Structural Determinants of Skeletal Muscle Ryanodine Receptor Gating*

Received for publication, November 4, 2012, and in revised form, January 4, 2013 Published, JBC Papers in Press, January 14, 2013, DOI 10.1074/jbc.M112.433789

Srinivas Ramachandran‡1, Asima Chakraborty‡1,2, Le Xu‡1, Yingwu Mei‡, Montserrat Samso´§3, Nikolay V. Dokholyan‡4, and Gerhard Meissner‡5

From the ‡Department of Biochemistry and Biophysics, School of Medicine, University of North Carolina, Chapel Hill, North Carolina 27599–7260 and the §Department of Physiology and Biophysics, Virginia Commonwealth University, Richmond, Virginia 23298

Significance: The results provide new insights into how RyR activity is regulated by Ca2+.

Background: The molecular basis of calcium release by ryanodine receptors (RyRs) is incompletely understood.

Results: Mutations predicted by a computational model alter Ca2+-dependent single RyR channel activity.

Conclusion: An interface between a pore-lining helix and a linker helix has a critical role in RyR channel gating.

RyR1 releases Ca2+ from intracellular stores upon nerve impulse to trigger skeletal muscle contraction. Effector binding at the cytoplasmic domain tightly controls gating of the pore domain of RyR1 to release Ca2+. However, the molecular mechanism that links effector binding to channel gating is unknown due to lack of structural data. Here, we used a combination of computational and electro-physiological methods and cryo-EM densities to generate structural models of the open and closed states of RyR1. Using our structural models, we identified an interface between the pore-lining helix (Tyr-4912–Glu-4948) and a linker helix (Val-4830–Val-4841) that lies parallel to the cytoplasmic membrane leaflet. To test the hypothesis that this interface controls RyR1 gating, we designed mutations in the linker helix to stabilize either the open (V4830W and T4840W) or closed (H4832W and G4834W) state and validated them using single channel experiments. To further confirm this interface, we designed mutations in the pore-lining helix to stabilize the closed state (Q4947N, Q4947T, and Q4947S), which we also validated using single channel experiments. The channel conductance and selectivity of the mutations that we designed in the linker and pore-lining helices were indistinguishable from those of WT RyR1, demonstrating our ability to modulate RyR1 gating without affecting ion permeation. Our integrated computational and experimental approach significantly advances the understanding of the structure and function of an unusually large ion channel.

*This work was supported, in whole or in part, by National Institutes of Health Grant R01GM08687 (to G. M. and N. V. D.). This work was also supported by American Heart Association Predoctoral Fellowship 09PRE2090068 (to S. R.) and Virginia Commonwealth University startup funds (to M. S.).

‡These authors contributed equally to this work.

§These authors contributed equally to this work.

‡1Present address: Centre for Cellular and Molecular Biology, Council for Scientific and Industrial Research, Hyderabad 500 007, Andhra Pradesh, India.

1To whom correspondence may be addressed. Tel.: 804-828-9492; E-mail: meissner@med.unc.edu.

2To whom correspondence may be addressed. Tel.: 919-843-2513; Fax: 864-828-9492; E-mail: dokh@unc.edu.

3To whom correspondence may be addressed. Tel.: 804-828-9492; E-mail: msamso@vcu.edu.

4To whom correspondence may be addressed. Tel.: 919-966-2513; Fax: 864-828-9492; E-mail: dokh@unc.edu.

5To whom correspondence may be addressed. Tel.: 919-966-5021; Fax: 919-966-2852; E-mail: meissner@med.unc.edu.

6The abbreviations used are: RyR1, ryanodine receptor type 1; 6-TMD, six-transmembrane domain; SR, sarcoplasmic reticulum; pS, picosiemens; TM, transmembrane.
In K⁺ channels, bending at a glycine in the inner helix has been associated with channel opening, suggesting that bending of the inner helix alone does not open the pore in RyR1. Samsøe et al. (3) determined the cryo-EM structure of open and closed RyR1 both complexed with the associated 12-kDa subunit FKBP12. As observed for the high resolution structures of K⁺ channels, the inner helix has an outward bend at a glycine (Gly-4934 or Gly-4941) in the open but not closed RyR1. K⁺ channels also have four pore-lining transmembrane helices (one from each subunit) undergoing a coordinated change from a straight to a kinked conformation while going from the closed to the open state of the pore (13, 15), supporting the idea that the RyR1 pore may have a gating mechanism similar to that displayed by the K⁺ channels. However, the mapped selectivity filter and the pore-lining helix comprise only ~20% of the residues that form the 6-TMD of RyR1. The rest of the 6-TMD is thought to be important in mediating signals from the cytosolic domain to control pore gating, but the location of these residues with respect to the RyR1 pore and their interactions with the pore remain unknown. Here, we report the structural determinants of RyR1 gating based on homology modeling and high resolution cryo-EM data, which have been successfully validated by single channel measurements of WT and mutant channels.

**EXPERIMENTAL PROCEDURES**

**Materials**—[³H]Ryanodine was obtained from PerkinElmer Life Sciences, and protease and inhibitor mixtures were from Sigma. Chemicals were from Sigma unless specified otherwise.

**Sequence Analysis**—The six transmembrane (TM) segments of RyR1 correspond to residues 4561–4948. However, unlike 6-TM potassium channels, there is an insertion of 100 residues between S2 and S3, which, according to the topology of the channel, will be present in the cytoplasmic side. The Pfam annotation (a database of protein families) (19) for the C-terminal region of RyR1 indicates that the last four TM helices (S3–S6, residues 4766–4952) are grouped as a domain belonging to the ion transport family (accession number PF00520), whereas the first two TM helices (residues 4381–4670) belong to another putative domain (accession number PF06459). We used fragment 4766–4952 of RyR1, which is predicted to form a contiguous domain, to perform PSI-BLAST (20–22). We performed PSI-BLAST for five iterations with stringent inclusion criteria (an E-value threshold of 0.001). By the sixth iteration, we observed no new hits, indicating convergence of PSI-BLAST calculations of change in channel open probability (Po) upon mutation, we defined ΔG as ΔGWT = ΔGWt, where ΔGWt is the stability of the mutant, and ΔGWt is the stability of the wild-type channel. Thus, a destabilizing mutation would result in a positive ΔDG. For the two mutations (G4834W and V4838W) that resulted in a very large increase in van der Waals repulsion energy, we have not reported ΔDG because, for these mutations, there is the possibility of backbone relaxation, and we did not allow backbone movement in our calculations (these are labeled as ND in Table 1). To predict change in channel open probability (Po) upon mutation, we considered the thermodynamic cycle (Scheme 1).

\[
\begin{align*}
\text{WT}_{\text{Closed}} & \xrightarrow{\Delta G_{\text{WT}}^{\text{Open}}} \text{WT}_{\text{Open}} \\
\Delta G_{\text{Open}}^{\text{WT}} & \xrightarrow{\Delta G_{\text{Mut}}^{\text{Open}}} \text{Mut}_{\text{Open}} \\
\Delta G_{\text{Mut}} & \xrightarrow{\Delta G_{\text{Open}}^{\text{Mut}}} \text{Mut}_{\text{Closed}}
\end{align*}
\]

In the above schematic, ΔGWt and ΔGMut refer to the stability change upon channel opening for the WT and mutants, respectively, which were determined experimentally.
S4-S5 Linker and S6 in RyR1 Gating

by $P_o$ measurements. $\Delta \Delta G^{\text{Mut}}_c$ and $\Delta \Delta G^{\text{Mut}}_o$ are the stability change upon mutation for the closed and open states, respectively, which were determined computationally using Medusa.

The structural models for the open and closed states can be correlated with experimental measurements by summing two sides of the thermodynamic cycle (Equation 1).

$$\Delta \Delta G^{\text{WT}}_\text{Open} + \Delta \Delta G^{\text{Mut}}_o = \Delta \Delta G^{\text{WT}}_c + \Delta \Delta G^{\text{Mut}}_\text{Open} \quad (\text{Eq. 1})$$

Equation 1 can be rearranged to Equation 2.

$$\Delta \Delta G^{\text{Mut}}_\text{Open} - \Delta \Delta G^{\text{WT}}_\text{Open} = \Delta \Delta G^{\text{Mut}}_c - \Delta \Delta G^{\text{Mut}}_o \quad (\text{Eq. 2})$$

The terms on the left side of Equation 2 could be determined experimentally using $P_o$ determined for WT and mutant RyR1. The terms on the right side of Equation 2 were determined with Medusa using structural models of the closed and open states of WT and mutant RyR1. In the case of homology models, such calculations are good predictors of the net effect of mutation (stabilization or destabilization), but the magnitude of $\Delta \Delta G$ is not highly accurate (40). Thus, in this study, we used the $\Delta \Delta G$ values to predict if there will be an increase or decrease in $P_o$ of mutant channels, but not the extent of change in $P_o$.

Cryo-EM Models of RyR1—The three-dimensional reconstructions of RyR1 in the closed and open states were obtained previously at a nominal resolution of 10.2 Å (Electron Microscopy Data Bank (EMDB) accession codes 1066 and 1067) (3). For Fig. 3 (B and C), 4S and S6 were further traced by following the high density of the helices through successive slices of the three-dimensional reconstruction. Docking of the structural models onto the cryo-EM map was performed first using the colored functionality in Situs (41). Colores aligns a generated Gaussian density map of the structural model (at 10 Å resolution) with the cryo-EM map through rigid-body fitting in six dimensions (three rotational and three translational). The best Situs fits were evaluated visually and refined using the fit-in-map functionality in Chimera.

Site-directed Mutagenesis—The full-length rabbit RyR1 cDNA was constructed as described previously (42). Mutations were introduced by Pfu PCR using mutagenic oligonucleotides following the QuikChange II site-directed mutagenesis kit protocol (Stratagene, La Jolla, CA). The C-terminal Clal/Xbal fragment (14443/15276) of RyR1 cDNA cloned into the pBluescript vector served as the template for mutagenesis. Mutated sequences were confirmed by sequencing, and mutated C-terminal fragments were reintroduced into the Clal and Xbal sites of the C-terminal fragment of RyR1. Mutated full-length expression plasmids were prepared by ligation of three fragments (Clal/Xhol, Xhol/EcoRI, and EcoRI/XbaI containing the mutated sequence) and the expression vector pCMV5 (Clal/XbaI) as described previously (42).

Expression and Preparation of Wild-type and Mutant Channels—Rabbit WT and mutant RyR1 cDNAs were transiently expressed in HEK 293 cells (11). Crude membrane fractions (42) and proteoliposomes containing purified recombinant WT and mutant RyR1 channels (43) were prepared as described.

Cellular Ca$^{2+}$ Release—Stored Ca$^{2+}$ release was determined using the fluorescent Ca$^{2+}$ indicator dye Fluo-4 AM as described previously (44). HEK 293 cells grown on glass coverslips were washed three times with Krebs-Ringer-Henseleit buffer and loaded with 5 μM Fluo-4 AM for 30 min at 1 h at 37 °C. After loading and rinsing with Krebs-Ringer-Henseleit buffer to remove non-hydrolyzed Fluo-4 AM, cellular Ca$^{2+}$ release was induced by ~5 mM caffeine and measured in individual cells using the program EasyRatioPro (Photon Technology International, Lawrenceville, NJ).

SDS-PAGE and Immunoblot Analyses—WT and mutant RyR1 in crude membrane fractions were detected using 3–12% gradient SDS-PAGE and transferred to nitrocellulose membranes. Membranes were blotted with 2% ECL Advance blocking reagent (Amersham Biosciences) in 0.5% Tween 20 and TBS (pH 7.4) at 24 °C and probed with primary anti-RyR1 polyclonal antibody and peroxidase-conjugated anti-mouse IgG (1:50,000; Calbiochem). Immunoblots were developed using enhanced chemiluminescence and quantified using ImageQuant TL analysis software.

$[^3]$H]Ryanodine Binding—The highly specific plant alkaloid ryanodine is widely used as a probe of RyR channel activity and content (45). $B_{\text{max}}$ values of $[^3]$H]ryanodine binding were determined by incubating crude membrane preparations for 4 h at 24 °C with a nearly saturating concentration of $[^3]$H]ryanodine (20 nM) in 20 mM imidazole (pH 7.0), 0.6 M KCl, 0.15 M sucrose, 100 μM Ca$^{2+}$, and protease inhibitors. Nonspecific binding was determined using a 1000-fold excess of unlabeled ryanodine. Aliquots of samples were diluted 9-fold with ice-cold water and placed on Whatman GF/B filters saturated with 2% polyethyleneimine. Filters were washed with three 5-ml volumes of ice-cold 0.1 M KCl and 1 mM K-Pipes (pH 7.0). Radioactivity remaining on the filters was determined by liquid scintillation counting to obtain bound $[^3]$H]ryanodine.

### TABLE 1

| Mutation | $\Delta \Delta G$ open state | $\Delta \Delta G$ closed state | Net $\Delta \Delta G$ |
|----------|------------------------------|-------------------------------|---------------------|
|          | kcal/mol                     | kcal/mol                      | kcal/mol            |
| V4830W   | 7.6 ± 0.9                    | 12.6 ± 0.8                    | -5.1 ± 1.1          |
| H4832W   | 4.3 ± 0.8                    | 2.3 ± 0.7                     | 2.0 ± 1.1           |
| G4834W   | ND                           | -2.3 ± 0.7                    | ND                  |
| V4838W   | ND                           | ND                            | ND                  |
| T4840W   | 17.1 ± 0.9                   | 24.6 ± 0.7                    | -7.5 ± 1.1          |
| V4841W   | 10.7 ± 0.9                   | 4.0 ± 0.6                     | 6.8 ± 1.1           |
| Q4947N   | 2.4 ± 0.9                    | -4.8 ± 0.7                    | 7.3 ± 1.2           |
| Q4947T   | 5.2 ± 0.9                    | -3.6 ± 0.7                    | 8.8 ± 1.2           |
| Q4947S   | 0.8 ± 0.9                    | -3.1 ± 0.7                    | 4.0 ± 1.2           |

The values denote means ± S.E. ND, not determined.
**Sequence Analyses Point to a Conserved 6-TM Topology**—To uncover the molecular determinants of RyR1 opening and closing, we sought to understand the role of residues outside the pore-forming region of the RyR1 6-TMD in controlling RyR1 gating by constructing and verifying structural models of the open and closed states of the 6-TMD. TM helix prediction suggested the presence of at least six TM segments (Fig. 1A) in the RyR1 C-terminal region (residues 4561–4948; UniProt accession number P11716). The presence of the six membrane-spanning segments is sufficient to support RyR1 ion channel activity as seen by single channel recordings of tryptic fragments (47) and deletion of the preceding putative TM segments (48). Overlapping the TM prediction on secondary structure prediction (Fig. 1B) further defined the boundaries of the six TM segments of RyR1. The boundaries of the TM segments combined with the known luminal or cytosolic location of intervening loops in between the TM segments (1, 48) enabled us to propose a TM topology for RyR1 (Fig. 1C), which underscores its similarity to the known structures of 6-TM cation channels (15, 23, 49). This topology for the 6-TMD features six TM segments (S1–S6), where the S4 segment is connected to the S5 segment through a S4–S5 linker helix (45L,4830VTHNGKQLVMTV4841). The S5 segment is connected to a short pore helix (P-helix) through a luminal loop. The P-helix leads to the selectivity filter and then the pore-lining S6 segment.

We then directly identified structural homologs to RyR1 by querying protein sequence databases with PSI-BLAST (20–22), where we found the voltage-gated potassium channel Kv1.2 (UniProt accession number P63142 and Protein Data Bank code 3LUT) (Fig. 2A) to feature highly statistically significant (E-value of $\approx 2 \times 10^{-4}$) alignment with RyR1, thus providing us with a structural template to model the RyR1 6-TMD. The

![Image](313x295 to 563x733)

**RESULTS**

**Sequence Analyses Point to a Conserved 6-TM Topology**—To uncover the molecular determinants of RyR1 opening and closing, we sought to understand the role of residues outside the pore-forming region of the RyR1 6-TMD in controlling RyR1 gating by constructing and verifying structural models of the open and closed states of the 6-TMD. TM helix prediction suggested the presence of at least six TM segments (Fig. 1A) in the RyR1 C-terminal region (residues 4561–4948; UniProt accession number P11716). The presence of the six membrane-spanning segments is sufficient to support RyR1 ion channel activity as seen by single channel recordings of tryptic fragments (47) and deletion of the preceding putative TM segments (48). Overlapping the TM prediction on secondary structure prediction (Fig. 1B) further defined the boundaries of the six TM segments of RyR1. The boundaries of the TM segments combined with the known luminal or cytosolic location of intervening loops in between the TM segments (1, 48) enabled us to propose a TM topology for RyR1 (Fig. 1C), which underscores its similarity to the known structures of 6-TM cation channels (15, 23, 49). This topology for the 6-TMD features six TM segments (S1–S6), where the S4 segment is connected to the S5 segment through a S4–S5 linker helix (45L,4830VTHNGKQLVMTV4841). The S5 segment is connected to a short pore helix (P-helix) through a luminal loop. The P-helix leads to the selectivity filter and then the pore-lining S6 segment.

We then directly identified structural homologs to RyR1 by querying protein sequence databases with PSI-BLAST (20–22), where we found the voltage-gated potassium channel Kv1.2 (UniProt accession number P63142 and Protein Data Bank code 3LUT) (Fig. 2A) to feature highly statistically significant (E-value of $\approx 2 \times 10^{-4}$) alignment with RyR1, thus providing us with a structural template to model the RyR1 6-TMD. The
structure of Kv1.2 has been solved in the open state (15), and the topology of its TM helices is identical to that of RyR1. For the closed state template, we utilized the structure of the cyclic nucleotide-gated K^+/H11001 channel MlotiK1 (UniProt accession number Q98GN8 and Protein Data Bank code 3BEH) (Fig. 2B) (23), which has a membrane topology identical to that of RyR1 and Kv1.2. Besides the changes corresponding to open-closed transitions in the S1–S6 helices, the conformations of the P- helix and the selectivity filter in the open state Kv1.2 and closed state MlotiK1 structures are highly similar.

RyR1 Homology Structural Models Match the Corresponding RyR1 Conformation Determined by Cryo-EM—We used the alignments between Kv1.2 and RyR1 and between MlotiK1 and RyR1 to build structural models for the RyR1 membrane-spanning domains in the closed and open states (Fig. 3A) using Medusa (35–37) and discrete molecular dynamics (32–34). To analyze the correspondence between these homology models and the actual structure of RyR1 in the closed and open states, we docked our structural models to the 10 Å resolution cryo-EM densities of RyR1 in the closed and open states (Fig. 3, B and C) (3). We observed a high similarity between the structural models and the cryo-EM maps, with cross-correlation values of 0.89 and 0.90 for the closed-to-closed and open-to-open comparisons, respectively. When comparing the cryo-EM maps with the pseudo-atomic maps filtered at 10 Å resolution. The size of the pore of the structural models also fits the observed pore size in the cryo-EM maps, with distances of 17 and 21–22 Å between the centers of opposite inner helices for the closed and open states, respectively (Table 2). The lack of higher resolution EM densities prevented us from evaluating the fit of the S1–S4 helices, but we observed significant overlap between the EM densities and the structural model in the region starting at 45L helix and ending at the S6 helix for both the open and closed states (Fig. 3, B and C). Importantly, the distinct orientation of the 45L and S6 helices in the open and closed structural models fits the distinct densities of the corresponding regions traced in the cryo-EM maps (Fig. 3, B and C). The close fit of 45L and S6 in the closed and open state structural models with the corresponding cryo-EM maps demonstrates the suitability of our structural models to model interactions involved in RyR1 gating.

45L Region Mediates RyR1 Gating—An important aspect of 6-TM K^+ channels is the role of 45L in channel gating (50–55). The open-closed transition in the K^+ channels is characterized by the movement of 45L while maintaining an interface with S6. In the closed state, S6 is a straight helix, whereas in the open state, S6 is bent at the gating hinge, which has been suggested to correspond to Gly-4934 in RyR1 (3). The position of 45L in the closed state precludes bending of S6, thus maintaining the closed state. We found that in our structural model, 45L partially overlaps with a cytoplasmic segment linking S4 and S5 according to the model of Du et al. (1, 48), where the use of GFP inserts predicted a S4-S5 loop of 10 amino acids in length (Thr-4825–Gly-4834). Earlier studies discovered that amino acid
substitutions to alanine in the N-terminal half of this region (4825TILSS4829) result in changes in RyR1 gating (56). In our model, those residues are present in the C-terminal end of the S4 helix and would be accessible from the cytoplasmic side of the channel. The S4 helix in K\textsubscript{170}H11001 channels is known to undergo structural transitions due to gating (53, 57). Hence, mutations in the S4 helix of RyR1 are also expected to affect channel gating.

If the predicted 45L-S6 interface were important for RyR1 gating, we would expect high evolutionary conservation of this interface. To explore the conservation of these regions, we generated a multiple sequence alignment from the PSI-BLAST hits that consisted of 368 protein sequences linked evolutionarily to rabbit RyR1. We observed nearly 100% conservation of residues in S6 that face 45L (Ile-4936, Phe-4940, Leu-4943, and Arg-4944), whereas the residues of 45L that face S6 are ∼70% conserved (Val-4830, Gly-4834, Leu-4837, Val-4838, and Val-4841) (Fig. 3, D and E). We also found that in addition to being ∼70% conserved, the S6-facing side of 45L also allows only conservative substitution (Fig. 3E). The high conservation of residues of the proposed 45L and S6 support the idea that they form a functional interface that is important in gating. Thus, through our evolutionary and structural analysis, we have discovered that a stretch of amino acids ∼70 residues away from the pore on the primary sequence may form an interface with the S6 helix to control its gating.

45L Mutants Relatively Stabilize Either the Open or Closed State of RyR1—To validate the proposed interface between 45L and S6 helices, we computationally determined the effect of tryptophan-scanning mutagenesis of 45L on RyR1 gating. The contacts between many of the residues in 45L with the S6 helix are different in the open and closed states of RyR1, implying mutations in 45L to have distinct effects on the closed and open states. The probability of channel opening (\(P_o\)) measured experimentally can reveal whether a 45L mutant features a stabilized open or closed state relative to WT. We wanted to test the hypothesis that mutating residues in 45L would affect the relative stability of open and closed states, which would be reflected in the \(P_o\) of the mutants being significantly higher or lower than that of WT RyR1. Hence, we first calculated the change in the relative stability of RyR1 open and closed states upon mutations in the 45L region using Medusa (35–37). We focused on four amino acid residues (Val-4830, Gly-4834, Val-4838, and Val-4841) that, according to our model, directly interacted with S6 and two residues (His-4832 and Thr-4840) that were not expected to directly interact with S6 (Fig. 3D). From our calculations (except for G4834W and V4838W, which featured a high extent of steric clashes upon mutation), we observed two
effects upon performing these mutations: relative stabilization of the closed state or relative stabilization of the open state (Table 1), which translates to an expected decrease or increase in \( P_o \), respectively. 

**Design 45L Mutants Display Altered Gating in Single Channel Measurements—Recombinant WT and six mutant RyR1 channels in 45L (V4830W, H4832W, G4834W, V4838W, T4840W, and V4841W) were expressed as functional RyRs as indicated by a cellular caffeine-induced Ca\(^{2+}\) response that, in most instances, correlated reasonably well with their RyR1 protein contents (Table 3). V4830W showed a caffeine response in a smaller number of cells compared with the other mutants. From ion conductance measurements using the planar lipid bilayer method, we observed that the mutations had no effect on K\(^+\) conductance and on Ca\(^{2+}\) selectivity \((P_{Ca}/P_{K})\, in\, the\, presence\, of\, 10\, mM\, SR\, luminal\, Ca^{2+}\) compared with WT (Table 3). Hence, we conclude that introduction of mutations in 45L does not markedly alter RyR1 ion conductance and selectivity. In contrast to ion conductance and selectivity, we observed major differences in Ca\(^{2+}\) sensitivity, Ca\(^{2+}\) dependence, and gating characteristics of mutant channels compared with WT (Fig. 4, A–C, and Table 4). V4830W exhibited a high \( P_o \) (~1) at 0.01 \( \mu \)M to 10 mM cytoplasmic Ca\(^{2+}\) (Fig. 4, A and B), which suggests that the mutant channel is constitutively active. T4840W also exhibited an increased \( P_o \) at 0.01 \( \mu \)M to 10 mM cytoplasmic Ca\(^{2+}\) (Fig. 4, A and B), whereas H4832W and G4834W had a reduced \( P_o \) compared with WT (Fig. 4, A and C). The two remaining mutant channels (V4838W and V4841W) exhibited a \( P_o \) and Ca\(^{2+}\) dependence that were not significantly different from those of WT (Fig. 4, A and C). The effects of mutations on channel activity at 2 \( \mu \)M cytoplasmic Ca\(^{2+}\) were analyzed by time analysis of WT and mutant channel recordings. The marked increase in \( P_o \) for V4830W and T4840W was accounted for by an increase in long open events (mean open times of 369.8 ± 70.8 and 19.9 ± 8.5 ms, respectively, versus 0.68 ± 0.06 ms for WT) (Table 4). In contrast, the reduced activities of H4832W and G4834W were associated with a decreased duration of the open events and an increased duration of the closed events. Consistent with no major change in \( P_o \), no significant differences in the mean open and closed time constants were obtained for V4838W and V4841W compared with WT. Our results indicate that the V4830W and T4840W mutations stabilize the open state, whereas the H4832W and G4834W mutations stabilize the closed state.

**45L Mutants That Stabilize the Open State Also Affect Ca\(^{2+}\) Sensitivity of RyR1—**RyR1 receptors are activated by the binding of Ca\(^{2+}\) to high affinity sites and are inhibited by Ca\(^{2+}\) binding to low affinity sites, giving rise to the bimodal Ca\(^{2+}\) dependence of channel activity (WT in Fig. 4, B and C). Mutagenesis and disease-causing mutations show that multiple regions located in the large cytosolic structure contribute to the regulation of RyRs (58, 59). We found that two of the six 45L mutants also altered Ca\(^{2+}\) regulation of RyR1. Whereas four of the mutants (H4832W, G4834W, V4838W, and V4841W) displayed a Ca\(^{2+}\) dependence of channel activity similar to that of WT (with a \( P_o \) of ~0 at 0.01 mM and 10 mM cytoplasmic Ca\(^{2+}\)) (Fig. 4C), V4830W maintained a \( P_o \) of ~1.0 at 0.01 mM cytoplasmic Ca\(^{2+}\) (Fig. 4B). A decrease in \( P_o \) was observed only at 10 mM Ca\(^{2+}\). These results indicate that the V4830W mutation leads to a decoupling of high affinity Ca\(^{2+}\) binding and channel opening and likely reduced Ca\(^{2+}\) binding to the inhibitory sites. The elevated channel open probabilities of T4840W at low and high cytoplasmic Ca\(^{2+}\) indicate partial decoupling of Ca\(^{2+}\) regulation and channel gating (Fig. 4B).

**S6 Mutations Also Affect RyR1 Gating by Relatively Stabilizing the Closed State—**Given the effect of the 45L mutations on RyR1 gating, we hypothesized that mutating residues in S6 that face the linker will also affect RyR1 gating. We found that mutations of Gln-4947 relatively stabilized the closed state (Table 1), which we characterized experimentally. Three mutants (Q4947N, Q4947T, and Q4947S) were expressed as functional RyRs as indicated by a robust cellular caffeine response (Table 5). Furthermore, the mutations had no effect on K\(^+\) conductance and on Ca\(^{2+}\) selectivity \((P_{Ca}/P_{K})\, in\, the\, presence\, of\, 10\, mM\, SR\, luminal\, Ca^{2+}\) compared with WT (Table 5), indicating that these mutations did not affect channel conductance and stability and supporting the model that the side chain of Gln-4947 faces away from the pore. However, channel gating was significantly different from WT, with reduced channel open probability, decreased durations of open times, and increased durations of closed times, indicating stabilization of the closed state (Fig. 4, D and E, and Table 4), in accordance with the model. 

**DISCUSSION**

Our models for the open and closed states of RyR1 provide the structural basis for altered gating behavior of 45L and S6 mutants. The V4830W mutation in the open state features increased contacts between the N-terminal end of 45L and the

### Table 3: Properties of 45L mutants

| RyR1    | Cells with caffeine response | \( B_{max} \) of \([^{1}H]ryanodine binding^a \) | Intensity on immunoblots | \( \gamma^b \) | \( P_{Ca}/P_{K}^c \) |
|---------|-----------------------------|-----------------------------------------------|--------------------------|--------------------|----------------------|
| WT      | NA                          | NA                                            | NA                       | 802 ± 8 (12)       | 6.6 ± 0.1 (18)       |
| V4830W  | 22.5 ± 4.3 (20)^d           | 20.4 ± 11.1 (6)^d                            | 51.5 ± 6.3 (14)^d        | 786 ± 6 (10)       | 6.7 ± 0.2 (5)        |
| H4832W  | 67.2 ± 12.0 (9)^d           | 79.4 ± 13.5 (4)                              | 70.3 ± 6.2 (8)^d         | 785 ± 7 (14)       | 6.4 ± 0.2 (5)        |
| G4834W  | 85.0 ± 10.5 (8)             | 49.0 ± 14.5 (3)^d                            | 123.6 ± 17.1 (7)         | 791 ± 6 (15)       | 6.6 ± 0.2 (6)        |
| V4838W  | 81.1 ± 10.9 (7)             | 58.5 ± 11.4 (3)^d                            | 104.5 ± 17.6 (13)        | 772 ± 4 (17)       | 6.8 ± 0.3 (4)        |
| T4840W  | 127.0 ± 8.5 (13)^d          | 30.0 ± 12.3 (5)^d                            | 71.8 ± 8.9 (10)^d        | 770 ± 6 (7)        | 6.7 ± 0.1 (5)        |
| V4841W  | 95.1 ± 5.3 (7)              | 43.2 ± 13.3 (4)^d                            | 63 ± 8.3 (8)^d           | 783 ± 7 (13)       | 6.3 ± 0.2 (5)        |

^a Maximum binding capacity.
^b K\(^+\) conductance.
^c Selectivity of Ca\(^{2+}\) over K\(^+\).
^d \( p < 0.05 \) compared with WT.
S6 helix from the same monomer and the S5 helix from the adjacent monomer due to the larger side chain of tryptophan compared with valine (Fig. 5A). The increased contacts lead to the stabilization of the open state of the mutant compared with the closed state because the closed state features no additional contacts with S6 due to the mutation. Experimentally, V4830W features a 356% increase in the $P_o$ and a 545% increase in the duration of the open state compared with WT RyR1.

**FIGURE 4.** Experimental characterization of 45L and S6 mutants. A, representative single channel traces for WT RyR1 and 45L mutants. B and C, effect of cytoplasmic Ca$^{2+}$ on the $P_o$ of WT RyR1 and 45L mutants. D, representative single channel traces for S6 mutants. E, effect of cytoplasmic Ca$^{2+}$ on the $P_o$ of S6 mutants.

**TABLE 4**

Properties of 45L and S6 mutants

| RyR1         | $0.1 \mu M$ Ca$^{2+}$ | $2 \mu M$ Ca$^{2+}$ | No. of events/min | $T_o^{a}$ | $T_c^{b}$ |
|--------------|------------------------|---------------------|-------------------|----------|----------|
|              |                        |                     |                   | ms       | ms       |
| WT           | 0.005 ± 0.002 (24)     | 0.27 ± 0.03 (29)    | 23,821 ± 2001 (19)| 0.68 ± 0.06 (19)| 2.39 ± 0.37 (19) |
| V4830W       | 0.99 ± 0.01 (16)      | 0.96 ± 0.03 (10)    | 318 ± 155 (9)     | 369.80 ± 70.4 (9) | 1.97 ± 0.87 (9)  |
| H4832W       | 0.015 ± 0.014 (13)    | 0.10 ± 0.03 (13)    | 13,647 ± 435 (11) | 0.32 ± 0.02 (11) | 2.75 ± 1.80 (11) |
| G4834W       | 0.006 ± 0.005 (14)    | 0.10 ± 0.04 (15)    | 9625 ± 2491 (10)  | 0.33 ± 0.02 (10) | 11.69 ± 2.96 (10) |
| V4838W       | 0.026 ± 0.013 (8)     | 0.38 ± 0.07 (17)    | 22,602 ± 2296 (14)| 1.72 ± 0.86 (14)| 2.22 ± 0.64 (14) |
| T4840W       | 0.69 ± 0.22 (3)       | 0.83 ± 0.09 (7)     | 4120 ± 942 (6)    | 19.9 ± 8.5 (6)  | 2.26 ± 0.66 (6)  |
| V4841W       | 0.005 ± 0.001 (3)     | 0.20 ± 0.06 (13)    | 15,982 ± 2516 (11)| 0.65 ± 0.14 (11)| 5.02 ± 1.25 (11) |
| Q4947N       | 0.001 ± 0.001 (4)     | 0.02 ± 0.01 (10)    | 3355 ± 943 (10)   | 0.36 ± 0.07 (10)| 107.63 ± 59.67 (10) |
| Q4947T       | 0.003 ± 0.002 (5)     | 0.03 ± 0.01 (10)    | 5335 ± 1238 (10)  | 0.55 ± 0.03 (10)| 24.76 ± 10.65 (10) |
| Q4947S       | 0.004 ± 0.0004 (4)    | 0.07 ± 0.03 (11)    | 10,182 ± 3534 (11)| 0.34 ± 0.03 (11)| 65.42 ± 41.82 (11) |

$a$ Mean open time at cytoplasmic 2 $\mu M$ Ca$^{2+}$.

$b$ Mean closed time at cytoplasmic 2 $\mu M$ Ca$^{2+}$.

$c p < 0.05$ compared with WT.

S6 helix from the same monomer and the S5 helix from the adjacent monomer due to the larger side chain of tryptophan compared with valine (Fig. 5A). The increased contacts lead to the stabilization of the open state of the mutant compared with the closed state because the closed state features no additional contacts with S6 due to the mutation. Experimentally, V4830W features a 356% increase in the $P_o$ and a 545% increase in the duration of the open state compared with WT RyR1. We
observed a similar effect in the structural models and experiments for the T4840W mutant (Fig. 6A). Thus, the mutations that stabilize the open state according to our calculations increase the channel $P_o$.

The G4834W mutation in our structural model leads to unfavorable steric clashes between the tryptophan side chain and the S6 helix (Gln-4947 and Glu-4948), indicative of a destabilizing effect on the open state. However, in the closed state, due to the movement of S6 toward the C-terminal side of 45L, position 4834 is farther away from S6, and the G4834W mutation results in slight stabilization (Fig. 5B). Experimentally, the $P_o$ of G4834W is only 37% of that of WT RyR1. Similarly, the H4832W mutation also leads to increased clashes in the open state but no such destabilization in the closed state, with the net effect being the relative stabilization of the closed state (Fig. 6B). Thus, mutations that stabilize the closed state according to the structural model decrease the channel $P_o$ (G4834W and H4832W).

On S6, we focused on Gln-4947, which faces away from the pore (Fig. 7, A and B) and is part of the S6 interface with 45L. From the structural models of the three S6 mutants (Q4947N, Q4947T, and Q4947S), we observed that the open state is destabilized due to reduced contacts of S6 with 45L in the open state, as the glutamine side chain is replaced with smaller side chain amino acids (Fig. 5C and Fig. 7, C and D). There is no such destabilization in the closed state because Gln-4947 is away from the 45L interface in the closed state (Figs. 5C and 7); thus, we observed the relative stabilization of the closed state due to these mutations. In accordance with the model, the three single S6 mutations Q4947N, Q4947T, and Q4947S have a reduced $P_o$ compared with WT (Table 4).

To directly compare the computational predictions with experimental data, we plotted $\Delta \Delta G$ from $P_o$ measurements (left side of Equation 2) against $\Delta \Delta G$ from Medusa calculations (right side of Equation 2). We observed a high correlation ($r = 0.88$) (Fig. 8), which is statistically significant ($p$ value of correlation $= 0.008$), clearly indicating the predictive power of our structural models.

Overall, our mutational data combined with evolutionary and structural analysis point to a 45L-S6 interface that controls $Ca^{2+}$-dependent gating of RyR1. Significantly, our structural model paves the way for the characterization of the functional

### TABLE 5

| RyR1 mutant | Cells with caffeine response | $B_{max}$ of [$^3$H]ryanodine binding$^a$ | $\gamma^b$ | $P_c/P_k^c$ |
|-------------|-----------------------------|------------------------------------------|-----------|-------------|
| Q4947N      | 69.7 ± 19.6 (6)$^d$         | 28.3 ± 16.1 (5)$^d$                     | 788 ± 8   | 6.3 ± 0.2 (4) |
| Q4947T      | 67.7 ± 10.1 (6)$^d$         | 47.9 ± 5.4 (4)$^d$                     | 783 ± 6   | 6.4 ± 0.2 (5) |
| Q4947S      | 59.8 ± 7.3 (5)$^d$         | 54.8 ± 22.0 (4)$^d$                     | 784 ± 7   | 6.9 ± 0.3 (4) |

$^a$ Maximum binding capacity.

$^b$ Conductivity.

$^c$ Selectivity of $Ca^{2+}$ over $K^+$.

$^d$ $p < 0.05$ compared with WT.
contribution of residues outside the predicted pore region of RyR1. Even in the context of the pore, our data point to residues in S6 that are important for gating but not for selectivity and conductance. In contrast to earlier studies, we were able to design both activating and deactivating mutants based on molecular interactions that lead to such changes in gating behavior. The importance of 45L residues in Ca$^{2+}$-dependent gating of RyR1 is highlighted by the decoupling of channel gating.

**FIGURE 6. Structural models of linker mutations in open and closed states.** A, the structural models of T4840W in the open and closed states depict relative stabilization due to the mutation in the open state caused by formation of new van der Waals contacts. Residues forming contacts with residue 4840 are shown with surface representation, with the largest such interaction observed for T4840W in the open state. B, the structural models of H4832W in the open and closed states depict destabilization of the open state due to clashes between residue 4832 and residues in the S5 helix from the adjacent monomer (shown as red dotted lines) and no such clashes in the closed state. Both mutants are compared with WT.

**FIGURE 7. Orientation of S6 residues and models of S6 mutations in open and closed states.** A, a helical wheel representation of the C-terminal part of the S6 helix depicts the face of the helix that forms the interface with 45L (shown in blue) and the face of S6 that lines the pore (shown in red). B, the position of Gln-4947 in the structural model when observed from the cytoplasmic side indicates that the residue faces away from the pore (marked in the center). The Gln-4947 side chain is shown as sticks, whereas the structural model of the open state is shown by ribbon representation. C and D, the structural models of Q4947T and Q4947S, respectively, show loss of van der Waals contacts due to shortened side chains in the open state but no such contacts in the closed state. Both mutants are compared with WT.
ing from Ca\(^{2+}\) binding in the cytosolic side that is observed in the V4730W and T4840W mutants. The validation of 45L mutants indicates the structural conservation of the 6-TM topology between RyR1 and voltage-gated K\(^{+}\) and Na\(^{+}\) channels, which leads to the intriguing possibility of a common origin for the ligand-gated RyR and voltage-gated cation channels.

Acknowledgments—We thank Daniel Pasek for expressing and purifying WT and mutant RyR1 and Jazmyne Heffelfinger for performing part of the Ca\(^{2+}\) imaging experiments.

REFERENCES

1. Du, G. G., Sandhu, B., Khanna V. K., Guo, X. H., and MacLennan, D. H. (2002) Topology of the Ca\(^{2+}\) release channel of skeletal muscle sarcoplasmic reticulum (RyR1). Proc. Natl. Acad. Sci. U.S.A. 99, 16725–16730
2. Radermacher, M., Rao, V., Grassucci, R., Frank, J., Timmerman, A. P., Fleischer, S., and Wagenknecht, T. (1994) Cryo-electron microscopy and three-dimensional reconstruction of the calcium release channel/ryanodine receptor from skeletal muscle. J. Cell Biol. 127, 411–423
3. Samsø, M., Feng, W., Pessah, I. N., and Allen, P. D. (2009) Coordinated movement of cytoplasmic and transmembrane domains of RyR1 upon gating. PLoS Biol. 7, e85
4. Lanner, J. T., Georgiou, D. K., Joshi, A. D., and Hamilton, S. L. (2010) Ryanodine receptors: structure, expression, molecular details, and function in calcium release. Cold Spring Harb. Perspect. Biol. 2, a003996
5. Franzini-Armstrong, C., and Protasi, F. (1997) Ryanodine receptors of striated muscles: a complex channel capable of multiple interactions. Physiol. Rev. 77, 699–729
6. Rios, E., and Pizarro, G. (1991) Voltage sensor of excitation-contraction coupling in skeletal muscle. Physiol. Rev. 71, 849–908
7. Williams, A. J. (1992) Ion conduction and discrimination in the sarcoplasmic reticulum ryanodine receptor/calcium-release channel. J. Muscle Res. Cell Motil. 13, 7–26
8. Meissner, G. (2004) Molecular regulation of cardiac ryanodine receptor ion channel. Cell Calcium 35, 621–628
9. Balshaw, D., Gao, L., and Meissner, G. (1999) Luminal loop of the ryanodine receptor: a pore-forming segment? Proc. Natl. Acad. Sci. U.S.A. 96, 3345–3347
10. Zhao, M., Li, P., Li, X., Zhang, L., Winkfein, R. J., and Chen, S. R. (1999) Molecular identification of the ryanodine receptor pore-forming segment. J. Biol. Chem. 274, 25971–25974
11. Gao, L., Balshaw, D., Xu, L., Tripathy, A., Xin, C., and Meissner, G. (2000) Evidence for a role of the luminal M3-M4 loop in skeletal muscle Ca\(^{2+}\) release channel (ryanodine receptor) activity and conductance. Biophys. J. 79, 828–840
12. Xu, L., Wang, Y., Gillespie, D., and Meissner, G. (2006) Two rings of negative charges in the cytosolic vestibule of type-1 ryanodine receptor modulate ion fluxes. Biophys. J. 90, 443–453
13. Jiang, Y., Lee, A., Chen, J., Cadene, M., Chait, B. T., and MacKinnon, R. (2002) The open pore conformation of potassium channels. Nature 417, 523–526
14. Alam, A., and Jiang, Y. (2009) High-resolution structure of open NaK channel. Nat. Struct. Mol. Biol. 16, 30–34
15. Long, S. B., Campbell, E. B., and MacKinnon, R. (2005) Crystal structure of a mammalian voltage-dependent Shaker family K\(^{+}\) channel. Science 309, 897–903
16. Doyle, D. A., Morais Cabral, J., Pfuetzner, R. A., Kuo, A., Gulbis, J. M., Cohen, S. L., Chait, B. T., and MacKinnon, R. (1998) The structure of the potassium channel: molecular basis of K\(^{+}\) conduction and selectivity. Science 280, 69–77
17. Jiang, Y., Lee, A., Chen, J., Cadene, M., Chait, B. T., and MacKinnon, R. (2002) Crystal structure and mechanism of a calcium-gated potassium channel. Nature 417, 515–522
18. Ludtke, S. J., Sersycheva, I. I., Hamilton, S. L., and Chiu, W. (2005) The pore structure of the closed RyR1 channel. Structure 13, 1203–1211
19. Punta, M., Coggill, P. C., Eberhardt, R. Y., Mistry, J., Tate, J., Boursnell, C., Pang, N., Forslund, K., Ceric, G., Clements, J., Hegner, A., Holm, L., Sonnhammer, E. L., Eddy, S. R., Bateman, A., and Finn, R. D. (2012) The Pfam protein families database. Nucleic Acids Res. 40, D290–D301
20. Altschul, S. F., Madden, T. L., Schäffer, A. A., Zhang, J., Zhang, Z., Miller, W., and Lipman, D. J. (1997) Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res. 25, 3389–3402
21. Altschul, S. F., Wootton, J. C., Gertz, E. M., Agarwala, R., Morgulis, A., Schäffer, A. A., and Yu, Y. K. (2005) Protein database search using compositionally adjusted substitution matrices. FEBS J. 272, 5101–5109
22. Schäffer, A. A., Aravind, L., Madden, T. L., Shaviv, S., Spouge, J. L., Wolf, Y. I., Koonin, E. V., and Altschul, S. F. (2001) Improving the accuracy of PSI-BLAST protein database searches with composition-based statistics and other refinements. Nucleic Acids Res. 29, 2994–3005
23. Clayton, G. M., Altieri, S., Heginbotham, L., Unger, V. M., and Morais-Cabral, J. H. (2008) Structure of the transmembrane regions of a bacterial cyclic nucleotide-regulated channel. Proc. Natl. Acad. Sci. U.S.A. 105, 1511–1515
24. Serojhius, A. W. R., Hegedus, T., Aleksandrov, A. A., He, L., Cui, L., Dokholyan, N. V., and Riordan, J. R. (2008) Phenylalanine 508 mediates a cytoplasmic-membrane domain contact in the CFTR 3D structure crucial to assembly and channel function. Proc. Natl. Acad. Sci. U.S.A. 105, 3526–3526
25. Bemser, A., Viklund, H., Fark, J., Lindahl, E., von Heijne, G., and Elofsson, A. (2008) Prediction of membrane-protein topology from first principles. Proc. Natl. Acad. Sci. U.S.A. 105, 7177–7181
26. Mitaku, S., Hirokawa, T., and Tsuji, T. (2002) Amphiphilicity index of protein topology predictions are achieved using hidden Markov models and positive-inside rule. Proteins: Struct. Funct. Bioinformatic 48, 515–522
27. Viklund, H., and Elofsson, A. (2008) OCTOPUS: improving topology predictions using support vector machines. BMC Bioinformatics 10, 159
28. Viklund, H., and Elofsson, A. (2004) Best \(\alpha\)-helical transmembrane protein topology predictions are achieved using hidden Markov models and evolutionary information. Protein Sci. 13, 1908–1917
29. Viklund, H., and Elofsson, A. (2008) OCTOPUS: improving topology prediction by two-track ANN-based preference scores and an extended topological grammar. Bioinformatics 24, 1662–1668
30. von Heijne, G. (1992) Membrane protein structure prediction. Hydrophobicity analysis and the positive-inside rule. J. Mol. Biol. 225, 487–494
31. Cuff, J. A., and Barton, G. J. (2000) Application of multiple sequence align-
ment profiles to improve protein secondary structure prediction. *Proteins* **40**, 502–511.

32. Ding, F., Tsao, D., Nie, H., and Dokholyan, N. V. (2008) *Ab initio* folding of proteins with all-atom discrete molecular dynamics. *Structure* **16**, 1010–1018.

33. Dokholyan, N. V., Buldyrev, S. V., Stanley, H. E., and Shakhnovich, E. I. (1998) Discrete molecular dynamics studies of the folding of a protein-like model. *Fold. Des.* **3**, 577–587.

34. Shirvanyants, D., Ding, F., Tsao, D., Ramachandran, S., and Dokholyan, N. V. (2012) Discrete molecular dynamics: an efficient and versatile simulation method for fine protein characterization. *J. Phys. Chem. B* **116**, 8375–8382.

35. Ding, F., and Dokholyan, N. V. (2006) Emergence of protein fold families through rational design. *PLoS Comput. Biol.* **2**, e85.

36. Yin, S., Ding, F., and Dokholyan, N. V. (2007) Modeling backbone flexibility improves protein stability estimation. *Structure* **15**, 1567–1576.

37. Yin, S., Ding, F., and Dokholyan, N. V. (2007) Eris: an automated estimator of protein stability. *Nat. Methods* **4**, 466–467.

38. Ramachandran, S., Kota, P., Ding, F., and Dokholyan, N. V. (2011) Automated minimization of steric clashes in protein structures. *Proteins* **79**, 261–270.

39. Kota, P., Ding, F., Ramachandran, S., and Dokholyan, N. V. (2011) Gaia: automated quality assessment of protein structure models. *Bioinformatics* **27**, 2209–2215.

40. Yin, S., Biedermannova, L., Vondrasek, J., and Dokholyan, N. V. (2008) MedusaScore: an accurate force-field based scoring function for virtual drug screening. *J. Chem. Inf. Model.* **48**, 1656–1662.

41. Chacón, P., and Wriggers, W. (2002) Multi-resolution contour-based fitting of macromolecular structures. *J. Mol. Biol.* **317**, 375–384.

42. Gao, L., Tripathy, A., Lu X., and Meissner, G. (1997) Evidence for a role of C-terminal amino acid residues in skeletal muscle Ca²⁺ release channel (ryanodine receptor) function. *FEBS Lett.* **412**, 223–226.

43. Lee, H. B., Xu, L., and Meissner, G. (1994) Reconstitution of the skeletal muscle ryanodine receptor-Ca²⁺ release channel protein complex into proteoliposomes. *J. Biol. Chem.* **269**, 13305–13312.

44. Wang, Y., Xu, L., Duan, H., Pasek, D. A., Eu, J. P., and Meissner, G. (2006) Knocking down type 2 but not type 1 casquestrin reduces calcium sequestration and release in C₂C₁₂ skeletal muscle myotubes. *J. Biol. Chem.* **281**, 15572–15581.

45. Sutko, J. L., Airey, J. A., Welch, W., and Ruest, L. (1997) The pharmacology of ryanodine and related compounds. *Pharmacol. Rev.* **49**, 53–98.

46. Schoenmakers, T. J., Visser, G. J., Flik, G., and Theuvenet, A. P. (1992) CHELATOR: an improved method for computing metal ion concentrations in physiological solutions. *BioTechniques* **12**, 870–879.

47. Callaway, C., Seryshev, A., Wang, J. P., Slavik, K. J., Needleman, D. H., Cantu, C., 3rd, Wu, Y., Jayaraman, T., Marks, A. R., and Hamilton, S. L. (1994) Localization of the high and low affinity [³H]ryanodine binding sites on the skeletal muscle Ca²⁺ release channel. *J. Biol. Chem.* **269**, 15876–15884.

48. Du, G. G., Avila, G., Sharma, P., Khanna, V. K., Dirksen, R. T., and MacLennan, D. H. (2004) Role of the sequence surrounding predicted transmembrane helix M4 in membrane association and function of the Ca²⁺ release channel of skeletal muscle sarcoplasmic reticulum (ryanodine receptor isofrom 1). *J. Biol. Chem.* **279**, 37566–37574.

49. Payandeh, J., Scheuer, T., Zheng, N., and Catterall, W. A. (2011) The crystal structure of a voltage-gated sodium channel. *Nature* **475**, 353–358.

50. Caprini, M., Fava, M., Valente, P., Fernandez-Ballester, G., Rapisarda, C., Ferroni, S., and Ferrer-Montiel, A. (2005) Molecular compatibility of the channel gate and the N terminus of S5 segment for voltage-gated channel activity. *J. Biol. Chem.* **280**, 18253–18264.

51. Decher, N., Chen, J., and Sanguinetti, M. C. (2004) Voltage-dependent gating of hyperpolarization-activated, cyclic nucleotide-gated pacemaker channels. Molecular coupling between the S4-S5 and C-linkers. *J. Biol. Chem.* **279**, 13859–13865.

52. Ferrer, T., Rupp, J., Piper, D. R., and Tristani-Firouzi, M. (2006) The S4-S5 linker directly couples voltage sensor movement to the activation gate in the human ether-à-go-go-related gene (hERG) K⁺ channel. *J. Biol. Chem.* **281**, 12858–12864.

53. Long, S. B., Campbell, E. B., and Mackinnon, R. (2005) Voltage sensor of Kv1.2: structural basis of electromechanical coupling. *Science* **309**, 903–908.

54. Lu, Z., Klem, A. M., and Ramu, Y. (2002) Coupling between voltage sensors and activation gate in voltage-gated K⁺ channels. *J. Gen. Physiol.* **120**, 663–676.

55. Tristani-Firouzi, M., Chen, J., and Sanguinetti, M. C. (2002) Interactions between S4-S5 linker and S6 transmembrane domain modulate gating of HERG K⁺ channels. *J. Biol. Chem.* **277**, 18994–19000.

56. Murayama, T., Kurebayashi, N., Oba, T., Oyamada, H., Oguchi, K., Saku- rai, T., and Ogawa, Y. (2011) Role of amino-terminal half of the S4-S5 linker in type 1 ryanodine receptor (RyR1) channel gating. *J. Biol. Chem.* **286**, 35571–35577.

57. Yarov-Yarovoy, V., DeCaen, P. G., Westenbroek, R. E., Pan, C. Y., Scheuer, T., Baker, D., and Catterall, W. A. (2012) Structural basis for gating charge movement in the voltage sensor of a sodium channel. *Proc. Natl. Acad. Sci. U.S.A.* **109**, E93–E102.

58. Seo, M. D., Velamakanni, S., Ishiyama, N., Stathopoulos, P. B., Rossi, A. M., Khan, S. A., Dale, P., Li, C., Ames, J. B., Ikura, M., and Taylor, C. W. (2012) Structural and functional conservation of key domains in InS, and ryanodine receptors. *Nature* **483**, 108–112.

59. Klein, A., Lifliis, S., Munteanu, I., Scoto, M., Zhou, H., Quinlivan, R., Straub, V., Manzur, A. Y., Roper, H., Jeannet, P. Y., Rakowicz, W., Jones, D. H., Jensen, U. B., Wraige, E., Trump, N., Schara, U., Lochmuller, H., Sarkozy, A., Kingston, H., Norwood, F., Damian, M., Kirschner, J., Longman, C., Roberts, M., Auer-Grumbach, M., Hughes, I., Bushby, K., Sewry, C., Robb, S., Abbis, S., Jungbluth, H., and Muntoni, F. (2012) Clinical and genetic findings in a large cohort of patients with ryanodine receptor 1 gene-associated myopathies. *Hum. Mutat.* **33**, 981–988.