural biology* 205: 180-188

736 Terwilliger TC, Ludtke SJ, Read RJ, Adams PD, Afonine PV (2020) Improvement of cryo-EM maps by density
737 modification. *Nature Methods* 17: 923-927

738 Tiso N, Stephan DA, Nava A, Bagattin A, Devaney JM, Stanchi F, Larderet G, Brahmabhatt B, Brown K, Bauce
739 B, Muriago M, Basso C, Thiene G, Danieli GA, Rampazzo A (2001) Identification of mutations in the cardiac
740 ryanodine receptor gene in families affected with arrhythmogenic right ventricular cardiomyopathy type 2
741 (ARVD2). *Hum Mol Genet* 10: 189-94

742 Treves S, Jungbluth H, Muntoni F, Zorzato F (2008) Congenital muscle disorders with cores: the ryanodine
743 receptor calcium channel paradigm. *Current opinion in pharmacology* 8: 319-26

744 Tu MK, Levin JB, Hamilton AM, Borodinsky LN (2016) Calcium signaling in skeletal muscle development,
745 maintenance and regeneration. *Cell calcium* 59: 91-7

746 Xiong H, Feng X, Gao L, Xu L, Pasek DA, Seok J-H, Meissner G (1998) Identification of a Two EF-Hand
747 Ca²⁺ Binding Domain in Lobster Skeletal Muscle Ryanodine Receptor/Ca²⁺ Release Channel. *Biochemistry*
748 37: 4804-4814

749 Xu L, Chirasani VR, Carter JS, Pasek DA, Dokholyan NV, Yamaguchi N, Meissner G (2018) Ca²⁺-mediated
750 activation of the skeletal-muscle ryanodine receptor ion channel. *The Journal of biological chemistry* 293:
751 19501-19509

752 Xu L, Harms FL, Chirasani VR, Pasek DA, Kortüm F, Meinecke P, Dokholyan NV, Kutsche K, Meissner G
753 (2020) Single-channel properties of skeletal muscle ryanodine receptor pore Δ(4923)FF(4924) in two brothers
754 with a lethal form of fetal akinesia. *Cell Calcium* 87: 102182

755 Xu L, Mann G, Meissner G (1996) Regulation of cardiac Ca²⁺ release channel (ryanodine receptor) by Ca²⁺,
756 H⁺, Mg²⁺, and adenine nucleotides under normal and simulated ischemic conditions. *Circulation research* 79:
757 1100-9

758 Xu L, Meissner G (1998) Regulation of cardiac muscle Ca²⁺ release channel by sarcoplasmic reticulum luminal
759 Ca²⁺. *Biophysical journal* 75: 2302-12

760 Yamaguchi N (2020) Molecular Insights into Calcium Dependent Regulation of Ryanodine Receptor Calcium
761 Release Channels. *Adv Exp Med Biol* 1131: 321-336

762 Zhang K (2016) Gctf: Real-time CTF determination and correction. *Journal of structural biology* 193: 1-12

763 Zheng SQ, Palovcak E, Armache J-P, Verba KA, Cheng Y, Agard DA (2017) MotionCor2: anisotropic
764 correction of beam-induced motion for improved cryo-electron microscopy. *Nature Methods* 14: 331-332

765 Ziman AP, Ward CW, Rodney GG, Lederer WJ, Bloch RJ (2010) Quantitative measurement of Ca²⁺(+) in the
766 sarcoplasmic reticulum lumen of mammalian skeletal muscle. *Biophysical journal* 99: 2705-14

767

768

769
770

Figure legends and Tables

771
772
773
774
775
776
777
778
779
780
781
782
783
784
785
786
787

Figure 1. RyR1 at high Ca²⁺ concentration exhibits a mixture of open and inactivated conformations.

(A) Ryanodine binding of rabbit skeletal SR microsomes showing the Ca²⁺-induced inactivation of RyR1; activators ATP or ACP (2 mM) increased channel open probability by 3- and 4.5-fold, respectively. Ca²⁺ concentrations of 100 μM -2 mM progressively decrease the probability of opening (P_o). Mean specific [³H]-ryanodine binding ± SEM from four independent experiments. (B) Pore profile of RyR1-ACP/EGTA, and of open and inactivated conformations in the RyR1-ACP/Ca_A²⁺ dataset calculated with the program HOLE (Smart *et al.*, 1993). The position of relevant landmarks is indicated with violet shadowing. Radius corresponding to a dehydrated Ca²⁺ ion shown with a dashed line. (C) Dotted surfaces of RyR1 ion permeation pathway in closed, open and inactivated conformations, color-coded according to pore radius. Overlaid coordinates of the S5, S6, pore helices and selectivity filter are shown for two diagonal protomers. Insets, cytoplasmic views of the Ile4937 constriction and corresponding pore diameter measured at the Ca backbone. Similar results were obtained for RyR1-ACP/Ca_B²⁺ (Figure 1-figure supplement 5).

789
790
791
792
793
794
795
796
797
798
799
800
801
802
803
804
805
806
807
808
809
810
811
812
813
814
815
816

Figure 2. Inactivation of RyR1 involves out-of-plane rotation of the central block and rearrangement around the Ca^{2+} activation site.

(A) The high-affinity Ca^{2+} binding site in the CD/CTD interface with electron density around the Ca^{2+} site contoured at 4σ . Contacts within 2.8 Å from Ca^{2+} , as well as additional contact Gln3970-Ser4029 within 3.6 Å, are represented by dashed lines. Channel axis is on the left. During the transition from open to inactivated conformations, the CD/CTD block tilts around the Ca^{2+} binding site such that the protruding fourth helix of the CD (h) and connected CTD tilt inward, while the CD-C' tilts upward and away from the SR membrane -with Gln3970 separating from Ca^{2+} by ~6 Å. Arrows and stationary reference lines illustrate the conformational changes undergone with respect to the panel on the left. The region represented relative to the channel is highlighted with a square in panel C. See Figure 2-figure supplement 1 for the corresponding solid sphere representation. (B) Heat map showing $\text{C}\alpha$ -backbone RMSD (in Å) between domain pairs from respective conformations after aligning them (pre-aligned), in their native conformation (unaligned), and after aligning the protomers through their respective CD (CD-aligned). The RMSD difference between unaligned and pre-aligned represents the change caused by domain relocation. (C) Overlaid structures of the CD-CTD-S6 domains in different conformations; only two protomers shown for clarity. Left, in the transition from RyR1-ACP/ Ca^{2+} open (gray) to RyR1-ACP/ Ca^{2+} inactivated (colored), the CD-CTD block, “connected” by Ca^{2+} coordination, undergoes an out-of-plane rotation around the Ca^{2+} binding site that pushes S6C' toward the pore axis, closing the channel. Right, in the transition from RyR1-ACP/ Ca^{2+} inactivated (colored) to RyR1-ACP/EGTA (green), Ca^{2+} unbinding disconnects the CD from the CTD. Structures at the bottom right of each panel show the comparison of the CD-CTD-S6C' of the central block after forcing superimposition of their respective CDs. Residue boundaries for relevant domains are specified. (D) Schematics of the conformational changes from RyR1-ACP/EGTA (closed), to RyR1-ACP/ Ca^{2+} open, to RyR1-ACP/ Ca^{2+} inactivated. Activation, where Ca^{2+} binds to the high affinity site, is required prior to inactivation. The CD-protruding fourth helix (3753-3769) is indicated by “h”. Colored arrows indicate the conformational change toward the following structure.

Figure 3. Two salt bridges between the EF hands and the S2-S3 loop are determinant for the inactivated state.

(A) Cytoplasmic SR view (top row) and side view (bottom row) highlighting the EF hand domain (blue) and S2-S3 loop (magenta) at the subunit interface. Counterclockwise in-plane and ascending out-of-plane rotations of the central region in the transition from RyR1-ACP/EGTA (closed) to RyR1-ACP/Ca²⁺ open brings the two domains closer together. Further rotation of the CD (tan color in top row) and its protruding EF hand domain in RyR1-ACP/Ca²⁺ inactivated brings the two domains in contact. The inter Cα-Cα contact distances illustrate the progressive approximation of the two domains. Two salt bridges, Lys4101-Asp4730 and Glu4075-Arg4736 form in the RyR1-ACP/Ca²⁺ inactivated conformation. The location of the domains in the context of RyR1 is shown on the right panels. (B) MH/CCD mutation sites in the interface between the EF hand domain and S2-S3 loop that abolish Ca²⁺-dependent inactivation (black), versus MH/CCD mutations without effect on inactivation (green). The two EF loops are indicated (EF1, EF2). Residues forming the salt bridges are labeled according to domain color. R4736, directly forming the salt bridge, is susceptible to MH mutation.

834
835
836

837
838
839
840
841
842
843
844
845
846
847
848
849

Figure 4. Changes in the interaction network of the nucleotide and complexed Ca^{2+} . The ATP binding pocket is formed by the U-motif, CTD and S6C'. (A) Cryo-EM densities (gray mesh) of ACP in the different states represented at map significance values (RMSD σ) above 12, 7 and 7, respectively. Residues within 4 Å of either ACP, Ca^{2+} or Zn^{2+} (from the CTD zinc finger) are color-coded according to domain. Notably, an interaction between His4983 (CTD) and ACP is only observed in the inactivated state. Fitted water densities (w) are represented in red. (B) Left, ACP goes deeper into the ATP binding pocket in RyR1-ACP/ Ca^{2+} inactivated (colored structure, blue ACP) as compared to the open state (gray structure, red ACP) due to a 6° rotation (see arrows). Right, absence Ca^{2+} in the closed state (green) causes release of the CTD from the CD, resulting in a 4° tilt of the CTD and U-motif (see arrows). Additional reorganization allows closer interaction between ACP and U-motif in the closed state.

A **B** **D** **C**

851
852
853
854
855
856
857
858
859
860
861
862
863
864
865
866
867

Figure 5. The nanodisc environment and visualization of lipids in a crevice of the TMD.

(A) Top, central slice of the side view of the RyR1-ACP/Ca²⁺_A open and RyR1-ACP/Ca²⁺_A inactivated cryo-EM maps highlighting the density corresponding to the upper and lower nanodisc belts. Bottom, corresponding views seen from the cytoplasmic direction. For clarity, the nanodisc density (green) was extracted and low-pass filtered to 7 Å resolution. The top belt of the nanodisc expands slightly to accommodate the conformational change; see also Figure 5 – Video 1. (B) Side and luminal views of the TMD of RyR1-ACP/Ca²⁺_A inactivated with putative lipid densities shown in blue. (C) Lipid binding pocket of RyR1-ACP/Ca²⁺_A inactivated lined by lipophilic amino acids from S3 and S4 of the voltage sensor-like domain (S1-S4; yellow) and core helices S5 and S6 (cyan) from two different protomers. Amino acids within 5 Å from the lipids are shown (sticks) with their corresponding side chain densities. Electron densities corresponding to the lipids contoured at 8σ (in blue) are modeled as a PC (16:0-11:0) for lipid-1 and a 16 C acyl chain for lipid-2. (D) Molecular lipophilicity potential of the surface lining the crevice, ranging from hydrophilic (cyan) to hydrophobic (golden). The hydrophobic tails of the lipids are shown in blue and the negative electrostatic surface potential of the polar lipid head is shown in red.

Figure 6. Local conformational changes in the transmembrane helices among closed, open and inactivated conformations.

(A) Top, residues per turn for S4 reveals a 3_{10} helix (4807-4813; blue dashed line) at its N', and helical changes among the three conformations. Residues per turn are computed for a 3-residue moving window. Bottom, a segment of S4 displaying 3_{10} helix in FFF (4807-4809). Uncoiling of the S4-C' in the open structure rotates the FFF region, including Phe4808 which interacts with lipid-2 in the inactivated structure. (B) Top, the N' region of S6 (residues 4920-4928) contains a tetra Phe motif (4920-4923) followed by a π -helix region. Bottom, the backbone hydrogen bonding network in the π -helix region in the three distinct conformations; dashed lines show $i \leftarrow i+5$ hydrogen bonds. (C) A Cys4876-Cys4882 disulfide bond formed between the luminal loop (4860-4878) and the pore helix (4879-4893) of the same subunit and predicted hydrogen bonds between the P-loop (pore helix) and S6 from the neighboring subunit likely contribute to stabilize the luminal mouth of RyR1. The cryo-EM densities are shown in mesh.

886 **Table 1. Summary of cryo-EM data collection and image processing parameters**

Dataset	RyR1 ACP/EGT A closed	RyR1 ACP/Ca ²⁺ _A inactivated	RyR1 ACP/Ca ²⁺ _A inactivated class1	RyR1 ACP/Ca ²⁺ _A inactivated class2	RyR1 ACP/Ca ²⁺ _A inactivated class3	RyR1 ACP/Ca ²⁺ _A inactivated CDTM	RyR1 ACP/Ca ²⁺ _A open	RyR1 ACP/Ca ²⁺ _A open CDTM	RyR1 ACP/Ca ²⁺ _B inactivated	RyR1 ACP/Ca ²⁺ _B open
Data Acquisition										
Microscope/Detector	Krios/K2	Krios/K3							Krios/K2	
Voltage (kV)	300	300							300	
Magnification	130000	81000							130000	
Defocus range (µm)	-1.2 to -2.2	-1.25 to -2.5							-1.25 to -2.5	
Pixel Size (Å) (calibrated)	1.06 (1.07)	1.08 (1.105)							1.06 (1.07)	
Total electron dose (e/Å ²)	70	70							70	
Exposure time (sec)	12	4.4							14	
Number of frames	60	50							50	
Total number of Micrographs	1,959	10002							1346	
Image Processing										
Total number of Particles selected	52,856	311258							35554	
Final number of Particles	21,551	90530	79277	159444	71499	90530	14012	14012	14669	4160
Reconstruction symmetry	C4	C4	C1	C1	C1	C4	C4	C4	C4	C4
Map Resolution, FSC (0.143) (Å) (symmetry expanded map)	4.3 (3.9)	3.8 (3.5)	3.7	3.3	3.8	3.5	4.6 (4.0)	4.4	4.1 (3.8)	5.8
Map sharpening B-factor (Å ³) (symmetry expanded map)	-132	-150 (-144)	-125	-102	-123	-142	-168 (-135)	-144	-111(-89)	-148
EMDB ID	22616, 22597	25828	25831	25830	25832	25828*	25829	25829*	25833	-

*Submitted as an additional map

887
888
889
890

Table 2. Summary of model refinement and validation statistics

Dataset	RyR1 ACP-EGTA	RyR1 ACP/Ca ²⁺ _A inactivated	RyR1 ACP/Ca ²⁺ _A inactivated class1	RyR1 ACP/Ca ²⁺ _A inactivated class2	RyR1 ACP/Ca ²⁺ _A inactivated class3	RyR1 ACP/Ca ²⁺ _A open
RMS Deviation (Bonds)	0.004	0.086	0.004	0.004	0.003	0.004
RMS Deviation (Angle)	0.919	0.812	0.893	0.788	0.848	0.766
Ramachandran Plot statistics (%)						
Preferred	92.39	93.38	93.85	95.54	94.13	94.90
Allowed	7.45	6.57	5.97	4.38	5.75	5.07
Outliers	0.16	0.05	0.19	0.08	0.12	0.03
Clash-score	7.58	7.65	5.62	3.90	4.94	4.60
MolProbity Score	1.89	1.85	1.71	1.49	1.65	1.59
PDB ID	7K0T	7TDG	7TDJ	7TDI	7TDK	7TDH

891
892

893
894
895
896
897
898
899
900
901
902
903
904
905
906
907
908
909
910

Table 3. Differences between closed, open and inactivated conformations.

Conformation	Closed	Open	Inactivated
Pore	closed	open	closed
Flexion angle (average)	-2.2°	-5.3°	-4.4°
In-plane rotation	0° (reference)	counterclockwise	counterclockwise
High affinity Ca ²⁺ site	empty	occupied	occupied
Ca ²⁺ bound to ACP	no	yes	yes
CD/CTD	disconnected	connected	connected
Position of CD-C'	down	up/intermediate	up
Position of CD-N'	up	down/intermediate	down
EF hand / S2-S3 loop	separated	separated	form 2 salt bridges
S6 TMD helix (4921-4928)	π	π wide-short	π narrow-long
Lipids in TMD crevice	yes	absent	present

Legends for the supplementary figures

Figure 1- figure supplement 1. Image Processing Scheme for the RyR1-ACP/Ca²⁺_A dataset

(A) Representative micrograph collected on a Titan Krios at 81,000x magnification with a K3 camera in counting mode. (B) Representative 2D class averages obtained by reference-free 2D classification in RELION. (C) Image processing workflow with tetrameric and single subunit-masked RyR1 particles in RELION. Overall resolution values obtained for the inactivated conformations after density modification in Phenix resolve. (D) Euler angle distribution of the particles contributing to the RyR1-ACP/Ca²⁺_A inactivated (3.8 Å resolution) and RyR1-ACP/Ca²⁺_A open (4.6 Å resolution) maps. (E) Left, Gold standard Fourier shell correlation prior to density modification. Right, Local resolution of the symmetry-expanded maps calculated with ResMap. (F) Main 3D classes obtained from the focused 3D classification after symmetry expansion with NTD and CD domains highlighted in yellow and green, respectively, with the pore axis on the right. Classification yielded one open conformation and three closed conformations that differ in the flexion angle of the cytoplasmic domain.

Figure 1 – Figure supplement 2. Correlation of RyR1-ACP/Ca²⁺_A inactivated model with the cryo-EM density

Model quality for RyR1-ACP/Ca²⁺_A inactivated in the consensus cryo-EM map; domains in the central and transmembrane regions are shown.

Figure 1 – figure supplement 3. Image processing scheme for the RyR1-ACP/Ca²⁺_B dataset

(A) Representative micrograph collected on a Titan Krios at 130,000x magnification with a K2 camera in super resolution mode. (B) Representative 2D class averages obtained by reference-free 2D classification in RELION. Overall resolution values obtained for inactivated 3D-class after a density modification in Phenix resolve. (C) Workflow of image processing with tetramer and single subunit-masked particles carried out in RELION. (D) Euler angle distribution of the particles contributing to the RyR1-ACP/Ca²⁺_B inactivated and RyR1-ACP/Ca²⁺_B open maps at 4.1 Å and 5.8 Å resolution, respectively. (E) Left, Gold standard Fourier shell correlation prior to density modification. Right, Local resolution of the symmetry-expanded map calculated with ResMap with flexion angle of the cytoplasmic domain indicated.

Figure 1 -figure supplement 4. Image processing scheme for the RyR1-ACP/EGTA dataset

(A) Representative micrograph collected on a Titan Krios at 130,000x magnification with a K2 camera in super-resolution mode. (B) Representative 2D class averages obtained by reference-free 2D classification in RELION. (C) Image processing workflow including 3D classification focused on RyR1's quarter carried out in RELION. (D) Euler angle distribution of the particles contributing to the 4.3 Å resolution 3D map. (E) Left, Gold standard Fourier shell correlation. Right, Local resolution of the symmetry-expanded map calculated with ResMap.

Figure 1 – figure supplement 5. Pore conformation in the RyR1-ACP/Ca²⁺_B dataset

(A) Dotted surfaces of RyR1 ion-permeation pathway in open and inactivated conformations. Inset, Fourfold cytoplasmic views of Ile4937 constriction and corresponding pore diameter (measured at Cα backbone) with respective cryo-EM maps (mesh). (B) Left, Pore profiles of inactivated and open conformations calculated with HOLE. Radius corresponding to a dehydrated Ca²⁺ ion is shown as a dotted line. Right, fourfold cytoplasmic views of the Gln4933 constriction in RyR1-ACP/Ca²⁺_A and RyR1-ACP/Ca²⁺_B pores with their respective cryo-EM maps.

Figure 2 – figure supplement 1. Reorganization of the high-affinity Ca²⁺ binding site at the CD/CTD interface under different conditions

Ca²⁺ binding site in the CD/CTD interface in different conformations with involved residues shown with spheres. Residues Glu3983, Glu3967, Gln3970 (CD), and Thr5001 (CTD), within 2.8 Å from Ca²⁺ in the open state, are shown. In going from RyR1-ACP/Ca²⁺_{open} to RyR1-ACP/Ca²⁺_{inactivated}, Gln3970 switches its interaction from Ca²⁺ to Ser4029 (interaction within 3.6 Å). Arrows and stationary reference lines illustrate the conformational changes undergone with respect to the panel on the left. The region represented relative to the channel is highlighted with a square in Figure 2, panel C; fourfold axis is on the left.

965 **Figure 2 – figure supplement 2. Rotation axes of the CD of RyR1 among the different conformational transitions**
966 (A) Reproducibility of the conformation of the “central block” of RyR1-ACP/Ca²⁺-inactivated from two independent
967 datasets (A and B, superimposed), with an RMSD of 0.95 Å over the Ca atoms. The central block comprises the CD, EF
968 hand, U-motif, S6C’ and CTD. (B) Transition from RyR1-ACP/Ca²⁺ open (gray) to RyR1-ACP/Ca²⁺ inactivated (colored)
969 involves an out-of-plane rotation of the CD around the yellow-colored axis. This results in inward tilt in CD-N’ (brown
970 arrows) and upward shift/tilt of CD-C’ and connected EF hand domain (blue arrows). Two orthogonal views are shown,
971 and the fourfold axis on the left panels, in front of the structure, is indicated. (C) Transition from RyR1-ACP/Ca²⁺
972 inactivated (colored) to RyR1-ACP/EGTA closed (gray) involves out-of-plane rotation of the CD of RyR1 around the
973 magenta-colored axis.

974
975 **Figure 4 – figure supplement 1. Map significance of the ACP and putative Ca²⁺ densities under different conditions**
976 Root Mean Square Deviation (RMSD σ) values of the densities attributed to Ca²⁺ and ACP in the cryo-EM maps of RyR1-
977 ACP/EGTA closed, RyR1-ACP/Ca²⁺ open and RyR1-ACP/Ca²⁺ inactivated.

978
979 **Figure 5 – figure supplement 1. Lipid binding pocket lined by the S3/S4 and S5⁺/S6⁺ helices in inactivated and closed**
980 **structures of RyR suggests a conserved functional site.**

981 (A) Cytoplasmic fourfold view of part of the TMD showing movement of the transmembrane helices S3, S4 and S5
982 (arrows) into the lipid pocket while transitioning from RyR1-ACP/Ca²⁺ open to inactivated conformations. No ordered
983 lipids were observed in the open channel. Each subunit is displayed with a different color. (B) Side view of the lipophilic
984 pocket in RyR1-ACP/Ca²⁺ open with overlaid lipid backbone extracted from the RyR1-ACP/Ca²⁺ inactivated conformation,
985 showing expected steric hindrance of lipid-1 and lipid-2 with S6 and S4, respectively, as indicated with asterisks. See also
986 Figure 6A, bottom panels. (C) Side view of RyR1-ACP/Ca²⁺ inactivated with resolved lipids. (D) Side view of the
987 lipophilic pocket in RyR1-ACP/EGTA, with lower resolution but foreseeable lack of steric hindrance between protein and
988 lipids. Overlaid lipid backbones are extracted from the inactivated conformation. (E) Side view of the lipophilic pocket in
989 RyR2 closed at 3.27 Å resolution (PDB ID: 6WOU (Iyer et al, 2020)) with lipids resolved in a comparable conformation to
990 that found for RyR1 inactivated. Corresponding residues of interest are indicated. The + sign indicates transmembrane
991 helices of the neighboring subunit.

992

993 **Figure 2 - Video 1. Ca²⁺-induced transitions in activation and inactivation**

994 During activation, Ca²⁺ coordination joins the CD and CTD-S6C’ region, separating the S6 four-helix bundle and opening
995 the channel. High Ca²⁺-induced inactivation promotes out-of-plane rotation of the CD-CTD-S6C’ block. This pushes the S6
996 helices towards the pore axis, closing the channel. Only two protomers in diagonal are shown.

997
998 **Figure 5 - Video 1. Gating-induced conformational changes in nanodisc**

999 Conformational change in upper nanodisc belt while morphing from RyR1-ACP/Ca²⁺ inactivated to open conformations.
1000 The nanodisc can be seen expanding when transitioning to the open state. Volumes are low pass filtered and helices are
1001 displayed at higher threshold for better visualization.

1002

A **B** **D** **C** **E**

closed
class-2

F

closed -class1

closed -class2

closed -class3

open

A **B** **D** **E** **C**

A **B** **D** **E** **C**

closed

open

inactivated

A **B**

ACP/ Ca^{2+} open to ACP/ Ca^{2+} inactivated

central block (side view)

CD (cytoplasmic view)

C

ACP/ Ca^{2+} inactivated to ACP/EGTA