Molecular basis for gating of cardiac ryanodine receptor explains the mechanisms for gain- and loss-of function mutations

Takuya Kobayashi, Akihisa Tsutsumi, Nagomi Kurebayashi, Kei Saito, Masami Kodama, Takashi Sakurai, Masahide Kikkawa, Takashi Murayama, Haruo Ogawa

Cardiac ryanodine receptor (RyR2) is a large Ca^{2+} release channel in the sarcoplasmic reticulum and indispensable for excitation-contraction coupling in the heart. RyR2 is activated by Ca^{2+} and RyR2 mutations are implicated in severe arrhythmogenic diseases. Yet, the structural basis underlying channel opening and how mutations affect the channel remains unknown. Here, we address the gating mechanism of RyR2 by combining high-resolution structures determined by cryo-electron microscopy with quantitative functional analysis of channels carrying various mutations in specific residues. We demonstrated two fundamental mechanisms for channel gating: interactions close to the channel pore stabilize the channel to prevent hyperactivity and a series of interactions in the surrounding regions is necessary for channel opening upon Ca^{2+} binding. Mutations at the residues involved in the former and the latter mechanisms cause gain-of-function and loss-of-function, respectively. Our results reveal gating mechanisms of the RyR2 channel and alterations by pathogenic mutations at the atomic level.
Cardiac ryanodine receptor (RyR2) is a Ca\textsuperscript{2+} release channel in the sarcoplasmic reticulum and plays a central role in cardiac muscle contraction\textsuperscript{1-2}. In cardiac excitation–contraction coupling, Ca\textsuperscript{2+} influx occurs through L-type voltage-dependent Ca\textsuperscript{2+} channels in the transverse (T) tubule membrane activates RyR2 to release a large amount of Ca\textsuperscript{2+}, a process known as Ca\textsuperscript{2+}-induced Ca\textsuperscript{2+} release (CICR)\textsuperscript{3,4}. In human RyR2, nearly 300 pathogenic mutations have been reported as arrhythmogenic heart diseases, including catecholaminergic polymorphic ventricular tachycardia (CPVT)\textsuperscript{5-8}, and idiopathic ventricular fibrillation (IVF)\textsuperscript{9-12}. Notably, these mutations divergently alter channel activity: mutations related to CPVT cause gain-of-function, whereas those for IVF lead to either gain- or loss-of-function\textsuperscript{13-14}.

RyR is a large tetrameric ion channel (~2.2 MDa), with each monomer composed of ~5000 amino acid residues and comprising a large N-terminal mushroom-like structure of the cytoplasmic domain and C-terminal transmembrane (TM) region\textsuperscript{15-18}. The opening of RyR channels is initiated by binding of Ca\textsuperscript{2+} to the Ca\textsuperscript{2+}-binding site, which was proposed at the interface between central and CTD domains by structural analysis using cryo-electron microscopy (EM)\textsuperscript{19} and, later, validated by functional analysis using site-directed mutagenesis\textsuperscript{20,21}. The mechanism of channel opening by Ca\textsuperscript{2+} binding has been extensively studied by structural analysis of the channel at the open state\textsuperscript{19,22-26}. These results demonstrated that Ca\textsuperscript{2+}-binding causes a large conformational change throughout the molecule via a large number of intra/inter-domain interactions, thereby the channel opens.

The activity of the RyR channels is modulated by various small molecules and associated proteins\textsuperscript{27}. The RyR structures in complex with regulatory molecules, such as ATP, caffeine, FKBP12, or calmodulin, have also been determined, and the mechanisms by which these regulatory molecules modulate RyR have been extensively discussed\textsuperscript{19,25,26}. In addition, the structures of three different gain-of-function mutants with mutations in the N-terminal domains have recently been reported\textsuperscript{28,29}. In all structures, slight slippages in the inter-domain interactions around the mutated site occur, and both groups concluded that these slippages may lead to the increase in the open probability in the channel through inter-domain interactions.

Although many structures of RyR have been so far determined, the molecular mechanism of channel gating of RyR still remains largely obscured, due to a lack of our knowledge about the key interactions for the conformational change, and how and in what order these multiple interaction-networks interlock for the channel opening. In addition, all the open structures obtained to date contain additional ligands (ATP, caffeine, and/or PCB95), which help the opening of the channel but might make it difficult to discriminate conformational changes by Ca\textsuperscript{2+}. To precisely trace the Ca\textsuperscript{2+}-induced conformational changes, it is necessary to obtain an open structure with Ca\textsuperscript{2+} alone.

In this study, we determine high-resolution closed and open structures of recombinant mouse RyR2. The open structure was successfully obtained by Ca\textsuperscript{2+} alone. We perform a functional analysis of the RyR2 channels carrying mutations in amino acid residues involved in the observed interactions. Finally, to support our hypothesis of gating mechanism, we determine the structures of a loss-of-function mutant. We demonstrate two fundamental mechanisms for channel gating of RyR2. In the resting state without Ca\textsuperscript{2+}, the channel is stabilized in the closed state by multiple interactions close to the channel pore. Upon Ca\textsuperscript{2+} binding, a series of interactions in the surrounding regions moves the S4-S5 linker outward to open the gate. Disruption of the former and the latter interactions causes gain-of-function and loss-of-function, respectively. Our results reveal mechanisms underlying channel opening upon Ca\textsuperscript{2+} binding and explain how pathogenic mutations alter channel activity.

Results

Overall conformational changes in RyR2 associated with Ca\textsuperscript{2+} binding. Recombinant mouse RyR2, expressed and purified from HEK293 cells using FKBP12.6 affinity chromatography\textsuperscript{30}, formed homogeneous tetrameric channels (Supplementary Fig. 1a, b). To precisely trace the Ca\textsuperscript{2+}-induced RyR2 structural changes, we adopted high salt conditions, in which the channel activity was markedly enhanced by Ca\textsuperscript{2+} alone but Ca\textsuperscript{2+}-dependent activation was preserved (Supplementary Fig. 1c-e)\textsuperscript{31,32}. We performed the cryo-EM single-particle analysis in the presence of 1 mM EGTA and 100 μM Ca\textsuperscript{2+} for closed and open states, respectively. Three-dimensional (3D) classification including focused classification analysis using the TM region revealed two and three classes in the presence of EGTA and Ca\textsuperscript{2+}, respectively (Supplementary Fig. 1f, g and Supplementary Table 1). No major differences were observed among the classes both in the closed and open states. Therefore, subsequent analysis was performed using the class with the highest resolution (before the final classification in the closed state and class 1 in the open state; Supplementary Table 1). The overall resolution of the final model was 3.3 Å and 3.45 Å for the closed and open states, respectively, and the local resolution for the TM region in the closed state was better than 2.9 Å (Supplementary Fig. 1f, g), which allowed us to build precise atomic models and identify specific residues important for channel gating (Supplementary Fig. 1h, i). In fact, it was confirmed that the pore was open only by the addition of 100 μM of Ca\textsuperscript{2+} (Supplementary Fig. 2a), and moreover, the boundary of FKBP12.6 is clearly visible in both the closed and open states (Supplementary Fig. 1j, k). Conformational changes between closed and open states were large and spanned the entire molecule (Fig. 1a, b, Supplementary Fig. 2b–d and Supplementary movie 1). Changes associated with Ca\textsuperscript{2+} binding were analyzed in three layers with different heights parallel to the membrane, i.e., the C-terminal domain (CTD), U-motif, and S4–S5 layers (Fig. 1c–e and Supplementary movie 1). Upon Ca\textsuperscript{2+} binding, CTD rotated clockwise (viewed from the cytoplasmic side) (Fig. 1c), U-motif rotated clockwise toward the S2–S3 linker domain (Fig. 1d), and S6 leaned outward from the center to open the gate accompanied by rearrangements of S1–S4 helices and outward movement of the S4–S5 linker (Fig. 1e). The C-terminal side of the Central domain (C-Central), U-motif, CTD, and cytoplasmic S6 (S6\textsubscript{cyto}) were tightly attached to each other in both closed and open states (Fig. 1f, Supplementary Fig. 2c, e, f and Supplementary movie 2) and rotated together 9.8° clockwise with respect to the axis approximately parallel to S6 (Fig. 1g). In contrast, the N-terminal side of the Central domain (N-Central) did not follow the rotation due to splitting the Central domain into two parts with G3987 as the pivot (Fig. 1f). Since the rotation axis was close to the Ca\textsuperscript{2+}-binding site, slight movements caused by Ca\textsuperscript{2+} binding resulted in very large movements around S6 (Fig. 1g).

Given these series of movements, we hypothesized that the rotation of the C-Central/U-motif/S6\textsubscript{cyto}/CTD complex upon Ca\textsuperscript{2+} binding leads to two independent downstream pathways for channel pore opening: (1) the S6\textsubscript{cyto} movement leads to the outward leaning of S6; (2) the U-motif movement leads to sequential movements of the S2–S3 linker domain and TM segments, causing outward movement of the S4–S5 linker and creating a space where S6 can lean into (Fig. 1h). To prove this hypothesis, we performed detailed structural analysis and functional studies.
Fig. 1 Conformational changes upon Ca\textsuperscript{2+} binding. a Overlay of RyR2 in the closed (light blue) and open (yellow) states viewed from the direction parallel to the lipid bilayer is shown as a ribbon model. Two facing protomers in the RyR2 tetramer are shown. b Magnified view of the dotted box in (a). In the left protomer, each domain is colored (N-Central, light pink; C-Central, purple; U-motif, magenta; S1–S5, wheat; S2–S3 linker domain, light green; S4–S5 linker, warm pink; S6, blue; CTD, orange). S4–S5 linker and S6 moved outside upon Ca\textsuperscript{2+} binding as indicated by the red arrows. Three regions parallel to the membrane are defined as CTD, U-motif, and S4–S5 layers. Ca\textsuperscript{2+}, shown as a cyan ball; Zn\textsuperscript{2+}, shown as a gray ball. c–e Cross-section views of CTD, U-motif, and S4–S5 layers. The closed state is colored in light blue and the open state is colored according to (b) or yellow. In (e), Cα representation overlaid with cylindrical TM helices is used. Ca\textsuperscript{2+} binding causes clockwise rotation of CTD (green arrow in (c)), U-motif (green arrow in (d)), and S1-S4 TM helices and outward movement of S4-S5 linker and S6 (red arrows in (e)). f C-Central/U-motif/S6\textsubscript{cyto}/CTD complex. Closed (light blue) and open (colored according to (b)) states are overlaid at the CTD. Central domain is split into two parts at G3987 which works as the pivot of the rotation upon Ca\textsuperscript{2+} binding. g Rotation of the C-Central/U-motif/S6\textsubscript{cyto}/CTD complex upon Ca\textsuperscript{2+} binding viewed from the rotation axis. h Scheme of channel opening upon Ca\textsuperscript{2+} binding. Two independent pathways via S6 and S4–S5 linker are hypothesized. Source data are provided as a Source Data file.
U-motif/S2–S3 linker domain interaction is a key for signal transduction in response to Ca2+ binding. In our hypothesis, the U-motif/S2–S3 linker domain interaction is important in transducing the rotation of the U-motif to TM segment movement (Fig. 1d, h and Supplementary movie 1, 2). We found three key interactions between the U-motif and S2–S3 linker domain formed by hydrogen bonds or salt bridges (Fig. 2a–c, Supplementary Fig. 3a, b and Supplementary movie 3). E4198-K4593-S4167 was evident in the open state, whereas Y4498-K4593 was only formed in the closed state, indicating that K4593 switches the interacting partner between the two states. Additionally, E4193-R4607 was stable in both states.

To clarify the roles of hydrogen bonds/salt bridges in channel gating, functional assays were conducted with recombinant RyR2 carrying mutations at the specific residues (Supplementary Table 2). Ca2+-dependent [3H]ryanodine binding was conducted as it reflects channel activity9,33. Wild-type (WT) RyR2 exhibited biphasic Ca2+-dependent [3H]ryanodine binding (Fig. 2d). This is explained by two independent Ca2+ binding sites; binding of Ca2+ to high-affinity site formed by N-Central and CTD opens the channel, whereas that to undetermined low-affinity site closes the channel27,34. Alanine substitution at K4593 markedly reduced the binding (Fig. 2d). Two pathogenic mutations, K4593Q and K4593R12, also led to the loss of binding (Fig. 2e and Supplementary Fig. 3c). In K4593R, two nitrogen atoms (Nε1 and Oε2) from E4198 in the open state, which prevents S4167-E4198 interaction essential for the channel opening. Similarly, binding was severely reduced after alanine substitutions (Y4498A, K4593A, S4167P)12 in the interacting pairs (Fig. 2d, e and Supplementary Fig. 3c). The behaviors of mutant RyR2 channels were also evaluated in live cells by monitoring cytoplasmic and endoplasmic reticulum (ER) luminal Ca2+ homeostasis9,35. In WT RyR2-expressing HEK293 cells, spontaneous Ca2+ oscillations occurred with a concomitant decrease in [Ca2+]ER in WT, while the K4593 mutant showed no Ca2+ oscillations with an increased [Ca2+]ER, indicating loss-of-function of the channel. Data were analyzed by one-way ANOVA with Dunnett’s multiple comparisons test. ***p < 0.001 from WT. Source data are provided as a Source Data file.

**Fig. 2** Key interactions between U-motif and S2–S3 linker domain upon channel opening. a–c Interface of U-motif and S2–S3 linker domain in the closed state (a), open state (b), and an overlay of both states (c) viewed parallel to the membrane is shown as a Ca model. Amino acid residues involved in key interactions are shown as stick models. The color of carbon atoms is the same as that of Ca; oxygen, red; nitrogen, blue. The TM region forming α-helices is overlaid with the cylinder model. Hydrogen bonds or salt bridges are shown as orange dotted lines. Density maps around side chains shown in (a) and (b) are superimposed and contoured at 0.025. d–g Functional analysis of mutants involved in U-motif/S2–S3 linker domain interaction. d Ca2+-dependent [3H]ryanodine binding of WT and representative mutants. Data are shown as means ± SD (n = 4). e Summary of [3H]ryanodine binding of WT and mutants at pCa 4.5. Data are shown as means ± SD (n = 6 and 4 for WT and mutants, respectively) and were analyzed by one-way ANOVA with Dunnett’s multiple comparisons test. ***p < 0.001 from WT. f Representative traces of cytoplasmic ([Ca2+]cyt) and ER ([Ca2+]ER) Ca2+ signals of HEK293 cells expressing WT or K4593A. Spontaneous Ca2+ oscillations occurred with a concomitant decrease in [Ca2+]ER in WT, while the K4593 mutant showed no Ca2+ oscillations with an increased [Ca2+]ER, indicating loss-of-function of the channel. g Summary of the upper level of [Ca2+]ER signals in WT and mutants. All mutants showed loss-of-function of the channel. Data are shown as box-and-whisker plots, with the median for all subjects shown as the center line, the box representing the 25–75 percentile, and the lines showing the range of the data (n = 97, 68, 76, 73, 46, 35, 44, 36, 46, 54, 63, and 53 for WT, S4167A, S4167P, E4193A, E4198A, Y4498A, K4593A, K4593Q, K4593R, R4607A, R4607Q, and R4607W, respectively). Data were analyzed by one-way ANOVA with Dunnett’s multiple comparisons test. ***p < 0.001 from WT. Source data are provided as a Source Data file.
suggest that both E4198-K4593-S4167 and Y4498-K4593 interactions are important for channel opening. We also evaluated the E4193-R4607 interaction and found that alanine substitutions (E4193A and R4607A) and pathogenic mutations (R4607Q and R4607W) led to a loss-of-function of the channel (Fig. 2d–g and Supplementary Fig. 3c). These residues and surrounding sequences are well conserved in all three mammalian RyR subtypes (Supplementary Fig. 3d). These findings indicate that three interactions above S2 in the U-motif/S2–S3 linker domain interface play a critical role in transducing the Ca$^{2+}$-binding signal to S2 and that loss of these interactions results in a loss of channel function.

Movements of the S1–S4 bundle lead to outward movement of the S4–S5 linker. We hypothesize that movement of the S2–S3 linker domain is an important step in the channel opening through the coordinated movement of transmembrane segments (Fig. 1d, e, h). Movement of the S2-S3 linker domain causes S2 movement (Fig. 2c and Supplementary movie 3). This leads to the coordinated 7.6° clockwise rotation of S1, S3, and S4 (Figs. 1e, 3a–c, Supplementary Fig. 4a–d and Supplementary movie 1, 4). S1, S2, S3, and S4 are arranged in a circle, placed at equal intervals in a clockwise direction (Fig. 3a–c and Supplementary Fig. 4a–d). We found interactions between S1 and S2 (S1/S2), S2/S3, S3/S4, and S1/S4, all of which were maintained in both closed and open states (Fig. 3a, b, Supplementary Fig. 4a–d and Supplementary movie 4). The hydrophobic interactions between F4497 and L4592 at S1/S2; the hydrogen bond between Y4589 and D4715 at S2/S3; hydrogen bond between Y4720 and D4744 at S3/S4; and the salt bridge between R4501 and D4744 at S1/S4 appear to bundle the four TM segments into one (i.e., S1–S4 bundle).

Alanine substitution of residues involved in S1/S2 (F4497 and L4592) and S2/S3 (Y4589 and D4715) interactions exhibited significantly reduced [3H]ryanodine binding (Fig. 3d, e and Supplementary Fig. 5a), loss of Ca$^{2+}$ oscillations, and increased ER Ca$^{2+}$ (Figs. 3f, g) — all indicative of loss-of-function. The pathogenic mutant F4497C also resulted in loss-of-function (Fig. 3e, g and Supplementary Fig. 5a). Thus, S1/S2 and S2/S3 interactions are necessary for channel opening. In contrast, alanine substitution or pathogenic mutations of residues involved in S1/S4 (R4501 and D4744) and S3/S4 (Y4720 and D4744) interactions caused gain-of-function of the channel with increased [3H]ryanodine binding and reduction in ER Ca$^{2+}$ (Fig. 3d–g and Supplementary Fig. 5a). Especially, D4744A, which is involved in both interactions, exhibited greatly increased [3H]ryanodine binding with an enhanced Ca$^{2+}$ sensitivity for activation and loss of Ca$^{2+}$ inactivation (Fig. 3d and Supplementary Fig. 5c, d). These residues are well conserved in all the mammalian RyR subtypes (Supplementary Fig. 5e).

In our hypothesis, rotation of the S1–S4 bundle causes movement of the S4–S5 linker to open the channel (Fig. 1e, h). We found that the upper part of S4 rotated 40° counterclockwise to form an α-helix upon channel opening, which dramatically changed the position of F4749 (Fig. 3h–j, Supplementary Fig. 4e–g and Supplementary movie 4). The S4–S5 linker, which is unfolded and significantly bent in the direction of S6 in the closed state, rewraps to an α-helix and moves outward (Supplementary Fig. 4e–h and Supplementary movie 4). Hydrophobic interaction between F4749 in the upper part of S4 and L4505 in S1 was identified in the closed state (Fig. 3h and Supplementary Fig. 4a, h).

Alanine substitutions of residues involved in hydrophobic interactions (L4505A and F4749A) and the pathogenic mutant L4505P caused gain-of-function with increased Ca$^{2+}$ sensitivity and loss of Ca$^{2+}$ inactivation (Fig. 3k–n and Supplementary Fig. 5b–d). Notably, no additive effects were observed upon binding of [3H]ryanodine to double mutant L4505A_F4749A (Supplementary Fig. 5b), supporting an interaction between the two residues. Whereas L4505 is conserved in all the mammalian RyR subtypes, F4749 is mutated to valine in the RyR1 subtype (Supplementary Fig. 5e). However, unlike the alanine mutant, F4749V exhibited biphasic Ca$^{2+}$ dependent [3H]ryanodine binding with a slightly reduced peak value (Supplementary Fig. 5b).

Taking these findings into consideration, we hypothesized a molecular mechanism in which a series of movements regulates channel opening. A brief summary of the roles of the key residues located on the S1–S4 bundle is shown as a scheme (Fig. 3o). In the absence of Ca$^{2+}$, the channel is stabilized in the closed state as the S4–S5 linker is unfolded and bent in the direction of S6 by the “stopper”, in the form of L4505-F4749 interaction. The L4505-F4749 interaction prevents the α-helix formation of the upper part of S4 and is supported by S1/S4 and S3/S4 interactions that keep the two residues appropriately placed. Upon Ca$^{2+}$ binding, clockwise rotation of the S1–S4 bundle induced by U-motif/S2–S3 linker domain interaction alters the relative positions of L4505 and F4749 to release the stopper. This allows the α-helix formation of the upper part of S4 and subsequent α-helix formation and outward movement of the S4–S5 linker to open the channel. Since the α-helix formation of the S4–S5 linker shortened its length in the open state (Fig. 3o, Supplementary Fig. 4g), we assume that the rewinding to α-helix in the upper part of S4 provides a margin for shortening of the S4–S5 linker. Loss of necessary interactions to release the stopper, i.e., U-motif/ S2–S3 linker domain, S1/S2, and S2/S3, leads to loss-of-function. Conversely, loss of interactions that support S4, i.e., S1/S4 and S3/ S4, spontaneously releases the stopper to cause a gain-of-function. This well explains the differential roles of TM helices on the channel gating (Fig. 3o).

U-motif plays a key role in stabilizing the channel in the closed state. U-motif is a key component for the RyR2 channel opening as described above (Fig. 1d, h). It constitutes the U-motif/S6cyto/CTD complex, which was stable in both the closed and open states (Fig. 1f). In the closed state, we found a close contact between U-motif and S6cyto (Fig. 4a, b, Supplementary Fig. 6a and Supplementary movie 5), which was similar as observed in the closed state structure of RyR1. In the closed state, F4173, V4176, and N4177 in the U-motif and Q4875 and V4879 in S6cyto faced each other forming van der Waals interactions, and were surrounded by Q4876 and Q4878 from S6 (Fig. 4b, Supplementary Fig. 6a and Supplementary movie 5). In the open state, S6cyto self-rotated (~30° clockwise at Q4875) and U-motif/S6cyto loosened and appeared unstable (Fig. 4c, Supplementary Fig. 6a–c and Supplementary movie 5). Buried surface area analysis calculated by CNS37 demonstrated that the U-motif/S6cyto interface in the open state (362 Å$^2$) was smaller than in the closed state (514 Å$^2$). The U-motif also interacted with CTD via F4888, where it penetrated partly into the hydrophobic pocket formed by U-motif F4171, I4172, and V4175 and CTD L4914 (Fig. 4d, Supplementary Fig. 6d and Supplementary movie 5). The U-motif/CTD interaction was stable in both states. We also noticed compaction of U-motif upon channel opening, which was induced by a 2.5 Å parallel shift of its N-terminal helix (4161–4178) toward the C-terminal side (4183–4205) (Fig. 4e–g). Upon binding of Ca$^{2+}$ to CTD, the upper part of S6cyto rotated together with CTD (Figs. 1g, 4g). Along with the rotation, S6cyto pushed the N-terminal helix of U-motif, thereby, the parallel shift of the N-terminal helix occurred. This parallel shift seems to be the key trigger for releasing the restraint of S6cyto from U-motif,
allowing further rotation and outward movement of S6_cyt (Fig. 4c, g and Supplementary Fig. 6a). Two hydrogen bonds (S4167-E4198 and E4198-K4593) newly formed upon opening seem to be the key for stabilizing this shift. Other important factors seem to be π–π interaction formed by F4171-F4191 and two consecutive glycines (G4179 and G4180) located on the loop connecting the N-terminal and the C-terminal helix of the U-motif (Fig. 4g and Supplementary Fig. 6e). The former may function as a guide for the shift of the N-terminal helix (Fig. 4g, inset), and the latter may function as the flexible hinge to enable the deformation of the U-motif.

All alanine-substituted mutants of residues interacting between U-motif and S6_cyt as well as two CPVT mutations (N4177S and N4177Y) led to gain-of-function (Fig. 4h–k) with mild enhancement...
Fig. 3 Key interactions in the transmembrane region upon channel opening. **a**-c** The S1–S4 bundle. Closed state (a), open state (b), and overlay of the structures in both states (c) are shown as a Ca model and overlaid with cylinder models. Hydrogen bonds/salt bridges are shown as orange dotted lines. Density maps around side chains shown in (a) and (b) are superimposed and contoured at 0.03. **d**-**g** Functional analysis of mutants involved in the S5/S2, S2/S3, S1/S4, or S3/S4 interaction. **d** Ca$$^{2+}$$-dependent [3H]ryanodine binding of WT and representative mutants. Data are shown as means ± SD (n = 4). **e** Summary of [3H]ryanodine binding of WT and mutants at pCa 4.5. Data are shown as means ± SD (n = 6 and 4 for WT and mutants, respectively) and were analyzed by one-way ANOVA with Dunnett’s test. ***p < 0.001 from WT. **f** Representative traces of [Ca$$^{2+}$$]ER signals of HEK293 cells expressing Y4589A or D4744A. **g** Summary of the upper level of [Ca$$^{2+}$$]ER signals in WT and mutants. Data are shown as box-and-whisker plots, with the median for all subjects shown as the center line, the box representing the 25–75 percentile, and the lines showing the range of the data (n = 97, 69, 65, 66, 35, 56, 74, 54, 63, 39, and 94 for WT, F4497A, F4497C, L4592A, Y4589A, D4715A, R4501A, Y4720A, Y4720C, and D4744A, respectively) and were analyzed by one-way ANOVA with Dunnett’s test. ***p < 0.001 from WT. **h** TI-M region around S4. Closed state (h), open state (i), and overlay of the structures in both states (j). Structures in both states were fitted to the bottom part of S4 helices and viewed along the axis of the S4 helix and from the cytoplasm; the Ca model overlaid with cylinder models. Main chain representation of the S4–S5 linker. Density maps around side chains shown in (h) and (i) are superimposed and contoured at 0.03. K Ca$$^{2+}$$-dependent [3H]ryanodine binding of WT and representative mutants. Data are shown as means ± SD (n = 4). **l** Summary of [3H]ryanodine binding of WT and mutants at pCa 4.5. Data are shown as means ± SD (n = 6 and 4 for WT and mutants, respectively) and were analyzed by one-way ANOVA with Dunnett’s test. ***p < 0.001 from WT. **m** Representative traces of [Ca$$^{2+}$$]ER signals in WT and mutants. Data are shown as box-and-whisker plots, with the median for all subjects shown as the center line, the box representing the 25–75 percentile, and the lines showing the range of the data (n = 97, 69, 65, 66, 35, 56, 74, 54, 63, 39, and 94 for WT, F4497A, F4497C, L4592A, Y4589A, D4715A, R4501A, Y4720A, Y4720C, and D4744A, respectively) and were analyzed by one-way ANOVA with Dunnett’s test. ***p < 0.001 from WT. **n** Scheme of the structure in the S1-S4 TM helices and S4-S5 linker. S1-S4 helices are drawn with a circle, and the positions of Ca atoms of S4-S5 linker are connected by a line. Amino-acid numbers associated with signal transduction and stabilization are shown in blue and red, respectively. While the S1/S2 and S2/S3 interactions are involved in signal transduction, the S1/S4 and S3/S4 interactions are involved in the stabilization of the channel in the closed state. Alteration of the relative positions of L4505 and F4749 to release the stopper shown as the green dotted box. This allows α-helix formation of the upper part of S4 and subsequent α-helix formation and outward movement of the S4–S5 linker to open the channel. Since α-helix formation of the S4–S5 linker shortened its length in the open state, the rewinding to α-helix in the upper part of S4 provides a margin for shortening of the S4–S5 linker. The position of the Ca atom of I4755 is shown as a black-filled circle. Source data are provided as a Source Data file.
It is interesting that the rotation due to Ca\textsuperscript{2+} binding occurred only in CTD and the upper part of S6\textsubscript{cyto} (Fig. 5c, g, Supplementary Fig. 9f). Since CTD is placed between 1-turn \(\beta\) sheet connected just before U-motif (Supplementary Fig. 9f), U-motif, CTD, and S6\textsubscript{cyto} are supposed to rotate together upon Ca\textsuperscript{2+} binding as observed in the open state (Fig. 1f, Fig. 5c, g, Supplementary Fig. 9f). The independent movement of CTD in K4593A indicates that CTD placed between 1-turn \(\beta\) sheet and U-motif have some degree of freedom in the rotation. The upper part of S6\textsubscript{cyto} rotated together with CTD because of a direct connection to CTD (Supplementary Fig. 9f). However, the rotation throughout S6 was blocked by the collision with the uncompacted U-motif in K4593A (Supplementary Fig. 9f).

**Summary of the Ca\textsuperscript{2+} induced movements in RyR2.** Here, we summarize the channel opening mechanism associated with Ca\textsuperscript{2+} binding of RyR2 determined by high-resolution cryo-EM structures: (1) binding of Ca\textsuperscript{2+} rotates the U-motif/S6\textsubscript{cyto}/CTD complex by 9.8° along with the axis which is 40 Å apart from S6\textsubscript{cyto}; (2) compaction of U-motif; (3) extruding the S2–S3 linker domain by the U-motif causes S2 to move; (4) movement of S2 rotates the S1–S4 bundle by 7.6° to release the stopper comprising F4749/L4505; (5) rotation of the upper part of S4 allows outward movement of the S4–S5 linker, creating a space where S6 can lean into; and (6) rotation of S6\textsubscript{cyto} by 30° causes the outward leaning of S6 by 7.8 Å (Fig. 5i). Thus, the sequential rotations of domains/\(\alpha\)-helices and domain-domain interactions may occur in conformation changes associated with Ca\textsuperscript{2+} binding. Rotation of
**U-motif in** K4593A *in the presence of Ca\(^{2+}\)* stopped in the middle of the transition from the closed state to the open state, that is, binding of Ca\(^{2+}\) rotates only U-motif by 4.2° along with the same axis as the rotation from closed state to open state (Fig. 5)).

**Discussion**

By combining high-resolution cryo-EM structures and quantitative functional analysis of mutant channels, we successfully clarified the gating mechanism of RyR2 upon Ca\(^{2+}\) binding. Figure 6a shows a schematic diagram of the channel core domain of RyR2 with details of the important interactions identified in this study. Mutations of residues involved in the signaling pathway for outward movement of the S4–S5 linker caused loss-of-function, whereas those supporting the stopper (i.e., S1/S3, S3/S4, and S1/S4 interactions) or the U-motif/CTD/S6\(_{\text{cyto}}\) complex led to gain-of-function. Given the locations of these mutations, it becomes apparent that interactions close to the channel pore are important for stabilizing the channel in the closed state and those in the surrounding region are essential for channel opening.

The present results can reasonably explain the gating mechanism of RyR2 channel and how pathogenic or alanine mutations affect the channel activity (Fig. 6b–d). In the closed state of the WT channel, the S4–S5 linker prevents outward leaning of S6 (Fig. 6b, left). In the open state of the WT, binding of Ca\(^{2+}\) moves CTD, which transmits signals into two downstream pathways to open the channel; S4–S5 linker is unlocked by sequential conformational changes beginning at the U-motif and S6 leans outward by the movement of S6\(_{\text{cyto}}\) (Fig. 6b, right). In addition to direct regulation of the channel gate, S6\(_{\text{cyto}}\) interacts with U-motif to restrict its movement toward the S2–S3 linker domain (blue T-shaped line, Fig. 6b, left). This negative regulation by S6\(_{\text{cyto}}\) is supported by hydrophobic interaction between CTD and U-motif (F4888) and U-motif (dotted line, Fig. 6b, left). Since the interaction is still maintained at the open state, the WT channel is not fully activated (Fig. 6b, right). Mutations in the U-motif/S2–S3 linker domain, S1/S2, or S2/S3 interface disrupt the interaction, which is essential for the channel opening (Fig. 6c, left). This interrupts Ca\(^{2+}\)-induced conformational changes, resulting in loss-of-function (Fig. 6c, right). Mutations in S3/S4 or S1/S4 interface unlock the S4–S5 linker (Fig. 6d, upper left). However, in the absence of Ca\(^{2+}\), this does not induce spontaneous outward movement of the S4–S5 linker and the channel pore is kept closed. In the presence of Ca\(^{2+}\), these channels will show gain-of-function since the S4–S5 linker is more mobile (Fig. 6d, upper right). Mutations in the U-motif/S6\(_{\text{cyto}}\) or the U-motif/CTD disrupt the interaction to make the U-motif and S6\(_{\text{cyto}}\) more mobile (Fig. 6d, lower left). Upon Ca\(^{2+}\) binding, these channels will show gain-of-function by increased mobility in the S4–S5 linker and S6\(_{\text{cyto}}\) (Fig. 6d, lower right). Considering that residues involved in the interactions are highly conserved and pathogenic mutations in RyR1 (1A405P, D4744H, and F4888Y; see Supplementary Table 2) also showed corresponding behaviors, the fundamental mechanism of channel gating may be common among three RyR subtypes.

Many structures of pig RyR2 have been determined\(^ {24–26}\). Among them, structures in the open state have been classified into two groups: PCB95 in addition to Ca\(^{2+}\) (Ca\(^{2+}/\text{PCB95}\)) and ATP/cafeine in addition to Ca\(^{2+}\) (Ca\(^{2+}/\text{ATP/caffeine}\)). We determined open structure of RyR2 with Ca\(^{2+}\) alone in high salt solution in which channel activity is greatly enhanced but Ca\(^{2+}\)-dependent activation is preserved (Supplementary Fig. 1c-e). We compared our structure in the open state with those structures by analyzing conformational changes upon Ca\(^{2+}\) binding at the CTD layer and at the U-motif layer (Supplementary Fig. 10a). In the Ca\(^{2+}/\text{PCB95}\) structure, rotation of CTD and U-motif occurred upon opening, thereby outward movement of S6 occurred (Supplementary Fig. 10a, top). In the Ca\(^{2+}/\text{ATP/caffeine}\) structure, in contrast, both CTD and U-motif made a translational outward movement toward caffeine (Supplementary Fig. 10a, middle). Conformational change in our structures was similar to that observed in the Ca\(^{2+}/\text{PCB95}\) structure (Supplementary Fig. 10a, bottom). Ca\(^{2+}/\text{PCB95}\) open structure was only determined in the absence of FKB12.6 because the addition of FKB12.6 closes the channel\(^ {26}\). This is in contrast to our open structure which binds FKB12.6 (Supplementary Fig. 1k).

Previous structures of RyR in the presence of Ca\(^{2+}\) alone failed to get an open state; the channel pore was closed\(^ {19,26}\). Instead, slight movements of CTD, U-motif, and S6 occurred upon Ca\(^{2+}\) binding (referred to as ‘primed’ state). Analysis of conformational changes in CTD and U-motif layers revealed slight rotation of CTD and U-motif and slight outward movement of S6 in the primed state, indicating an intermediate state between closed and open states (Supplementary Fig. 10b, top). In contrast, our K4593A(Ca\(^{2+}\)) structure showed no movement of U-motif and S6 at all, reflecting disruption of transduction signal from Ca\(^{2+}\) binding (Supplementary Fig. 10b, bottom). Thus, K4593A(Ca\(^{2+}\)) is a particular state caused by a specific mutation and is different from the reported primed state.
The outward movement of the S4–S5 linker is important in the channel opening, as it creates the space where S6 can lean into. des Georges et al. provided a more detailed description about the movement of the S4–S5 linker in RyR1; it is bent in the direction of S6 in the closed state, but it straightens and alters its contacts with S6, thereby opening the channel pore. We showed similar conformational changes in the S4–S5 linker in RyR2 and further demonstrated that rewinding of the upper part of S4 to an α-helix, which is caused by rotation of the S1–S4 bundle, drives the S4–S5 linker movement (Fig. 3h–j and Supplementary Fig. 4e–h). Regulatory mechanisms of interaction between the S4–S5 linker and S6 will be the next subject for a complete understanding of channel gating.
In RyR1, rearrangement of the salt bridges between S1 (R4563), S3 (Y4791) and S4 (D4815) has been reported to occur upon channel opening. We showed that the corresponding salt bridges between R4501, Y4720, and D4744, which are critical to keeping S4 in place for appropriate positioning of the stopper (L4505-F4749 interaction), were maintained in both closed and open states in RyR2 (Fig. 3 and Supplementary Fig. 4). Interestingly, a recent study showed that diaminocicatides bind to the pocket within the S1–S4 bundle of RyR1, thereby disrupting the salt bridge to open the channel. Our results reasonably explain why such insecitcides activate the RyR channel.

The U-motif is proposed to be important in channel gating as it forms a stable complex with S6cyclo and CTD, which move together when the channel opens. This movement is accomplished by a rigid shift in the S2–S3 linker domain, which has been proposed to eventually open the channel pore. We found that the interaction of the U-motif with the S2–S3 linker domain is critically important in the channel opening; disruption of the interaction causes loss-of-function of the channel (Fig. 2 and Supplementary Fig. 3). In addition, we demonstrated that the U-motif/S6cyclo interaction stabilizes the channel to the closed state (Fig. 4 and Supplementary Figs. 6, 7). Stabilization of the channel is important not only for the prevention of the hyperactivity but also for regulation by various stimuli or modifications that activate the channel, e.g., phosphorylation or oxidation/S-nitrosylation.

The U-motif/S6cyclo interaction is a stabilization mechanism that is identified at the molecular level and thus may contribute to the regulation of the RyR channel. In RyRs, the large cytoplasmic region is believed to play an important role in regulating channel opening. Indeed, a number of pathogenic mutations are located in the cytoplasmic region. The question arises as to how these mutations, located far from the core domain, cause changes in the channel activity. We demonstrated that the C-Central domain forms a tight complex with the U-motif/S6cyclo/CTD to move together upon Ca2+ binding (Fig. 1f and Supplementary Fig. 2f, j). The N-Central domain also moves upon Ca2+ binding (Fig. 1f). Through the N-/C-Central domains, the core domain has a series of interactions with the N-terminal domains (NTD), HANDLE, and HD1 domains which consist a major part of the large cytoplasmic region. Thus, it is possible that the conformational changes of the cytoplasmic region by pathogenic mutations affect the channel gating through these interactions. Recently, RyR1 and RyR2 structures carrying gain-of-function mutations at N-terminal domains (NTD) were reported. Determining the structures of various mutant RyRs will help to elucidate the long-range allosteric gating mechanism in the future.

In conclusion, we have revealed the gating mechanism of RyR2 upon Ca2+ binding and provided structural insights into the effects of pathogenic mutations on channel activity. These findings may greatly help develop more effective drugs to treat RyR2-associated diseases.

Methods
Expression and purification of SBP-FKBPI2.6. Expression and purification of SBP-tagged FKBPI2.6 were performed as described previously with some modifications. Briefly, cDNA for SBP-tagged human FKBPI2.6 was subcloned into the pET28 vector (Novagen) containing a 6×His-tag at the N-terminus. Escherichia coli BL21 cells (Novagen) transformed with the above expression vector were grown at 37°C in 500 mL LB medium containing ampicillin at a final concentration of 80 μg/mL. After reaching an OD600 of 0.9–1.2, SBP-FKBPI2.6 expression was induced by adding 1 mM IPTG (Wako) for 3 h at 37°C. Cells were then harvested, resuspended in 20 mM MOPS-Na (pH 7.4), 300 mM NaCl, 20 mM imidazole, and a cocktail of protease inhibitors (antipain (Peptide Institute), aprotinin (Nacalai), chymostatin (Peptide Institute), leupeptin (Peptide Institute) and pepstatin A (Peptide Institute), each 2 μg/mL), and lysed using a sonicator in an ice bath. The lysate was centrifuged at 100,000 × g for 30 min at 4°C and the pellet was discarded. The supernatant was incubated for 1 h at 4°C with 0.5 mL Profinity IMAC affinity resin (Bio-Rad Laboratories, Hercules, CA) in a buffer containing 20 mM MOPS-Na (pH 7.4), 300 mM NaCl, and 20 mM imidazole. The resin was washed five times with the buffer and SBP-FKBPI2.6 was eluted with buffer containing 300 mM imidazole. The eluted protein was quickly frozen in liquid nitrogen and stored at –80°C.

Construction of WT and mutant RyR2 cDNA expression vector and generation of stable HEK293 cell lines. cDNA for RyR2 was cloned from mouse ventricle and inserted into pcDNA5/FRT/TO vector (ThermoFisher). Alanine substitution or pathogenic mutations were introduced by inverse PCR using F5.
Fig. 6 Details of interactions and schematic diagram of the channel gating of WT and mutant RyR2 upon Ca\textsuperscript{2+} binding. **a** Details of interactions identified in this study. Amino-acid residues shown in red letter and blue letter indicate gain-of-function and loss-of-function by alanine-substituted or pathogenic mutations, respectively. Arrows indicate interactions. Purple and green arrows indicate interactions only found in the closed and open states, respectively. **b–d** Schematic diagram of the channel gating of WT and mutant RyR2 upon Ca\textsuperscript{2+} binding. The left and right diagrams show the states in the absence and presence of Ca\textsuperscript{2+}, respectively. The black lines, the domain interactions; The dotted line, CTD/U-motif interaction via F4888; The blue T-shaped lines, U-motif/S6cyto interaction that acts as a suppressor; The black lines with arrowhead, signals for channel opening. The domains shown in gray-scale and colored indicate the domains in the inactive and active states, respectively. **b** In WT., the channel pore is closed, since the movement of S4-S5 linker is locked. Ca\textsuperscript{2+} binding unlocks the S4-S5 linker and induces the outward leaning of S6, resulting in the pore opening. **c** Loss-of-function (LOF) mutants. Mutations in the U-motif/S2-S3 linker domain (#1), S1/S2 (#2) or S2/S3 (#3) interface cause disconnection of signal transduction. The binding of Ca\textsuperscript{2+} therefore cannot induce the outward movement of the S4-S5 linker and the channel pore is kept closed. **d** Gain-of-function (GOF) mutants. (Upper panels) Mutations in S3/S4 (#4) or S1/S4 (#5) interface unlock the S4-S5 linker to be activated. In the absence of Ca\textsuperscript{2+}, the channel pore is kept closed, since the outward leaning of S6 does not occur spontaneously. Binding of Ca\textsuperscript{2+} causes hyperactivity of the channel, since the S4-S5 linker is more active than WT. (Lower panels) Mutations in U-motif/S6cyto (#6) or CTD/U-motif (#7) reduce or lose U-motif/S6cyto interaction. The binding of Ca\textsuperscript{2+} causes hyperactivity of the channel, since S6 and the S4-S5 linker are more active than WT.

Preparation of microsomes from RyR2-expressing HEK293 cells. HEK293 cells stably expressing mouse RyR2 (wild type or mutants) were grown in sixty 150-mm cell culture dishes. At 70–80% confluency, protein expression was induced by 2 µg/mL doxycycline (SIGMA) for 48 h. Cells were then harvested, rinsed with cold phosphate-buffered saline (PBS) (GIBCO), and microsomes were prepared as described by Inesi et al.\textsuperscript{19}. Briefly, the cell pellet was resuspended in 60 mL of 10 mM NaHCO\textsubscript{3} in the presence of protease inhibitors and processed for nitrogen cavitation for 30 min at 1000 psi. The suspension was diluted with 60 mL of 0.6 M sucrose, 0.3 M KCl, 40 mM MOPS-Na (pH 7.4), and protease inhibitors and then centrifuged at 1000 × g for 10 min. The supernatant was supplemented with 30 mL of 2 M KCl, 0.3 M sucrose, 20 mM MOPS-Na (pH 7.4), and the protease inhibitor cocktail, and ultracentrifuged again. Finally, the pellet was resuspended in 12 mL of 0.3 M sucrose, 20 mM MOPS-Na (pH 7.4), and the protease inhibitor cocktail, followed by quick freezing in liquid nitrogen and storage at −80 °C until further use.
Purification of RyR2. RyR2 was purified using SBP-FKBP12.6 affinity chromatography with some modifications. The microsomes were solubilized with 2% (w/v) CHAPS (Dejindo) and 1% (w/v) soybean lecithin (Avanti Polar Lipids) in 1 M NaCl, 20 mM MOPS (pH 7.4), 2 mM dithiothreitol, and the protease inhibitor cocktail for 30 min on ice. After centrifugation at 100,000 × g for 30 min at 4°C, the supernatant was diluted with four volumes of 20 mM MOPS (pH 7.4), 2 mM dithiothreitol, and the protease inhibitor cocktail, after which it was passed through a 0.45-μm filter unit and dialyzed against 80 mM NaCl, 20 mM MOPS–Na (pH 7.0), 2 mM dithiothreitol, 1 mM AMP, and 1 mM MgCl2. Free Ca2+ was adjusted to 10 mM EGTA using WEBMAG MAXC. A program on the web (https://somapp.ucdmc.ucdavis.edu/pharmacology/bers/maxchelator/webmaxc/webmaxcS.htm). The [3H]ryanodine binding data (B) were normalized to the maximal fluorescence intensity (Bmax) which was separately determined by Scatchard plot analysis using various concentrations (5–20 nM) of [3H]ryanodine in a high-salt medium. The resultant B/Bmax represents the averaged activity of each mutant.

Single-cell Ca2+ imaging. Single-cell Ca2+ imaging was performed using HEK293 cells expressing WT or mutant RyR2. Na+ signals from the cyto-plasm (F Ca2+ Ca2+) and ER lumen (F Ca2+ ER) were monitored using G-GEOLI.1 (a gift from Robert Campbell from the University of Alberta; Addgene plasmid #32445) and R-CEPIA1er (a gift from Masamitsu Iino, Nihon University, Tokyo, Japan; Addgene plasmid #58216), respectively. Cells were transfected with cDNAs for these Ca2+ indicators 26–28 h before measurement, and at the same time, detergents (2 μg/mL) were added to the culture medium to induce RyR2 expression. Ca2+ signals were measured in HEPES-buffered Krebs solution (140 mM NaCl, 5 mM KCl, 2 mM CaCl2, 1 mM MgCl2, 11 mM glucose, and 5 mM HEPES at pH 7.4) for 5 min and then in 10 mM caffeine-containing Krebs solution for 1.5 min. At the end of each experiment, Fmim and Fmax were obtained with 0Ca-Krebs solution containing 20 μM ionomycin, 5 mM BAPTA, and 20 μM cyclopiazonic acid and 20Ca-Krebs solution containing 20 μM ionomycin and 20 mM CaCl2, respectively. The fluorescence signal (F − Fmim) was normalized to the maximal fluorescence intensity (Fmax − Fmim). Measurements were carried out at 26°C.

Statistics. Data are presented as means ± SD. One-way analysis of variance (ANOVA), followed by Dunnett’s multiple comparisons test, was performed to compare the multiple groups. Statistical analysis was performed using Prism 9 (GraphPad Software, Inc., La Jolla, USA).

Reporting summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability. All data generated or analyzed in this study are available within the article and its Supplementary Information. All raw data supporting the findings of this study are available from the corresponding author upon request. Atomic coordinates and cryo-EM density maps have been deposited in the Protein Data Bank (PDB) and the Electron Microscopy Data Bank (EMDB) under the accession codes 7VML [https://doi.org/10.2210/pdb7VML/pdb] and EMDB-30688 (closed state before classification), 7VMN [https://doi.org/10.2210/pdb7VMN/pdb] and EMDB-30689 (closed state class 2), 7VMO [https://doi.org/10.2210/pdb7VMO/pdb] and EMDB-30691 (open state class 3), 7VMP [https://doi.org/10.2210/pdb7VMP/pdb] and EMDB-30692 (open state class 4), 7VMQ [https://doi.org/10.2210/pdb7VMQ/pdb] and EMDB-30693 (open state class 3), 7MR [https://doi.org/10.2210/pdb7MR/pdb] and EMDB-32036 (K4593A mutant in the presence of 1 μM EGTA), 7VMS [https://doi.org/10.2210/pdb7VMS/pdb] and EMDB-32037 (K4593A mutant in the presence of 100 μM of Ca2+). Source data are provided with this paper.

Received: 20 November 2021; Accepted: 28 April 2022; Published online: 20 May 2022

References
1. Bers, D. M. Macromolecular complexes regulating cardiac ryanodine receptor function. J. Mol. Cell Cardiol. 37, 417–429 (2004).
Santulli, G., Lewis, D., des Georges, A., Marks, A. R. & Frank, J. Ryanodine receptor structure and function in health and disease. *Subcell. Biochem.* 87, 320–352 (2018).

Endo, M. Calcium-induced calcium release in skeletal muscle. *Physiol. Rev.* 89, 1153–1176 (2009).

Fabião, A. & Fabião, F. Calcium-induced release of calcium from the sarcoplasmic reticulum of skinned cells from adult human, dog, cat, rabbit, rat, and frog hearts and from fetal and newborn rat ventricles. *Ann. NY Acad. Sci.* 307, 491–522 (1978).

Kawamura, M. et al. Genetic background of catecholaminergic polymorphic ventricular tachycardia in Japan. *Circ. J.* 77, 1705–1713 (2013).

Pérez-Riera, A. R. et al. Catecholaminergic polymorphic ventricular tachycardia, an update. *Ann. Nominus. Electrocardiol.* 23, e12512 (2018).

Pedraza, S. G. et al. Clinical and molecular characterization of patients with catecholaminergic polymorphic ventricular tachycardia. *Circulation* 106, 74–94 (2002).

Testor, D. J., Spoon, D. B., Valdivia, H. H., Makielski, J. C. & Ackerman, M. J. Targeted mutational analysis of the RyR2-encoded cardiac ryanodine receptor in sudden unexplained death: a molecular autopsy of 49 medical examiner/coroner’s cases. *Mayo Clin. Proc.* 79, 1380–1384 (2004).

Fujii, Y. et al. A type 2 ryanodine receptor variant associated with reduced Ca2+ release and short-coupled torsades de pointes ventricular arrhythmia. *Heart Rhythm* 14, 98–107 (2017).

Paech, C. et al. Ryanodine receptor mutations presenting as idiopathic ventricular fibrillation: a report on two novel familial compound mutations, c.6224 T > C and c.13781 A > G, with the clinical presentation of idiopathic ventricular fibrillation and sudden death. *Proc. Natl Acad. Sci. USA* 104, 18309–18314 (2007).

Priori, S. G. et al. Clinical and molecular characterization of patients with inherited dysfunction of sarcoplasmic reticulum Ca2+ handling and arrhythmogenesis. *Circ. Res.* 108, 871–883 (2011).

Clarke, O. B. & Hendrickson, W. A. Structures of the colloidal RyR1 calcium release channel. *Curr. Opin. Struct. Biol.* 39, 144–152 (2016).

Hirose, S. et al. Loss-of-function mutations in cardiac ryanodine receptor channel cause various types of arrhythmias including long QT syndrome. *European* 24, 497–510 (2022).

Benkusky, N. A., Farrell, E. F. & Valdivia, H. H. Ryanodine receptor channelopathies. *Biochem. Biophys. Res. Commun.* 332, 1280–1285 (2004).

Priori, S. G. & Chen, S. R. Inherited dysfunction of sarcoplasmic reticulum Ca2+ handling and arrhythmogenesis. *Circ. Res.* 108, 871–883 (2011).

Brünger, A. T. et al. Crystallography & NMR system: a new software suite for structural biology. *Acta Crystallogr D.* 54, 105–109 (1998).

Ma, R. et al. Structural basis for diamide modulation of ryosensitive transition. *Nat. Chem. Biol.* 16, 1246–1254 (2020).

Epley, S. & Inesi, G. Phospholipid orientation in sarcoplasmic membranes: spin-label ESR and proton NMR studies. *Biochim. Biophys. Acta* 282, 174–179 (1972).

Woll, K. A., Haji-Ghassemi, O., Van & Petegem, F. Pathological conformational changes of disease mutant ryanodine receptors revealed by cryo-EM. *Nat. Commun.* 12, 807 (2021).

31. Meissner, G. Ryanodine receptor/Ca2+ release channels and their regulation by endogenous effectors. *Annu. Rev. Physiol.* 56, 485–508 (1994).

Ogawa, Y. Role of ryanodine receptors. *Crit. Rev. Biochem. Mol. Biol.* 29, 229–274 (1994).

Murayama, T. et al. A tropospheric residue in the caffeine-binding site of the ryanodine receptor regulates Ca2+ sensitivity. *Commun. Biol.* 1, 90 (2018).

Murayama, T. & Kurebayashi, N. Two ryanodine receptor isoforms in nonmammalian vertebrate skeletal muscle: possible roles in excitation–contraction coupling and other processes. *Prog. Biophys. Mol. Biol.* 105, 134–141 (2011).

Uehara, A. et al. Extensive Ca2+ leak through K475Q cardiac ryanodine receptors caused by cytosolic and luminal Ca2+ hypersensitivity. *J. Gen. Physiol.* 149, 199–218 (2017).

Yan, Z. et al. Structure of the rabbit ryanodine receptor RyR1 at near-atomic resolution. *Nature* 517, 50–55 (2015).

Brünger, A. T. et al. Crystallography & NMR system: a new software suite for macromolecular structure determination. *Acta Crystallogr D.* 54, 905–921 (1998).

Ma, R. et al. Structural basis for diamide modulation of ryosensitive transition. *Nat. Chem. Biol.* 16, 1246–1254 (2020).

Epley, S. & Inesi, G. Phospholipid orientation in sarcoplasmic membranes: spin-label ESR and proton NMR studies. *Biochim. Biophys. Acta* 282, 174–179 (1972).

Woll, K. A., Haji-Ghassemi, O., Van & Petegem, F. Pathological conformational changes of disease mutant ryanodine receptors revealed by cryo-EM. *Nat. Commun.* 12, 807 (2021).

Acknowledgements

We thank Mariko Kurakata for her assistance with cell culture. We thank Kizu Hiraga and the Laboratory of Radiosotope Research, Research Support Center, Juntendo University Graduate School of Medicine for technical assistance. We also thank the staff scientists at the University of Tokyo’s cryo-EM facility. This study was partly supported by the Japan Science for the Promotion of Sciences KAKENHI (grant numbers 19K07015 and 22K06652 to T.K., 19H03404 and 22H02805 to T.M. and JP16H04748 and 21H02411 to H.O.); the Platform Project for Supporting Drug Discovery and Life Science Research (Basis for Supporting Innovative Drug Discovery and Life Science Research [BINDS]; grant number JP20am0101080 to H.O. and JP19am011115 [support number (0064)]; the Practical Research Project for Rare/Intractable Diseases from the Japan Agency for Medical Research and Development (AMED; grant number 19dk0109202 to N.K.); an Intramural Research Grant (2–5) for Neurological and Psychiatric Disorders of NCNP (to T.M.); and the Vehicle Racing Commemorative Foundation (6114, 6237, and 6303) to T.M. and H.O. interpreted the data. T.M. and H.O. supervised the project. H.O. and T.K. performed cell culture. T.K. performed protein purification and approved the final version of the manuscript.

Competing interests

The authors declare no competing interests.
