The Predicted TM10 Transmembrane Sequence of the Cardiac Ca\(^{2+}\) Release Channel (Ryanodine Receptor) Is Crucial for Channel Activation and Gating*

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The predicted TM10 transmembrane sequence, 4841FDFITFVFFVILLAIQGL14867, has been proposed to be the pore inner helix of the ryanodine receptor (RyR) and to play a crucial role in channel activation and gating, as with the inner helix of bacterial potassium channels. However, experimental evidence for the involvement of the TM10 sequence in RyR channel activation and gating is lacking. In the present study, we have systematically investigated the effects of mutations of each residue within the 24-amino acid TM10 sequence of the mouse cardiac ryanodine receptor (RyR2) on channel activation by caffeine and Ca\(^{2+}\). Intracellular Ca\(^{2+}\) release measurements in human embryonic kidney 293 cells expressing the RyR2 wild type and TM10 mutants revealed that several mutations in the TM10 sequence either abolished caffeine response or markedly reduced the sensitivity of the RyR2 channel to activation by caffeine. By assessing the Ca\(^{2+}\) dependence of \(^{3}H\)ryanodine binding to RyR2 wild type and TM10 mutants we also found that mutations in the TM10 sequence altered the sensitivity of the channel to activation by Ca\(^{2+}\) and enhanced the basal activity of \(^{3}H\)ryanodine binding. Furthermore, single I4862A mutant channels exhibited considerable channel openings and altered gating at very low concentrations of Ca\(^{2+}\). Our data indicate that the TM10 sequence constitutes an essential determinant for channel activation and gating, in keeping with the proposed role of TM10 as an inner helix of RyR. Our results also shed insight into the orientation of the TM10 helix within the RyR channel pore.

Muscle contraction is initiated by the release of Ca\(^{2+}\) from the sarcoplasmic reticulum. In cardiac muscle, it is believed that sarcoplasmic reticulum Ca\(^{2+}\) release is triggered by Ca\(^{2+}\) influx via a mechanism known as Ca\(^{2+}\)-induced Ca\(^{2+}\) release (1–4). Specifically, the voltage-gated Ca\(^{2+}\) channel or dihydropyridine receptor located in the transverse tubular membrane opens upon membrane depolarization. The resulting Ca\(^{2+}\) entry through the dihydropyridine receptor activates the cardiac Ca\(^{2+}\) release channel (ryanodine receptor (RyR)) located in the sarcoplasmic reticulum, leading to a large Ca\(^{2+}\) release and, subsequently, muscle contraction. Activation of RyR2 by Ca\(^{2+}\) is therefore an essential step in the process of excitation-contraction coupling in heart muscle, and alterations in Ca\(^{2+}\) activation of RyR2 have been implicated in diseases, including heart failure and arrhythmias (5–7). Despite its physiological importance, the molecular mechanism of Ca\(^{2+}\) activation of RyR remains poorly understood.

Activation of RyR by Ca\(^{2+}\) is likely to be mediated by the binding of Ca\(^{2+}\) ions to high affinity Ca\(^{2+}\) binding sites in the channel protein (8, 9), but the locations of these Ca\(^{2+}\) activation sites in the RyR sequence are unknown. Structure-function studies of RyR have provided important information about the molecular determinants of Ca\(^{2+}\) activation. Expression of an NH\(_{\text{terminally}}\)-terminally truncated RyR containing the last −1,000 COOH-terminal amino acid residues in Chinese hamster ovary cells yielded a Ca\(^{2+}\)-activated Ca\(^{2+}\) release channel, indicating that the Ca\(^{2+}\) sensor of RyR resides within the last −1,000 amino acid residues (10). Within this region, several EF-hand-like Ca\(^{2+}\) binding sites have been identified, although their significance in RyR function has yet to be demonstrated (11, 12). Furthermore, deletion of residues 4,274–4,535 increased the sensitivity of RyR1 to activation by caffeine and Ca\(^{2+}\), indicating that this region is unlikely to contain the Ca\(^{2+}\) activation sites (13). We have demonstrated recently that a single point mutation, E3987A in RyR2 (14) and E3885A in RyR3 (15), reduces the sensitivity of the RyR channel to Ca\(^{2+}\) activation by 1,000–10,000-fold. Moreover, coexpression of the RyR2 or RyR3 wild type (wt) and the corresponding Ca\(^{2+}\)-sensing mutant proteins produces single individual channels that exhibit intermediate sensitivity to Ca\(^{2+}\) activation. These observations have led us to propose that the Glu3987 residue in RyR2 and the Glu3885 residue in RyR3 constitute an essential determinant of Ca\(^{2+}\) activation (14, 15).

In addition to these identified residues, other determinants of Ca\(^{2+}\) activation probably exist particularly in the channel pore region of RyR because the signal or conformational changes resulting from the binding of Ca\(^{2+}\) ions to the activation sites must be transduced into the pore region to open the channel. However, the exact signal transduction mechanism for Ca\(^{2+}\) activation is currently unknown because of, in part, the lack of detailed structural information about the RyR channel pore. Sequence analysis and functional expression studies have suggested that the ion conduction pore of RyR is located in the COOH-terminal region (10, 16, 17). Recent site-directed

* This work was supported by research grants from the Canadian Institutes of Health Research and the Heart and Stroke Foundation of Alberta, Northwestern Territories, and Nunavut (to S. R. W. C.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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‡ The abbreviations used are: RyR, ryanodine receptor; CHAPS, 3-(3-cholamidopropyl)dimethylammonio)-1-propanesulfonic acid; HEK, human embryonic kidney; TM, transmembrane; wt, wild type.
mutagenesis studies have revealed further insights into the location of the conduction pathway of RyR. We have shown that a single substitution of alanine for glycine at position 4824 in the mouse RyR2 decreases the single channel conductance by 97% (18). The Gly\(^{4824}\) residue is located in a highly conserved luminal loop linking the predicted transmembrane helices TM8 and TM10, and the loop linking these two transmembrane helices form a major part of the RyR channel pore.

The predicted secondary structure of this putative pore-forming region of RyR shares a high degree of similarity with that of the bacterial potassium channel KcsA, whose three-dimensional structure has been resolved (21). The crystal structure of both the Ca\(^{2+}\)- and voltage-gated bacterial potassium channels have also been determined (22–24), and a comparison of these three-dimensional structures shows an almost identical pore architecture among both the ligand- and voltage-gated bacterial potassium channels. Based on sequence analysis and mutational studies, Williams et al. (25) have suggested that the RyR pore may share a basic common architecture with that of potassium channels, and they proposed a structural model for the pore-forming region of RyR using the three-dimensional pore structure of KcsA as a framework. In this hypothetical model, the TM8 and TM10 sequences are thought to correspond, respectively, to the outer and inner helices of the KcsA pore, while the luminal loop linking TM8 and TM10 contain motifs equivalent to the pore helix and selectivity filter of KcsA.

Comparative analysis of the three-dimensional structures of closed and open potassium channels reveals that the inner helices are involved in coupling the conformational changes in the gating domain to channel opening via a gating hinge located near the middle of the inner helix (22). This structural feature is apparently conserved among a wide range of ligand- and voltage-gated potassium channels (22–24). On the basis of these findings and the proposed pore model of RyR, it is anticipated that the putative inner helix of the RyR channel pore, TM10, would be involved in channel activation and gating.

In line with this hypothesis, we have recently shown that the TM10 sequence contains an important determinant for ryanodine interaction (26) and that ryanodine is able to sensitize the RyR channel to Ca\(^{2+}\) activation (27), suggesting the involvement of TM10 in Ca\(^{2+}\) activation. To test this hypothesis directly, we have mutated each residue in the TM10 sequence and systematically characterized the effects of these mutations on channel activation by caffeine or by Ca\(^{2+}\). These studies demonstrate that mutations in the TM10 sequence can alter the sensitivity of the channel to activation by caffeine and Ca\(^{2+}\), the basal channel activity, and the gating properties, indicating that the TM10 sequence plays an important role in RyR channel activation and gating.

**EXPERIMENTAL PROCEDURES**

**Materials**—\(\text{H}^{3}\)Iryanodine was obtained from PerkinElmer Life Sciences. Ryanodine was purchased from Calbiochem. Brain phosphatidylserine, heart phosphatidylethanolamine, and plant phosphatidylcholine were purchased from Avanti Polar Lipid. CHAPS, caffeine, and other reagents were purchased from Sigma.

**Site-directed Mutagenesis and DNA Transfection**—Single point mutations within the proposed TM10 transmembrane sequence of the mouse RyR2 were generated by the overlap extension method as described previously (26). HEK293 cells grown on 100-mm tissue culture dishes in Dulbecco’s modified Eagle’s medium, supplemented with 0.1 mM nonessential amino acids, 4 mM l-glutamine, 100 units of penicillin/ml, 100 \(\mu\)g of streptomycin/ml, 4.5 g of glucose/filter, and 10% fetal calf serum, for 18–20 h after subcultures were transfected with 12 \(\mu\)g of wt or mutant RyR2 cDNA using the method of Ca\(^{2+}\) phosphate precipitation (28).

**Ca\(^{2+}\) Release Measurements in Transfected HEK293 Cells**—Free cytosolic Ca\(^{2+}\) concentration in transfected HEK293 cells was measured using the fluorochrome 

**RESULTS**

**Effect of Mutations in the TM10 Sequence on the Response of RyR2 to Activation by Caffeine**—To investigate the role of the proposed pore inner helix, TM10, in RyR channel activation, we have mutated each amino acid residue within the TM10 sequence to either alanine or glycine, the latter only in the case of the single alanine residue, Ala\(^{4862}\). A total of 24 TM10 mutants were generated. We have shown that all of the TM10 mutants were expressed in HEK293 cells and that their levels of expression were comparable with that of the wt (26). To screen the impact of this large number of mutations on channel activation quickly, we employed a simple and quick Ca\(^{2+}\) release assay based on the use of the HEK293 cell expression system and using caffeine as a functional probe. Caffeine is the most widely used activator of RyRs and is believed to stimulate RyR activity by enhancing Ca\(^{2+}\) activation (9). In this assay, we monitored the level of intracellular Ca\(^{2+}\) release induced by sequential additions of increasing concentrations of caffeine in HEK293 cells transfected with wt cDNA or the TM10 mutants cDNA using the fluorescence 

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**H\text{I}Ryanodine Binding—Equilibrium \(\text{H}^{3}\)Iryanodine binding to cell lysates was performed as described previously (14) with some modifications. \(\text{H}^{3}\)Iryanodine binding was carried out in a total volume of 300 \(\mu\)l of binding solution containing 30 \(\mu\)l of cell lysate, 500 mM KCl, 25 mM Tris, 50 mM Hepes (pH 7.4), a wide range of Ca\(^{2+}\) concentrations or various channel modulators as indicated, 5 mM \(\text{H}^{3}\)Iryanodine, and a protease inhibitor mix at 37°C for 2–3 h. The binding mix was diluted with 5 ml of ice-cold washing buffer containing 25 mM Tris, pH 8.0, and 250 mM KCl and immediately filtered through Whatman GF/F filters presoaked with 1% polyethylenimine. The filters were washed, and the radioactivities associated with the filters were determined by liquid scintillation counting. Nonspecific binding was determined by measuring \(\text{H}^{3}\)Iryanodine binding in the presence of 20 \(\mu\)M unlabelled ryanodine. All binding assays were done in duplicate.

**Single Channel Recordings—Recombinant RyR2 wt and the I4862A mutant proteins were partially purified from whole cell lysate by sucrose density gradient centrifugation and used for single channel recordings as described previously (14).**

**RESULTS**
addition of caffeine from 0.05 mM up to 1.0 mM and then decreased with further additions of caffeine (2.5 and 5.0 mM). The reduced level of Ca$^{2+}$ release seen after the additions of 2.5 and 5.0 mM caffeine, compared with that seen after the addition of 1.0 mM caffeine, is likely the result of the depletion of the intracellular Ca$^{2+}$ stores by the previous additions of caffeine (0.025–1.0 mM).

The responses to this repeated caffeine stimulation of HEK293 cells expressing each TM10 mutant are shown in Figs. 1 and 2. Based on the patterns of their caffeine responses, the TM10 mutants can be grouped into four classes. The first class includes I4844A[1], I4845A[2], F4846A[3], I4848A[5], T4849A[6], G4864A[21], A4860G[17], and Q4863A[20] belong to the third class of TM10 mutants. These mutants showed a pattern of response to repeated caffeine stimulation quite different from that observed with RyR2 wt. As seen in Fig. 2A, HEK293 cells expressing RyR2 wt were considerably activated by the first addition of 0.25 mM caffeine, and the level of Ca$^{2+}$ release generated by the first addition of caffeine (0.25 mM) was comparable with that detected with the second and the third additions of caffeine (0.5 and 1.0 mM, respectively). In contrast, the amount of Ca$^{2+}$ release induced by the first and second additions of caffeine (0.25 and 0.5 mM, respectively) to HEK293 cells expressing these mutants was lower than that observed with the third (1.0 mM) or subsequent additions (2.5 or 5.0 mM) of caffeine, indicating that these mutants require higher concentrations of caffeine for maximal activation than RyR2 wt. On the other hand, using the same set of caffeine concentrations (from 0.25 to 10 mM) (Fig. 2A), the response of the first class of TM10 mutants to repeated caffeine stimulation was indistinguishable from that of RyR2 wt (not shown), which is consistent with the data shown in Fig. 1A. The fourth class of TM10 mutants includes D4847A[4], F4850A[7], L4858A[15], L4859A[16], and I4866A[23]. HEK293 cells expressing these mutants displayed no or little Ca$^{2+}$ release in response to stimulation by various concentrations of caffeine (Fig. 2B). These mutations apparently have a severe impact on channel activation. Because all TM10 mutants were expressed in HEK293 cells at a comparable level, the reduced level or lack of caffeine-induced Ca$^{2+}$ release in the second and fourth classes of TM10 mutants was not the result of poor expression.

**Fig. 1.** Intracellular Ca$^{2+}$ release in HEK293 cells expressing RyR2 (wt) and the TM10 mutants in response to repeated caffeine stimulation. HEK293 cells were transfected with RyR2 (wt) (Aa), I4844A[1] (Ab), I4845A[2] (Ac), F4846A[3] (Ad), I4848A[5] ( Ae), T4849A[6] (Af), I4857A[14] (Ag), I4861A[18] (Ah), G4864A[21] (Ai), L4865A[22] (Aj), V4856A[13] (Ba), I4862A[19] (Bb), or I4867A[24] (Bc) cDNA. The fluorescence intensity of fluo 3-loaded cells was monitored continuously before and after consecutive additions of various concentrations of caffeine (from 0.025 to 5.0 mM). The decreases in fluorescence immediately after the addition of caffeine seen in some traces were the result of fluorescence quenching by caffeine. Traces shown are from representative experiments that have been repeated three times. Similar results were obtained in all replicates.

**Fig. 2.** Role of TM10 in channel activation and gating of intracellular Ca$^{2+}$ release.
Mutations in the TM10 Sequence Reduce the Sensitivity of RyR2 to Activation by Caffeine—To assess quantitatively the effect of the TM10 mutations on caffeine activation of RyR2, we determined the sensitivity of the TM10 mutants to activation by caffeine. In the light of the results of our initial screening (Figs. 1 and 2), we focused on the third class of TM10 mutants that appeared to have reduced caffeine sensitivity. HEK293 cells were transfected with each of the third class of the TM10 mutants. The transfected cells expressing the same mutant were pooled from multiple plates and aliquoted. The levels of intracellular Ca\(^{2+}\) release from each aliquot of transfected cells were then determined after a single addition of different concentrations of caffeine. Fig. 3 shows examples of individual Ca\(^{2+}\) release events in HEK293 cells expressing RyR2 wt (Fig. 3Aa) and F4852A[9] (Fig. 3Ab), induced by a single dose of caffeine of various concentrations. The peak values of caffeine-induced Ca\(^{2+}\) release were then used to generate the caffeine activation curves for RyR2 wt and each of the class III TM10 mutants. As shown in Fig. 3B, the sensitivity of all the class III TM10 mutants, in particular mutants F4851A[8], F4852A[9], I4855A[12], and A4860G[17], to caffeine activation was reduced compared with that of the wt, consistent with the observations seen in Fig. 2. Analyses of the concentration dependence of caffeine activation using the Hill equation yielded the apparent EC\(_{50}\) values for caffeine activation of 0.30 ± 0.02 mM (n = 3) for RyR2 wt, 5.10 ± 0.23 mM (n = 4) for F4851A[8], 1.25 ± 0.08 mM (n = 3) for F4852A[9], 0.55 ± 0.06 mM (n = 3) for F4853A[10], 0.79 ± 0.28 mM (n = 4) for F4854A[11], 2.40 ± 0.10 mM (n = 3) for I4855A[12], 1.75 ± 0.47 mM (n = 4) for A4860G[17], and 0.56 ± 0.20 mM (n = 4) for Q4863A[20] (inset in Fig. 3B). Taken together, these data indicate that mutations within the proposed TM10 pore inner helix markedly alter channel activation by caffeine.

Effects of the TM10 Mutations on the Sensitivity of RyR2 to Activation by Ca\(^{2+}\)—Because caffeine activates RyR by sensitizing the channel to Ca\(^{2+}\) activation (9), and mutations that alter Ca\(^{2+}\) activation can in turn affect caffeine activation (14), the observed changes in caffeine response suggest that the TM10 mutations may also alter Ca\(^{2+}\) activation. To test this possibility directly, we examined the response of the TM10 mutants to Ca\(^{2+}\) activation using [\(^{3}\)H]ryanodine binding. [\(^{3}\)H]Ryanodine binding has been used widely to assess RyR channel activities because ryanodine has access to its binding site only when the channel is in the open state (29–31). We have shown previously that about half of the 24 mutations made within the TM10 sequence severely curtailed or abolished [\(^{3}\)H]ryanodine binding (26). Hence the Ca\(^{2+}\) response of these mutants could not be assessed using the [\(^{3}\)H]ryanodine binding assay. On the other hand, 13 of the 24 TM10 mutants exhibited a considerable amount of [\(^{3}\)H]ryanodine binding. Subsequently, the Ca\(^{2+}\) response of these mutants was determined by measuring the amount of bound [\(^{3}\)H]ryanodine at a wide range of Ca\(^{2+}\) concentrations. Analysis of the Ca\(^{2+}\) response using the Hill equation yielded an EC\(_{50}\) value of 0.18 ± 0.02 mM (n = 4) for Ca\(^{2+}\) activation of RyR2 wt (Fig. 4A). Both increased and decreased EC\(_{50}\) values for Ca\(^{2+}\) activation were observed among the TM10 mutants. For instance, the V4854A[11] mutation substantially increased the EC\(_{50}\) value for Ca\(^{2+}\) activation to 0.75 ± 0.03 mM (n = 4), whereas the I4862A[19] mutation considerably reduced the EC\(_{50}\) value to 0.09 ± 0.02 mM (n = 5). Thus, mutations in the TM10 sequence can influence the sensitivity of the channel to Ca\(^{2+}\) activation. The EC\(_{50}\) values for other TM10 mutants were 0.62 ± 0.09 mM (n = 4) for I4844A[1], 0.24 ± 0.05 mM (n = 3) for I4845A[2], 0.48 ± 0.18 mM (n = 3) for I4845A[5], 0.43 ± 0.01 mM (n = 3) for F4852A[9], 0.39 ± 0.10 mM (n = 4) for F4853A[10], 0.14 ± 0.03 mM (n = 4) for I4857A[14], 0.34 ± 0.03 mM (n = 3) for A4860G[17], 0.40 ± 0.12 mM (n = 4) for I4861A[18], 0.13 ± 0.02 mM (n = 5) for G4864A[21], 0.22 ± 0.02 mM (n = 3) for L4865A[22], and 0.42 ± 0.03 mM (n = 3) for I4867A[24]. The B\(_{\text{max}}\) values for RyR2 wt and the TM10 mutants were 0.63 ± 0.29 pmol/mg (n = 4) for RyR2 wt, 0.28 ± 0.17 pmol/mg (n = 4) for F4851A[8], 0.25 ± 0.17 pmol/mg (n = 4) for F4852A[9], 0.39 ± 0.10 pmol/mg (n = 4) for F4853A[10], 0.14 ± 0.03 pmol/mg (n = 4) for I4857A[14], 0.34 ± 0.03 pmol/mg (n = 3) for I4862A[19], 0.42 ± 0.03 pmol/mg (n = 3) for I4867A[24], and 0.42 ± 0.03 pmol/mg (n = 3) for I4867A[24]. The
Fig. 3. Mutations in the TM10 sequence reduce the sensitivity of RyR2 to caffeine activation. HEK293 cells grown on 20 tissue culture dishes (100-mm diameter) were transfected with RyR2 (wt) (○), F4853A[10] (△), V4854A[11] (×), I4862A[19] (+), I4866G[17] (●), or Q4863A[20] (■) cDNA. HER293 cells transfected with the same DNA were pooled, loaded with fluo 3-AM, and aliquoted. Fluorescence intensity of an aliquot of fluo 3-AM-loaded cells was monitored continuously before and after a single addition of various concentrations of caffeine (0.01 to 10 mM). A shows examples of individual Ca^{2+} release events induced by different concentrations of caffeine in aliquots of HEK293 cells expressing RyR2 (wt) (Aa) and F4853A[9] (Ab). The levels of intracellular Ca^{2+} release triggered by various concentrations of caffeine in HEK293 cells expressing each mutant were normalized to the maximal level of Ca^{2+} release induced by 10 mM caffeine (100%) and fitted with the Hill equation (B). Data points shown are from representative experiments that have been repeated three or four times. The EC_{50} values of caffeine activation for wt and mutants are indicated as the mean ± S.E. (n = 3–4).

for I4844A[1], 0.29 ± 0.13 pmol/mg (n = 3) for I4845A[2], 0.40 ± 0.10 pmol/mg (n = 3) for I4848A[5], 0.41 ± 0.04 pmol/mg (n = 3) for F4852A[9], 0.56 ± 0.10 pmol/mg (n = 4) for F4853A[10], 0.12 ± 0.04 pmol/mg (n = 4) for V4854A[11], 0.33 ± 0.06 pmol/mg (n = 4) for I4857A[14], 0.27 ± 0.12 pmol/mg (n = 3) for A4860G[17], 0.23 ± 0.05 pmol/mg (n = 4) for I4861A[18], 0.24 ± 0.04 pmol/mg (n = 5) for I4862A[19], 0.52 ± 0.13 pmol/mg (n = 5) for G4864A[21], 0.35 ± 0.07 pmol/mg (n = 3) for L4865A[22], and 0.23 ± 0.06 pmol/mg (n = 3) for I4867A[24].

Mutations in the TM10 Sequence Enhance the Basal Activity of [3H]Ryanodine Binding to RyR2—In addition to alterations in Ca^{2+} sensitivity, Fig. 4 also shows that mutations in TM10 can induce considerable [3H]ryanodine binding to RyR2 at very low Ca^{2+} concentrations. At Ca^{2+} concentrations between 0.1 and 10 mM, 11.2 ± 1.9% (n = 4), 33.1 ± 8.7% (n = 5), and 11.5 ± 2.6% (n = 5) of maximal binding were observed in the I4857A[14], I4862A[19], and G4864A[21] mutants, respectively, whereas ~5% of maximal binding was detected in wt. This enhanced basal activity of [3H]ryanodine binding is unlikely to result from nonspecific [3H]ryanodine binding, as the basal activity was modulated by various ligands. As shown in Fig. 5, the basal [3H]ryanodine binding activity of the I4862A[19] mutant at ~3 mM Ca^{2+} was inhibited by Mg^{2+} and ruthenium red and was activated by Ca^{2+}, ATP, and caffeine. Furthermore, the response of the I4862A[19] mutant to different modulators at this Ca^{2+} concentration was similar to that observed at a higher concentration of Ca^{2+} (~43 mM). Because [3H]ryanodine binding reflects channel activity, the enhanced specific basal [3H]ryanodine binding to some of the TM10 mutants suggests that mutations in the TM10 sequence can lead to channel openings at very low concentrations of Ca^{2+}, probably by destabilizing the closed state of the channel.

Single Channel Properties of the I4862A[19] Mutant at Low Ca^{2+} Concentrations—To examine directly the possibility that the I4862A[19] mutation alters channel gating, we incorporated single wt and I4862A[19] mutant channels into planar lipid bilayers and assessed their channel properties at low Ca^{2+} concentrations. As shown in Fig. 6A, a single wt channel displayed very little channel activity at ~45 nM Ca^{2+}. The average open probability (Po), mean open time (To), and mean closed time (Tc) of single wt channels under these conditions were 8.3 × 10^{-5} ± 5.7 × 10^{-5}, 0.69 ± 0.26 ms, and 4.1 × 10^{3} ± 1.3 × 10^{3} ms (n = 9), respectively. On the other hand, under the same conditions, considerable opening events and altered gating were detected in single I4862A[19] mutant channels (Fig. 6B). The average Po, To, and Tc of single I4862A[19] mutant channels were 4.1 × 10^{-3} ± 3.8 × 10^{-3}, 1.8 ± 0.8 ms, and 350 ± 120 ms (n = 7), respectively. Hence, the I4862A[19] mutation increased the channel Po by ~50-fold and mean open time by ~2-fold, and decreased the mean closed time by ~90%. However, the single channel conductance of the I4862A[19] mutants (796 ± 25 pico Siemens, n = 5) was indistinguishable...
from that of the wt (−800 picosiemens). The marked decrease in mean closed time observed in single I4862A[19] mutant channels, compared with that of RyR2 wt, is consistent with the view that the I4862A[19] mutation may destabilize the closed state of the RyR2 channel. A destabilized closed state would be expected to cause spontaneous channel openings, and, consequently, depletion of the intracellular Ca\(^{2+}\) stores in HEK293 cells expressing the I4862A[19] mutant. This may account for the low level of caffeine-induced intracellular Ca\(^{2+}\) release observed in HEK293 cells expressing the I4862A[19] mutant (Fig. 1B). Taken together these observations indicate that mutations in TM10 can affect the gating properties of RyR2.

**DISCUSSION**

In the present study we have investigated the role of the predicted TM10 transmembrane sequence in channel activation by caffeine and Ca\(^{2+}\) and in channel gating. Each residue within the 24-amino acid TM10 sequence was mutated, and the effect of each mutation on channel function was assessed. These studies have demonstrated that mutations in the TM10 sequence can reduce the sensitivity of the channel to activation by caffeine or severely curtail caffeine activation. Using \[^{3}H\]ryanodine binding and single channel analysis we have shown that mutations in the TM10 sequence can influence the sensitivity of the channel to activation by Ca\(^{2+}\) and alter channel gating. We have also demonstrated recently that mutations in the TM10 sequence reduce or abolish \[^{3}H\]ryanodine binding. Together, these results indicate that the TM10 sequence is involved in both Ca\(^{2+}\) activation and ryanodine interaction. This dual role of TM10 may provide an explanation for the action of ryanodine on channel open probability and Ca\(^{2+}\) sensitivity (27). These data provide the first evidence that the TM10 sequence constitutes an essential determinant of RyR channel activation and gating.

It is likely that the TM10 sequence plays an important role in communicating the signals of binding of ligands such as Ca\(^{2+}\), caffeine, and ryanodine to channel opening, but the exact mechanisms underlying the ligand gating of RyR are unknown. Fundamental insights into the gating mechanism of potassium channels have recently been revealed by determining the three-dimensional structures of several bacterial potassium channels (22–24). A wide range of potassium channels seems to share a common structural basis for gating. Both ligand- and voltage-gated potassium channels contain a gating hinge located near the middle of the pore inner helix. It is believed that conformational changes in the gating domain induced by ligand binding or membrane voltage are transduced to the inner helices, causing the gating hinge to bend and resulting in opening of the inner helix bundle and the channel pore (22). Although it is unclear whether the RyR channel adopts a gating mechanism similar to that seen with potassium channels, sequence analysis and functional studies suggest that the putative pore region of RyR may share a basic architecture with that of the bacterial potassium channel KcsA. With this rationale, Williams et al. (25) have proposed a structural model for the RyR channel pore. In this model, the TM10 sequence of RyR...
would be equivalent to the inner helix of KcsA. Thus, being analogous to the inner helix of KcsA, the putative inner helix of the RyR channel, TM10, would have a major role in channel gating. Our observations that mutations in the TM10 sequence can alter channel activation and gating corroborate this view.

According to the hypothetical model of the RyR channel pore, the TM10 helices of each monomer would line a water-filled cavity of the tetrameric channel pore. In such an arrangement, one side of the TM10 helix would face the water-filled cavity, and the opposite side would interact with other helices or the lipid membrane. The amino acid residues that contribute to these two unique faces of the TM10 helix are unknown, but it is of interest to know that mutations that severely curtail caffeine or Ca\(^{2+}\) activation are predominantly clustered on one side of the TM10 helix. These mutations include I4844A[1], D4847A[4], F4850A[7], F4851A[8], F4852A[9], V4854A[11], I4855A[12], L4858A[15], L4859A[16], and I4866A[23]. On the other hand, most of the mutants located on the other side of the TM10 helix retain considerable channel activity. This side of the helix also contains residues Ala\(^{4860}\)[17] and Gly\(^{4864}\)[21], which have small side chains, and residues Thr\(^{4849}\)[6] and Gln\(^{4863}\)[20], which possess low relative hydrophobicity. If the TM10 helices do indeed form the water-filled cavity, one would expect that mutations of large and hydrophobic residues to a smaller and less hydrophobic residue, alanine, would have relatively mild effects on channel activity if the residue is facing the aqueous pore, but would impair channel function if the residue is involved in hydrophobic interactions. One would also expect that large and highly hydrophobic side chains pointing toward the aqueous pore would tend to impede ion conduction.

On the basis of these rationales and the results of our mutational studies, we propose a model for the orientation of the TM10 helix in relation to the channel pore. As shown in Fig. 7, we hypothesize that the side of the TM10 helix containing smaller and less hydrophobic residues is oriented toward the water-filled cavity, whereas the opposite side of the helix is involved in hydrophobic interactions with other helices of the channel. In support of this model, we have shown that TM10 residues such as Phe\(^{4846}\)[3], Thr\(^{4849}\)[6], Val\(^{4856}\)[13], and Gln\(^{4863}\)[20] are critical for interaction with ryanodine (26). These residues are located on the proposed pore-lining side of the TM10 helix, which is consistent with the notion that the high affinity ryanodine binding site is likely located in the outer vestibule of the RyR channel pore (31). Systematic investigations of the accessibility of the TM10 residues should provide further insight into the orientation of the TM10 helix within the channel pore.

In potassium channels the water-filled cavity formed by the inner helices not only provides a pathway for ion conduction, but also contains receptor sites for the N-type inactivation gate and large, organic blocking cations (32). Interestingly, permeant cation translocation in the RyR channel can be blocked by both potassium channel inactivation peptides (33) and large tetraalkylammonium cations such as tetrabutylammonium (34), suggesting that the water-filled cavity of the RyR channel, presumably formed by the putative inner helix, TM10, may also contain receptor sites for peptides and organic cations. In this context, it will be of interest to determine whether the TM10 helix and, in particular, the proposed pore-lining face of the TM10 helix, is involved in interactions with various compounds and peptides that have been shown to alter the activity and conduction of RyR.

In summary, mutations in the predicted TM10 transmembrane sequence can influence the activation and gating of the RyR2 channel. These data are in agreement with a structural model of the RyR pore in which the TM10 sequence constitutes the pore inner helix that lines the water-filled cavity and plays an important role in channel activation and gating. Further investigation of the orientation of the TM10 helix, the molecular determinants of the gating hinge and gating domain, and the interactions between them should help toward an understanding of the mechanisms of gating and Ca\(^{2+}\) activation of the RyR channel.

Acknowledgments—We thank Dr. Alan Williams for helpful discussions and suggestions on modeling the RyR2 channel pore and for critical reading of the manuscript.

Fig. 7. Hypothetical models of the TM10 sequence and the RyR2 channel pore. A, the TM10 sequence is modeled as an \(\alpha\)-helix. One side of the TM10 helix mainly contains residues that have large side chains with high relative hydrophobicity. Most of the mutations located on this side of the helix (indicated by blue circles) severely curtailed caffeine or Ca\(^{2+}\) activation of the RyR2 channel. The opposite side of the TM10 helix contains residues that have small side chains and less relative hydrophobicity (depicted by green circles). Most of the mutants located on this side of the helix retain considerable channel activities. Numbers beside each circle indicate the positions of amino acid residues in the TM10 sequence, running from the NH\(_2\)-terminal end (luminal side) to the COOH-terminal end (cytoplasmic side). The linear sequence of TM10 (boxed) and positions of the TM10 residues in the RyR2 sequence (top numbers) are also illustrated. B, the TM10 helices from each RyR2 monomer are proposed to line the tetrameric channel conduction pore. We hypothesize that the side of the TM10 helix which contains small and less hydrophobic residues (indicated by the green area) faces the water-filled cavity, whereas the opposite side of the helix (depicted by the blue area) interacts with other transmembrane helices (TMs) of the channel.
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The Predicted TM10 Transmembrane Sequence of the Cardiac Ca\(^{2+}\) Release Channel (Ryanodine Receptor) Is Crucial for Channel Activation and Gating

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J. Biol. Chem. 2004, 279:3635-3642. doi: 10.1074/jbc.M311367200 originally published online October 30, 2003

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