Mutation of Divergent Region 1 Alters Caffeine and Ca\textsuperscript{2+} Sensitivity of the Skeletal Muscle Ca\textsuperscript{2+} Release Channel (Ryanodine Receptor)*

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Replacement of amino acids 4187–4628 in the skeletal muscle Ca\textsuperscript{2+} release channel (skeletal ryanodine receptor (RyR1)), including nearly all of divergent region 1 (amino acids 4254–4631), with the corresponding cardiac ryanodine receptor (RyR2) sequence leads to increased sensitivity of channel activation by caffeine and Ca\textsuperscript{2+} and to decreased sensitivity of channel inactivation by elevated Ca\textsuperscript{2+} (Du, G. G., and MacLennan, D. H. (1999) J. Biol. Chem. 274, 26120–26126). In further investigations, this region was subdivided by the construction of new chimeras, and alterations in channel function were detected by measurement of the caffeine dependence of \textit{in vivo} Ca\textsuperscript{2+} release and the Ca\textsuperscript{2+} dependence of \textsuperscript{[3H]}ryanodine binding. Chimera RF10a (amino acids 4187–4381) had a lower EC\textsubscript{50} value for activation by caffeine, and RF10c (4557–4628) had a higher EC\textsubscript{50} value, whereas the EC\textsubscript{50} value for chimera RF10b (4382–4556) was unchanged. Chimeras RF10b and RF10c were more sensitive to activation by Ca\textsuperscript{2+}, whereas RF10a was less sensitive to inactivation by Ca\textsuperscript{2+}, implicating RF10b and RF10c in Ca\textsuperscript{2+} activation and RF10a in Ca\textsuperscript{2+} inactivation. Deletion of much of divergent region 1 sequence to create mutant D4274–4535 led to higher caffeine and Ca\textsuperscript{2+} sensitivity of channel activation and to lower Ca\textsuperscript{2+} sensitivity for inactivation. Thus, deletion results demonstrate that caffeine, Ca\textsuperscript{2+}, and ryanodine binding sites are not located in amino acids 4274–4535. Nevertheless, the properties of the deletion and chimeric mutants demonstrate that amino acids 4274–4535 and three shorter sequences in this region (F10a, amino acids 4187–4381; F10b, 4382–4556; and F10c, 4557–4628) in RyR1 modulate Ca\textsuperscript{2+} and caffeine sensitivity of the Ca\textsuperscript{2+} release channel.

The mechanism of excitation-contraction coupling differs in cardiac and skeletal muscles: in cardiac muscle, contraction requires extracellular Ca\textsuperscript{2+} influx through the dihydropyridine receptor to activate the Ca\textsuperscript{2+} release channel (ryanodine receptor (RyR))\textsuperscript{3} in the sarcoplasmic reticulum membrane and cause Ca\textsuperscript{2+}-induced Ca\textsuperscript{2+} release from the sarcoplasmic reticulum; in skeletal muscle, contraction does not require extracellular Ca\textsuperscript{2+} and appears to be induced through the dihydropyridine receptor via physical interaction with the ryanodine receptor (1, 2). Despite these physiological differences, Ca\textsuperscript{2+} is a basic modulator of both RyR1 and RyR2. Both channels are also modulated by other endogenous and exogenous modulators, such as ATP, calmodulin, Mg\textsuperscript{2+}, ruthenium red, and ryanodine, but the extent of modulation by Ca\textsuperscript{2+}, ATP, Mg\textsuperscript{2+}, and ruthenium red differs between RyR1 and RyR2 (3–5). The molecular mechanisms underlying the interactions of these modulatory ligands with RyRs are not yet known. The amino acid sequences of RyR1 and RyR2, deduced from cDNA cloning (6–9), are 66% identical, with regions of high sequence identity and regions of high diversity. If different physiological functions and pharmacological properties could be correlated with different sequences, binding sites for modulating agents in RyR might be identified, leading to a better understanding of structure/function relationships within the molecule.

Structure/function analysis of ryanodine receptors has identified several important regions in the molecule. Malignant hyperthermia and central core disease mutations, found in the sequences lying between amino acids 35 and 614 and 2163 and 2458 (10), alter sensitivity of the channel to caffeine and halothane (11, 12). Another central core disease mutation has been found in the C terminus in predicted transmembrane 9 (TM9) or its adjacent luminal domain (13). Evidence that the Ca\textsuperscript{2+} sensor lies in TM2 has been presented by Chen et al. (14), who showed that mutation of Glu\textsubscript{3987} in RyR3 (equivalent to Glu\textsubscript{4032} in RyR1) caused a huge decrease in Ca\textsuperscript{2+} sensitivity. Other mutations of acidic amino acids in TM2, TM7, and TM10 have also been shown to block caffeine and 4-chloro-m-cresol activation and high affinity ryanodine binding, but single channel function was not analyzed (15). Evidence that TM9 is a Ca\textsuperscript{2+} channel pore has been presented by Chen and co-workers (16), showing that a single mutation, G4824A, reduced single channel conductance from 798 pS for the wild type channel to 22 pS. The mutant channel was modulated by Ca\textsuperscript{2+}, Mg\textsuperscript{2+}, ATP, caffeine, ruthenium red, and ryanodine. Co-expression of wild type and G4824A mutant proteins yielded single channels with intermediate unitary conductances. This is in line with observations in the central core disease mutation (13). Deletion of the N-terminal sequences of RyR1 revealed that one-fifth of the C-terminal sequence contains structures sufficient to form a functional Ca\textsuperscript{2+} release channel, but the N-terminal sequence also regulates the release channel (17). Deletion of 3 amino acids at the C terminus of RyR1 resulted in decreased activities, whereas deletion of 15 amino acids yielded an inactive RyR (18).

In earlier studies, we used chimeric molecules to exploit the differences between Ca\textsuperscript{2+} inactivation profiles of RyR1 and RyR2, allowing us to localize the low affinity Ca\textsuperscript{2+} binding site(s) that inactivates the channel between amino acids 3726...
Divergent Region 1 Mutants

The construction of a deletion mutant involving the D1 region was carried out in \( \text{RYR1} \) cDNA cassette 10 (\( \text{Nhel-ClaI} \)) (Fig. 1). In \( \text{C10} \), there are five \( \text{NarI} \) sites (12819, 12840, 12873, 13107, and 13605). To obtain \( \Delta4274–4535\)-\( \text{C10} \), \( \text{C10} \) was digested with \( \text{NarI} \) to delete DNA sequence 12819–13605 and self-ligated with T4 DNA ligase. The deletion \( \text{C10} \) was inserted into the corresponding region of \( \text{BBS-RF1} \) and the resulting cDNA sequence was then excised and inserted into pcDNA3.1(−) with \( \text{XhoI} \) and \( \text{HindIII} \) to form \( \Delta4274–4535\)-\( \text{R1} \). These chimeric inserts and the deletion mutant were confirmed by DNA sequencing and restriction enzyme-mapping digestion.

Cell Culture and DNA Transfection—Culture of HEK-293 cells and cDNA transfection by the calcium phosphate precipitation method (28), was carried out as described previously (27).

Fluorescence Measurements—A microfluorosystem unit (Photon Technologies Inc.) was used to monitor the Fura-2 AM fluorescence changes in transiently transfected or nontransfected HEK-293 cells, as described previously (21).

Solubilization of Transfected HEK-293 Cells and Measurement of \(^{3} \text{H}\)Ryanodine Binding—Transfected HEK-293 cells grown in 100-mm Petri dishes were solubilized with 1% CHAPS and 5 mg/ml phosphatidylincholine and analyzed with the \(^{3} \text{H}\)ryanodine binding assay described previously (21). In brief, 25-μl aliquots of solubilized total cellular protein were diluted 10-fold in binding buffer composed of 0.5 mM KCl, 1 mM ATP, 100 μM free \( \text{Ca}^{2+} \), 0.2 mM EGTA, 50 mM Heps, pH 7.1, a protease inhibitor mix (0.1 mM AEBSF, 1 mM benzamidine, 1 μg/ml of each leupeptin, pepstatin, aprotinin, and E64), and various concentrations of \(^{3} \text{H}\)ryanodine. Nonselective binding was determined using a 1000-fold excess of unlabeled ryanodine. After 2 h at 37 °C, the 0.25-ml samples were diluted with 1 ml of ice-cold washing buffer composed of 25 mM Heps, pH 7.1, and 0.25 mM KCl and placed on Whatman GF/B membrane filters presoaked with 1% polyethyleneimine in washing buffer. Filters were washed three times with 6 ml of washing buffer. The filters were bound to the filter was quantified by liquid-scintillation counting. All binding assays were carried out in duplicate. To assess the effects of \( \text{Ca}^{2+} \) on high-affinity ryanodine binding, protocols were modified by removal of ATP from the binding buffer and addition of different concentrations of \( \text{Ca}^{2+} \) with 2.5 mM \(^{3} \text{H}\)ryanodine. Free \( \text{Ca}^{2+} \) was calculated using the apparent binding constants described by Fabiato (29).

SDS-Polyacrylamide Gel Electrophoresis and Immunoblotting—About 50 μg of proteins from cells lysed with CHAPS were separated by 5% SDS-polyacrylamide gel electrophoresis (30). \( \text{RyR} \) proteins were detected by immunoblotting (31) as described previously (26).

Protein Assay—Protein concentration was determined by dye binding using bovine serum albumin as a standard (32).

Data Analysis—Data were analyzed with Microcal Origin software (Microcal Software Ltd., Northampton, MA). Scatchard analysis was used to determine the dissociation constant (\( K_d \)) and maximum binding capacity (\( B_{\max} \)) from equilibrium binding data. \( E_{\text{cp}} \) and \( I_{\text{cp}} \) values were obtained by fitting the curves with an equation for logistic dose response. Data are expressed as mean ± S.E. An unpaired Student \( t \) test was used for evaluation of the mean values between groups. A value of \( p \leq 0.05 \) was considered to be statistically significant.

RESULTS

In earlier studies, we showed differences in the curves of \( \text{Ca}^{2+} \) dependence for \(^{3} \text{H}\)ryanodine binding to recombinant \( \text{RyR1} \) and \( \text{RyR2} \) that could be equated with differences in \( \text{Ca}^{2+} \) inactivation (21). We exploited this difference to locate the \( \text{Ca}^{2+} \) inactivation sites of \( \text{RyR1} \) in the \( \text{COOH} \) terminus, in particular, in fragments between amino acids 3726–4186 (F9) and 4187–4381 (F10). We also observed that chimeras containing F9 and F10 were more sensitive to \( \text{Ca}^{2+} \) and caffeine activation, implying that these two sequences might be involved in \( \text{Ca}^{2+} \) and caffeine activation of \( \text{RyR} \). Because F10 encompasses the most divergent region (D1) between \( \text{RyR1} \) and \( \text{RyR2} \), and because we were interested in the role of this region in \( \text{Ca}^{2+} \) release channel function, we subdivided the D1 region of \( \text{RyR1} \) into three smaller chimeras by substitution with the corresponding regions of \( \text{RyR2} \) (Fig. 1). We also constructed a deletion mutant in the D1 region of \( \text{RyR1} \) (Fig. 1). These constructs were then transiently expressed in HEK-293 cells, and the \( \text{Ca}^{2+} \) dependence of \(^{3} \text{H}\)ryanodine binding (21) was used as an indirect measurement of \( \text{Ca}^{2+} \) activation and inactivation.
The chimeric or deletion-mutant RyR, compared with RyR1. The mutant was subdivided, there was no obvious difference in mobility when sequence have a higher mobility than RyR1 in SDS gels. When F10 confers high level expression. In addition, caffeine-induced Ca^{2+} release was measured in vivo by Ca^{2+} photometry using HEK-293 cells transfected with chimeric or deletion-mutant RYR cDNA.

**Transient Expression of Chimeric and Deletion-mutant RyR cDNAs—** Immunostaining of CHAPS-solubilized cell lysates, using monoclonal antibody 34C against an epitope located in RyR1 amino acids 2756–2803 (11), was used to detect the expression of RyR proteins in transfected HEK-293 cells. Fig. 2 shows the absence of RyR immunostaining in pcDNA-transfected cells. Because the chimeric and deletion-mutated proteins all retained the RyR1 epitope, immunostaining with monoclonal antibody 34C was used as a measure of RyR expression. Immunostaining demonstrated that the chimeras RF10a, RF10b, and the deletion mutant Δ4274–4535-R1 were expressed at levels comparable to wild type RyR1 and that RF10a and Δ4274–4535-R1 were expressed at levels higher than that of RyR1. It has been demonstrated that RyR1 and RyR2 are not expressed with equal efficiency, and F10 and F11 from RyR2 have been shown to be the key sequences conferring high levels of expression in HEK-293 cells (19). In this study, a smaller fragment, F10a from RyR2, was also shown to confer high level expression.

We have observed that RyR2 and chimeras containing the F10 sequence have a higher mobility than RyR1 in SDS gels. When F10 was subdivided, there was no obvious difference in mobility when compared with RyR1. The mutant Δ4274–4535-R1 had a slightly higher mobility due to deletion of 262 amino acids.

**Fluorescence Measurements of Caffeine-induced Ca^{2+} Release in Vivo—** We used Fura-2 fluorescence to measure the properties of caffeine-induced Ca^{2+} release in the chimeric or deletion-mutant proteins expressed in HEK-293 cells (21). No significant Ca^{2+} release occurred with caffeine up to 30 mM in pcDNA-transfected cells (21), but caffeine-induced Ca^{2+} release was observed in cells transfected with each of the constructs. The peak fluorescence amplitude was measured during the course of incremental application of 0.03 to 30 mM caffeine and normalized to the peak amplitude for maximal Ca^{2+} release induced by 30 mM caffeine. EC_{so} values were then calculated by fitting the caffeine dose-response curves with an equation for logistic dose response. As described previously (21), EC_{so} values measuring the caffeine sensitivity of Ca^{2+} release were higher for recombinant RyR2 than for recombinant RyR1 (Fig. 3). Dose response curves for RyR1, RyR2, RF10, and chimeric and deletion-mutant proteins are shown in Fig. 3. EC_{so} values are summarized in the inset to Fig. 3.

Chimera RF10b had an EC_{so} value for caffeine that was similar to RyR1. However, chimeras RF10, RF10a and mutant Δ4274–4535-R1 had lower caffeine EC_{so} values, and RF10c had a higher caffeine EC_{so} value. Because caffeine activation was retained after deletion of sequences in the D1 region, caffeine activation sites are unlikely to be located in this region. The higher caffeine sensitivity exhibited by the RF10 and RF10a chimeras and the deletion mutant Δ4274–4535-R1 and the lower sensitivity by the RF10c chimera might be explained by induced conformations that modulate the Ca^{2+} release channel function, either negatively or positively. The Hill coefficients for chimeras RF10, RF10a, and RF10b resembled that for RF10 (Fig. 3, inset), and the Hill coefficients for other constructs were similar to that of RyR1, indicating that the F10a (and possibly F10b) sequence of RF10 can partially suppress the co-operative interactions that occur in RyR1.

**High Affinity Equilibrium Binding of [3H]Ryanoidine to Chimeric and Deletion-mutated RyRs—** We measured the equilibrium binding properties of [3H]ryanoidine to the chimeric and mutated RyR proteins to determine whether the high affinity ryanoidine binding site was preserved. We also used [3H]ryanoidine binding to determine expression levels, because 1 mol of a tetrameric RyR molecule binds 1 mol of ryanoidine with high affinity (21, 26, 33). Scatchard analysis showed a single binding site in all of the chimeras and the deletion mutant (Fig. 4), effectively ruling out the possibility that the high affinity ryanoidine binding site is located between amino acids 4274 and 4535. K_{d} values for these mutants were similar to those for wild type RyR2 and RyR1 (Ref. 21 and Fig. 4, inset), ranging from 1.6 nM for RF10 to 3.3 nM for RF10a. These data indicate that the high affinity binding site for ryanoidine is unchanged in all of these mutants. The B_{max} values ranged from 0.22 to 1.42 pmol/mg of protein in these chimeras and mutants (Fig. 4, inset), reflecting different expression levels. These results show that RF10a expression, like RF10 expression, was increased 5–6-fold over RyR1 expression. The expression of Δ4274–4535-R1 was increased 3-fold, confirming results from immunoblotting. As reported previously (21), there was no significant binding in lysates isolated from pcDNA-transfected HEK-293 cells.

**Ca^{2+} Activation and Inactivation of High Affinity [3H]Ryanoidine Binding to Chimeric and Deletion-mutant RyRs—** Dose-response curves for Ca^{2+} activation and inactivation of [3H]ryanoidine binding to wild type RyR1 and RyR2 and RF10 were shown previously (19, 21). The dose-response curves for Ca^{2+} activation and inactivation of [3H]ryanoidine binding to the mutants are shown in Fig. 5, where they are compared with
those of RF10. At Ca\(^{2+}\) concentrations below pCa 7, there was little binding of \[^{3}H\]ryanodine to the RyR proteins, except for D\(^{4274–4535}\)R1, which bound nearly 0.14 pmol of \[^{3}H\]ryanodine per mg of protein in the absence of Ca\(^{2+}\). This level of activation was observed even in the presence of 1 mM EGTA (data not shown). As with wild type RyR1 and RyR2, \[^{3}H\]ryanodine binding was activated by increasing Ca\(^{2+}\) concentrations, with maximal binding occurring between pCa 5.7 and pCa 4 for most of the constructs. EC\(_{50}\) values, expressed in pCa units, were similar for wild type RyR1 and RyR2, and the EC\(_{50}\) value for RF10a did not differ from that of either RyR1 or RyR2 (Fig. 5 and Table I). However, in chimeras RF10b and RF10c and especially in the mutant D\(^{4274–4535}\)R1, activation of \[^{3}H\]ryanodine binding was observed with lower Ca\(^{2+}\) concentra-

**Fig. 3.** Dose-response curves obtained from fluorescence measurements of \textit{in vivo} Ca\(^{2+}\) release induced by incremental concentrations of caffeine in HEK-293 cells transfected with \textit{RyR1}, \textit{RyR2}, chimeric \textit{RyR1/RyR2}, and deletion-mutated \textit{RyR1} cDNAs. Cells on a glass coverslip were loaded with 1 μM Fura-2-AM and mounted on the stage of an inverted microscope. Selected fields containing about 30 cells were challenged with incremental concentrations of caffeine added on the top of the cells immersed in media. Caffeine was washed out to restore resting Ca\(^{2+}\) concentrations after the peak amplitude (peak of change in the ratio of fluorescence at 340/380 nm, indicating a peak change in [Ca\(^{2+}\)]) was obtained. Individual peak amplitudes (fluorescence ratio at the highest response to caffeine minus the ratio at rest) were collected and normalized to the maximal amplitude of the peak response in 340/380 nm fluorescence ratio caused by 30 mM caffeine in each experiment. The resulting dose-response curves were fitted with an equation for logistic dose response to obtain EC\(_{50}\) values and Hill coefficients (for details, see Ref. 21). Values are the averages of 4–6 separate experiments for the chimeras and mutants and are presented in the inset table. a, p \(\leq 0.05\) when compared with RyR1. Values for RyR1, RyR2, and RF10 were from our previous studies (19, 21).

**Fig. 4.** Scatchard analysis of \[^{3}H\]ryanodine binding to solubilized \textit{RyR1/RyR2} chimeras and \textit{RyR1} mutant expressed in transfected HEK-293 cells. Solubilized proteins (25 μl) were incubated with various concentrations of \[^{3}H\]ryanodine (0.031 to 20 nM) in binding buffer (pCa 4.7) containing 0.1% CHAPS and 1 mM ATP at 37 °C for 2 h. Specific \[^{3}H\]ryanodine binding was determined by filtration, as described under “Experimental Procedures.” \[^{3}H\]Ryanodine bound/free (pmol/mg of protein/nM) was plotted as a function of \[^{3}H\]ryanodine bound (pmol/mg of protein). Linear fitting yielded the \(K_d\) and \(B_{\text{max}}\) values from three or four separate experiments, which are presented in the inset table.
trations, and EC_{so} values were significantly higher than those for wild type RyR1 and RyR2 (Fig. 5 and Table I). In addition, the slope for RF10b and Δ4274–4535-R1 was decreased below 0.7, indicating that the co-operativity of Ca^{2+} activation that is observed in wild type RyR1 and RyR2 is absent in these mutants and is lowered below 2 in mutants RF10 and RF10c.

Ca^{2+} inactivation was studied indirectly through measurement of the inhibition of [3H]ryanodine binding by elevated Ca^{2+}. IC_{50} values for the chimeric and deletion mutant RyR proteins, expressed in pCa units, are also presented in Table I and illustrated in Fig. 5, where they are compared with values for wild type RyR1, RyR2, and RF10. The IC_{50} for chimeras RF10b and F10c did not differ from that of wild type RyR1. IC_{50} values were significantly reduced, however, for chimera RF10a (pCa 1.90), associating the RF10a sequence (amino acids 4187–4381) with the low affinity Ca^{2+} binding site. The slopes for the curves of inactivation were not changed for chimeras and mutant Δ4274–4535-R1, when compared with RyR1. Mutant Δ4274–4535-R1 also had a lower IC_{50} (pCa 2.01) when compared with RyR1, suggesting some involvement of this sequence with the low affinity Ca^{2+} binding site, perhaps in its region of overlap with RF10a.

DISCUSSION

In an earlier study, we measured alterations in Ca^{2+} release channel function that resulted from exchange of RyR1 sequences with corresponding sequences in RyR2 (19). In this study, we substituted shorter sequences in RyR1 with corresponding sequences in RyR2 and measured alterations in channel sensitivity to Ca^{2+} and caffeine. This strategy allowed us to map part of the Ca^{2+} inactivation site to the F10a sequence (amino acids 4187–4381). Decreased sensitivity to inactivation by Ca^{2+} in mutant Δ4274–4535-R1 may be due either to the deletion of the Ca^{2+} inactivation site and rearrangement of overlap with RF10a or to conformational changes induced by the deletion. Increased sensitivity to activation by Ca^{2+} or caffeine was also observed in all chimeras. This is unlikely to involve the activation sites for Ca^{2+} and caffeine, because the deletion mutant Δ4274–4535-R1 exhibited increased sensitivity to Ca^{2+} and caffeine. In fact, high affinity Ca^{2+} binding sites are suggested to be in hydrophobic sequences (14, 15, 34), which were not analyzed in this study. Chen et al. (14) have presented evidence for a Ca^{2+} activation site involving a residue in RyR3 that corresponds to Glu^{4632}, located in TM2 of RyR1. They showed that the mutant channel retained normal conductance, but sensitivity to activating Ca^{2+} was reduced by 10,000-fold, and heterotetrameric forms of wild type and mutant chimeras, created by coexpression, had intermediate Ca^{2+} sensitivities. Therefore, it is most likely that the increased sensitivity observed in the chimeras was due to conformational changes that might have altered function through long range effects.
The use of antibodies against several domains in the D1 region has been associated with Ca\(^{2+}\) activation of the Ca\(^{2+}\) release channel. Polyclonal antibodies against the junctional face membrane of skeletal muscle sarcoplastic reticulum and purified ryanodine receptor from skeletal muscle blocked Ca\(^{2+}\)-induced Ca\(^{2+}\) release and decreased single channel open probability and conductance (35, 36). Some of the epitopes recognized by these anti-RyR antibodies have been mapped to amino acids 4445–4586 and 4760–4877. Polyclonal antibodies raised against amino acids 4380–4621 and 4425–4621 in the C terminus of RyR1 decreased Ca\(^{2+}\)-induced Ca\(^{2+}\) release and doxorubicin-induced Ca\(^{2+}\) release from isolated terminal cisternae (37). An antibody raised against amino acids 4478–4512 increased the Ca\(^{2+}\) sensitivity of the Ca\(^{2+}\) release channel (38). After the antibody was purified with a Pro-Glu repeat peptide sequence (amino acids 4490–4499), the purified antibody inhibited Ca\(^{2+}\)- or caffeine-activated channel activity but did not inhibit ATP-activated channel activity (39). The major epitopes for the antibody made against amino acids 4478–4512 were shown not to be located in the Pro-Glu repeat region. From these results, it might be deduced that the domains involving Ca\(^{2+}\) activation are associated with or lie close to the cytoplasmic loop between proposed transmembrane sequences 2 and 5 in the Zozrato numbering scheme. These earlier results are, therefore, consistent with our current results. However, the Ca\(^{2+}\) activation site itself is not likely to be located between amino acids 4274 and 4535, as discussed above. Because deletion of amino acids 4274–4535 increased channel sensitivity to activation by Ca\(^{2+}\) and caffeine, this sequence in RyR1 could form a complex domain, which modulates RyR1 channel function, presumably by suppressing channel activation.

The sequences in RyR1 that were exchanged or deleted in this study, with the exception of RP10c, would be likely to form a cytoplasmic loop between M2 and M5 in the topological model of Zorzato et al. (7). Although M3 (amino acids 4277–4299), M4 (amino acids 4342–4362), and M5 (amino acids 4559–4580) were predicted to be transmembrane sequences (7), M3 and M4 are not highly conserved among RyR isoforms and are no longer considered to be transmembrane sequences (15). M5 is one of the most hydrophobic sequences in RyR1 and is almost certain to be a transmembrane sequence (6, 7). The fact that channel function was not destroyed after deletion of amino acids 4274–4535, which includes the sequences formerly designated M3 and M4, provides further reason to think that these sequences do not form any part of the channel pore.

Nothing is known of the structure of this probable cytoplasmic loop region, although Gly-, Ala-, and Pro-rich sequences between amino acids 4274 and 4535 (6, 7) might limit the extent of helical structure. Most of this sequence is hydrophilic, implying that at least part of the sequence is surface-exposed and antigenic (39, 40). Binding domains for several peptides have been mapped on RyR1 by cryoelectron microscopy (41–43). Binding sites for calmodulin have been identified between structural domains 3 and 7 in RyR1 (42, 43), and calmodulin binding sites have been localized to amino acids 4303–4328 and 4534–4552 (44, 45), which lie in the D1 region. Thus, it is possible that the D1 region lies near structural domain 3 and 7. Deletion of more than 200 amino acids might be apparent in cryoelectron microscopy of this mutant form of RyR1, providing a means to localize it in RyR1. Knowledge of the location of regulatory domains relative to the channel forming domains could be helpful in understanding the regulation of the Ca\(^{2+}\) release channel.

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