The central domain of cardiac ryanodine receptor governs channel activation, regulation, and stability

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Structural analyses identified the central domain of ryanodine receptor (RyR) as a transducer converting conformational changes in the cytoplasmic platform to the RyR gate. The central domain is also a regulatory hub encompassing the Ca2+-, ATP-, and caffeine-binding sites. However, the role of the central domain in RyR activation and regulation has yet to be defined. Here, we mutated five residues that form the Ca2+ activation site and 10 residues with negatively charged or oxygen-containing side chains near the Ca2+ activation site. We also generated eight disease-associated mutations within the central domain of RyR2. We determined the effect of these mutations on Ca2+, ATP, and caffeine activation and Mg2+ inhibition of RyR2. Mutating the Ca2+ activation site markedly reduced the sensitivity of RyR2 to Ca2+ and caffeine activation. Unexpectedly, Ca2+ activation site mutation E3848A substantially enhanced the Ca2+-independent basal activity of RyR2, suggesting that E3848A may also affect the stability of the closed state of RyR2. Mutations in the Ca2+ activation site also abolished the effect of ATP/caffeine on the Ca2+-independent basal activity, suggesting that the Ca2+ activation site is also a critical determinant of ATP/caffeine action. Mutating residues with negatively charged or oxygen-containing side chains near the Ca2+ activation site significantly altered Ca2+ and caffeine activation and reduced Mg2+ inhibition. Furthermore, disease-associated RyR2 mutations within the central domain significantly enhanced Ca2+ and caffeine activation and reduced Mg2+ inhibition. Our data demonstrate that the central domain plays an important role in channel activation, channel regulation, and closed state stability.

Ryanodine receptor type 2 (RyR2) is an intracellular Ca2+ release channel that is expressed predominantly in the heart and brain and plays an essential role in many cellular processes, including muscle contraction, learning, and memory, by governing the release of Ca2+ from intracellular Ca2+ stores (1–9). RyR2 is normally activated through a mechanism known as Ca2+-induced Ca2+ release (CICR), in which an elevation of cytosolic Ca2+ opens the RyR2 channel, leading to a large Ca2+ release from the sarcoplasmic reticulum and the endoplasmic reticulum (10–12). Thus, activation of RyR2 by cytosolic Ca2+ is a critical step in the mechanism of CICR (13–15). Consistent with its important physiological roles, impaired Ca2+ activation of RyR2 has been associated with diseases in both the heart and brain, such as cardiac arrhythmias, cardiomyopathies, and intellectual disability (16–19). However, despite its physiological and pathological significance, the molecular mechanism underlying Ca2+ activation of RyR2 is not well defined.

It has long been recognized that RyR2 contains a high-affinity cytosolic Ca2+ activation site that mediates Ca2+ activation of RyR2 and CICR (10–12, 20–24). Major efforts have been focused on identifying residues that are responsible for Ca2+ activation of RyR2. Through site-directed mutagenesis and single-channel analyses, we showed that a point mutation E3987A in RyR2 dramatically reduced the sensitivity of RyR2 to activation by Ca2+ (25). We also reported that point mutation E3885A in RyR3 (corresponding to E3987A in RyR2) markedly decreased the Ca2+ sensitivity of RyR3 (26). Furthermore, point mutation E4032A in RyR1 (corresponding to E3987A in RyR2) abolished Ca2+-dependent activation of RyR1 (27, 28). These findings indicate that residue Glu-3987 in RyR2 and the corresponding residue Glu-4032 in RyR1 and Glu-3885 in RyR3 play an important role in Ca2+ activation of RyRs.

Recent success in solving near-atomic resolution 3D structures of RyR1 and RyR2 using cryo-EM has greatly advanced our understanding of the molecular basis of RyR activation (29–36). Comparisons of the open and closed states of RyR1 and RyR2 reveal that the central domains of RyR1 (amino acids 3668–4251) and RyR2 (amino acids 3613–4207) play an important role in channel activation (32–36). The putative Ca2+ binding site in RyR1 formed by residues Glu-3893, Glu-3967, Gln-3970, His-3895, and Thr-5001 in the core solenoid domain of RyR1, and the putative Ca2+ binding site in RyR2 formed by residues Glu-3848, Glu-3922, Gln-3925, His-3850, and Thr-4931 in the central domain of RyR2 have been identified (34–36). Interestingly, the RyR1–Glu-4032 or RyR2–Glu-3987 residue is located just beside the Ca2+ activation site. Although the RyR1–Glu-4032 or RyR2–Glu-3987 residue does not directly contribute to Ca2+ coordination, it is involved in H-bonding between the central domain and the C-terminal domain (CTD) that may stabilize the Ca2+ binding pocket. Furthermore, the putative ATP- and caffeine-binding sites have also been localized to regions very close to the Ca2+ activation site (34–36). Recent functional studies are consistent with the proposed locations of the Ca2+ and caffeine activation sites (37). For instance, mutations in the Ca2+ activation site (E3848A or E3922A in RyR2) diminished Ca2+ activation of RyR2, whereas mutation W4644A or W4644R in the caffeine-binding site abolished caffeine activation of RyR2 (37). Thus, the central domain is critically involved in RyR2 channel
activation and regulation. It is also known that RyR is inactivated by Mg\(^{2+}\) (20, 22–24, 38), but the molecular basis of Mg\(^{2+}\)-dependent inhibition of RyR is not well understood.

In addition to those Ca\(^{2+}\)-coordinating residues, the central domain also contains a number of residues with negatively charged or oxygen-containing side chains clustered near the Ca\(^{2+}\) activation site (29–36). The significance of these residues in channel activation and regulation is unknown. The central domain also harbors a number of RyR2 mutations associated with catecholaminergic polymorphic ventricular tachycardia (CPVT) and sudden unexplained death (17, 39), but their functional effect has yet to be determined. It is also unclear how ATP and caffeine modulate Ca\(^{2+}\) activation of RyR2. To address these important questions, in the present study, we performed a systematic site-directed mutagenesis and structure-function relationship analysis of the central domain of RyR2. Our results revealed that the central domain plays a critical role not only in channel activation by Ca\(^{2+}\), ATP, and caffeine, but also in channel regulation by Mg\(^{2+}\) and in determining the stability of the closed state of the channel in the near absence of Ca\(^{2+}\). Our data also showed that disease-associated RyR2 mutations located within the central domain enhanced Ca\(^{2+}\) activation and reduced Mg\(^{2+}\) inhibition of RyR2. Thus, the central domain controls the activation and regulation of the RyR2 channel.

**Results**

**Contribution of Ca\(^{2+}\)-coordinating residues to Ca\(^{2+}\) and caffeine activation and basal activity of RyR2**

Recent 3D structural analyses revealed the RyR2 Ca\(^{2+}\) activation site that is formed by residues Glu-3848, Glu-3922, Gln-3925, His-3850, and Thr-4931 (35, 36) (Fig. 1A). To assess the role of these Ca\(^{2+}\)-coordinating residues in RyR2 function, we mutated each of these residues to alanine (i.e. E3848A, E3922A, Q3925A, H3850A, and T4931A) in the mouse RyR2 and determined the Ca\(^{2+}\)-dependent \[^{3}H\]ryanodine binding to each of these RyR2 mutants with a wide range of Ca\(^{2+}\) concentrations (∼0.1–100 mM). Note that the mouse and human RyR2 proteins share >97% amino acid sequence identity. Because ryanodine only binds to the open state of RyR, \[^{3}H\]ryanodine binding assay has widely been used to monitor RyR channel activity (27, 40–42). As shown in Fig. 1, there was little or no \[^{3}H\]ryanodine binding to RyR2 WT in the near absence of Ca\(^{2+}\) (∼0.1 mM). This indicates that there is little or no Ca\(^{2+}\)-independent basal activity of RyR2 WT. \[^{3}H\]ryanodine binding to RyR2 WT was activated at ∼100 nM Ca\(^{2+}\) with an EC\(_{50}\) of 0.21 μM, maximized at ∼1 μM Ca\(^{2+}\), and slightly inactivated at >10 μM Ca\(^{2+}\) (Fig. 1, B, C, and E and Table 1). This is consistent with previous studies (25, 43). Unlike WT, the E3848A mutant displayed a complex Ca\(^{2+}\) dependence of \[^{3}H\]ryanodine binding. In the near absence of Ca\(^{2+}\), the E3848A mutant exhibited a substantially higher level of \[^{3}H\]ryanodine binding than WT (p < 0.0001) (Fig. 1, C and E). This indicates that the E3848A mutation markedly increases the Ca\(^{2+}\)-independent basal activity of RyR2. Interestingly, different from WT, elevating Ca\(^{2+}\) concentration from ∼1 μM to ∼10 mM decreased (rather than increased) \[^{3}H\]ryanodine binding to the E3848A mutant. This suggests the existence of a putative Ca\(^{2+}\) inactivation site(s) in RyR2 that is independent of residue Glu-3848. However, at Ca\(^{2+}\) concentrations >10 mM Ca\(^{2+}\), \[^{3}H\]ryanodine binding to the E3848A mutant increased but did not saturate even at 100...
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Table 1

Effects of mutations on [3H]ryanodine binding to RyR2

| Mutation | EC50 (μM) of Ca2⁺ activation | Adjusted p value | Basal activity (pmol/mg) | Adjusted p value | n number of separate experiments performed |
|----------|-------------------------------|-----------------|-------------------------|-----------------|------------------------------------------|
| RyR2 WT  | 0.21 ± 0.02                   | –               | 3.1 × 10⁻³ ± 1.7 × 10⁻³ | –               | 4                                        |
| E3848A   | >1.0 × 10⁻⁶                   | –               | 28.0 × 10⁻³ ± 2.8 × 10⁻³ | <0.0001         | 3                                        |
| E3848A/E3922A | 0.92 ± 0.01               | –               | 5.29 × 10⁻³ ± 4.1 × 10⁻³ | <0.0001         | 3                                        |
| H3850A   | 0.78 ± 0.01                   | <0.0001         | 0.4 × 10⁻³ ± 0.4 × 10⁻³ | 0.4700          | 3                                        |
| E3922A   | 1.38 × 10⁻⁸ ± 0.25 × 10⁻³    | 0.0055          | 12.2 × 10⁻³ ± 3.1 × 10⁻³ | 0.0002          | 4                                        |
| Q3925A   | 1.58 × 10⁻⁷ ± 0.24 × 10⁻³    | 0.0031          | 0.2 × 10⁻³ ± 0.2 × 10⁻³ | 0.3416          | 4                                        |
| T4931A   | 0.28 ± 0.04                   | 0.2359          | 1.5 × 10⁻³ ± 1.1 × 10⁻³ | 0.8839          | 3                                        |

EC50 ANOVA summary: F = 426.3, p value < 0.0001; Basal activity ANOVA summary: F = 231.1, p value < 0.0001

Data are presented as mean ± S.D. The significance of differences in EC50 and basal activity between WT and mutants was evaluated by performing one-way ANOVA with Dunnett’s multiple comparisons post hoc testing. A p value < 0.05 was considered statistically significant.

mm Ca2⁺ (Fig. 1E and Table 1). Because we could not estimate the maximal (saturated) [3H]ryanodine binding to E3848A, we cannot accurately determine the EC50 of Ca²⁺-dependent activation of E3848A, but it is likely to be greater than 10 μM (Fig. 1E and Table 1). We propose that this increase in [3H]ryanodine binding to RyR2 at concentrations >10 μM reflects the Ca²⁺-dependent activation of the E3848A mutant, which would be dramatically reduced compared with that of the WT. The E3922A mutant also displayed a significantly higher level of basal [3H]ryanodine binding than the WT in the near absence of Ca²⁺ (~0.1 μM). In addition, [3H]ryanodine binding to E3922A was activated at ~10mM Ca²⁺ (Fig. 1, B, C, and E and Table 1). This indicates that, like the E3848A mutation, the E3922A mutation also significantly increases the Ca²⁺-independent basal activity and dramatically decreases the Ca²⁺-dependent activation of RyR2. We also assessed the effect of the E3848A/E3922A double mutation on the Ca²⁺ dependence of [3H]ryanodine binding. The E3848A/E3922A double mutation nearly completely abolished Ca²⁺-dependent activation of RyR2 (even by 100 μM Ca²⁺). The E3848A/E3922A mutant also displayed elevated Ca²⁺-independent basal [3H]ryanodine binding and Ca²⁺-dependent inhibition (by ~1 μM to 10 μM Ca²⁺) of [3H]ryanodine binding (Fig. 1, B, C, and E and Table 1). The Q3925A and H3850A mutations also significantly increased the EC50 (158 μM for Q3925A and 0.78 μM for H3850A) of Ca²⁺-dependent activation of [3H]ryanodine binding to RyR2 but did not significantly affect Ca²⁺-independent basal [3H]ryanodine binding (Fig. 1, B, C, and F and Table 1). The T4931A mutation did not significantly alter the EC50 or basal activity of [3H]ryanodine binding, mutations E3848A, E3922A, Q3925A, and H3850A also markedly inhibited caffeine-induced Ca²⁺ release in HEK293 cells, shifting the caffeine response curve to the right (Fig. 1, D, G, and H, and Fig. 2). Interestingly, the T4931A mutation also markedly inhibited caffeine activation (Fig. 1, D and H, and Fig. 2), despite
the basal activity (at and I) of [3H]ryanodine binding. This suggests that the activation of RyR2 WT and Ca2⁺-coordinating residues can exert different effects on the Ca2⁺-coordinating residues and that the Ca2⁺-coordinating residues are interconnected with the ATP- and caffeine-binding sites.

Effect of ATP and caffeine on [3H]ryanodine binding to RyR2 WT and Ca2⁺-coordinating mutation site mutants

Structural analyses also revealed that the Ca2⁺-coordinating site is located near the ATP- and caffeine-binding sites and that the Ca2⁺-coordinating and caffeine-binding sites are interconnected through the CTD (Fig. 1A). Thus, it is possible that mutations in the Ca2⁺-coordinating site may alter the actions of ATP and caffeine in RyR2 channel gating. To test this idea, we assessed the effect of ATP/caffeine on [3H]ryanodine binding to Ca2⁺-coordinating site mutants. We found that ATP (3 mM) plus caffeine (3 mM) markedly increased the sensitivity of RyR2 WT to Ca2⁺-binding (Ca2⁺ sensitivity) and the basal activity (in the near absence of Ca2⁺, ~0.1 nM) of RyR2 WT (Fig. 3, A, H, and I and Table 3). Similarly, ATP/caffeine enhanced both the Ca2⁺ sensitivity and basal activity of the T4931A mutant (Fig. 3, B, H, and I and Table 3). ATP/caffeine also dramatically increased the basal activity (at ~0.1 nM Ca2⁺) of the E3848A, E3922A, or E3848A/E3922A mutants. The effect of ATP/caffeine on Ca2⁺ activation of these mutants could not be accurately determined because of their nonsaturated [3H]ryanodine binding. Nevertheless, the estimated thresholds of Ca2⁺ activation of [3H]ryanodine binding to these mutants in the absence and presence of ATP/caffeine appeared to be similar (~10 mM) (Fig. 3, C–E, H, and I and Table 3). On the other hand, ATP/caffeine significantly enhanced the Ca2⁺ sensitivity of the H3850A and Q3925A mutants without significantly altering their basal activity (Fig. 3, F–I and Table 3). These observations indicate that ATP/caffeine significantly affects both the Ca2⁺-independent basal activity and the Ca2⁺-dependent activation (i.e. Ca2⁺ sensitivity) of RyR2. Our data also indicate that mutations of the Ca2⁺-coordinating residues can exert different effects on the actions of ATP and caffeine.

Effect of mutating residues with negatively charged or oxygen-containing side chains near the Ca2⁺-coordinating residues

In addition to the Ca2⁺-coordinating residues, there are a number of residues with negatively charged or oxygen-containing side chains near the Ca2⁺-coordinating site (Fig. 4A). These include Thr-3929, Gln-3932, Gln-3933, Ser-3984, Asn-3989, Glu-4146, Tyr-4149, Thr-4934, Gln-4936, and Glu-4937. The functional significance of these residues is unclear. To this end, we mutated each of these residues and determined their effect on Ca2⁺ activation and Mg2⁺ inhibition of RyR2 using the [3H]ryanodine binding assay. As shown in Table 2, the mutations resulted in a wide range of effects on basal activity and Ca2⁺ sensitivity.

Table 2

| Effects of mutations on caffeine activation of RyR2 |
|-----------------------------------------------|
| Apparent EC50 (mM) Adjusted p value | n number |
|-----------------------------------------------|
| A. Mutations of Ca2⁺-coordinating residues |
| RyR2 WT | 0.18 ± 0.02 | – | 5 |
| E3848A | 2.69 ± 0.22 | <0.0001 | 4 |
| E3848A/E3922A | 2.68 ± 0.17 | <0.0001 | 4 |
| H3850A | 3.98 ± 0.60 | <0.0001 | 4 |
| E3922A | 3.99 ± 0.03 | <0.0001 | 4 |
| Q3932A | 5.33 ± 0.00 | <0.0001 | 4 |
| T4931A | 4.88 ± 0.13 | <0.0001 | 4 |
| ANOVA summary: F = 427.1, p value < 0.0001 |
| B. Mutations of residues with negatively charged or oxygen-containing side chains |
| RyR2 WT | 0.18 ± 0.01 | – | 5 |
| E3848A | 2.69 ± 0.22 | <0.0001 | 4 |
| E3848A/E3922A | 2.68 ± 0.17 | <0.0001 | 4 |
| H3850A | 3.98 ± 0.60 | <0.0001 | 4 |
| E3922A | 3.99 ± 0.03 | <0.0001 | 4 |
| Q3932A | 5.33 ± 0.00 | <0.0001 | 4 |
| T4931A | 4.88 ± 0.13 | <0.0001 | 4 |
| ANOVA summary: F = 427.1, p value < 0.0001 |

Data are presented as mean ± S.D. The significance of differences in caffeine activation between WT and mutants was evaluated by performing one-way ANOVA with Dunnett’s multiple comparisons post hoc testing. A p-value < 0.05 was considered statistically significant.
Figure 3. Effects of ATP and caffeine on \(^{3}H\)ryanodine binding to mutants of Ca\(^{2+}\)-coordinating residues. \(^{3}H\)ryanodine binding to cell lysates prepared from HEK293 cells expressing the RyR2 WT (A) or RyR2 mutants (B–G) was carried out at various Ca\(^{2+}\) concentrations (0.1 nm–100 mM) in the absence (control) or the presence of 3 mM ATP and 3 mM caffeine (caff). \(^{3}H\)ryanodine binding to each construct was performed using the same amount of the same RyR2 WT or mutant cell lysate in the absence or presence of ATP/caffeine. The amount of \(^{3}H\)ryanodine binding to RyR2 WT and each RyR2 mutant at various Ca\(^{2+}\) concentrations in the absence or presence of ATP/caffeine was normalized to the maximal binding observed among these two conditions (i.e. with or without ATP/caffeine). \(H\), EC\(_{50}\) values of Ca\(^{2+}\) activation and \(I\) basal activity (in the near absence of Ca\(^{2+}\), ~0.1 nm) of \(^{3}H\)ryanodine binding to RyR2. Data points shown are mean ± S.D. (error bars) from three separate experiments. *, \(p < 0.05\); **, \(p < 0.01\).

Table 3

| Effect of ATP/caffeine on \(^{3}H\)ryanodine binding to Ca\(^{2+}\)-coordinating residue mutants of RyR2 | \(EC_{50}\) (\(\mu M\)) | Basal activity (pmol/mg) | \(p\) value | EC\(_{50}\) Control + ATP/Caff | Basal activity Control + ATP/Caff | \(p\) value | \(n\) number |
|---|---|---|---|---|---|---|---|
| RyR2 WT | 0.21 ± 0.02 | 0.03 ± 0.003 | 0.0043 | 3.3 \(\times 10^{-3}\) ± 0.7 \(\times 10^{-3}\) | 53.9 \(\times 10^{-3}\) ± 2.4 \(\times 10^{-3}\) | 0.0003 | 3 |
| E3848A | >1.0 \(\times 10^{4}\) | >1.0 \(\times 10^{4}\) | – | 28.0 \(\times 10^{-3}\) ± 2.8 \(\times 10^{-3}\) | 122.0 \(\times 10^{-3}\) ± 6.1 \(\times 10^{-3}\) | 0.0002 | 3 |
| E3848A/E3922A | – | – | – | 51.1 \(\times 10^{-3}\) ± 4.3 \(\times 10^{-3}\) | 150.4 \(\times 10^{-3}\) ± 23.2 \(\times 10^{-3}\) | 0.0153 | 3 |
| Q3925A | 0.78 ± 0.09 | 0.33 ± 0.12 | 0.0234 | 0.2 \(\times 10^{-3}\) ± 0.4 \(\times 10^{-3}\) | 3.6 \(\times 10^{-3}\) ± 2.8 \(\times 10^{-3}\) | 0.1685 | 3 |
| H3850A | >1.0 \(\times 10^{3}\) | >1.0 \(\times 10^{3}\) | – | 8.2 \(\times 10^{-3}\) ± 1.6 \(\times 10^{-3}\) | 154.6 \(\times 10^{-3}\) ± 22.1 \(\times 10^{-3}\) | 0.0073 | 3 |
| Q3925A | 1.54 \(\times 10^{2}\) ± 0.26 \(\times 10^{2}\) | 0.22 \(\times 10^{7}\) ± 0.11 \(\times 10^{7}\) | 0.0059 | 0.002 \(\times 10^{-3}\) ± 0.003 \(\times 10^{-3}\) | 4.5 \(\times 10^{-4}\) ± 2.7 \(\times 10^{-4}\) | 0.1000 | 3 |
| T4931A | 0.43 ± 0.09 | 0.13 ± 0.04 | 0.0153 | 1.6 \(\times 10^{-3}\) ± 0.5 \(\times 10^{-3}\) | 37.9 \(\times 10^{-3}\) ± 9.3 \(\times 10^{-3}\) | 0.0210 | 3 |

Data are presented as mean ± S.D. The significance of difference in EC\(_{50}\) and basal activity with and without 3 mm ATP/3 mm caffeine was assessed by two-tailed Student’s t tests. A \(p\) value <0.05 was considered statistically significant.

Thus, these Ca\(^{2+}\) noncoordinating residues Thr-3929, Gln-3932, Glu-4146, Thr-4934, Glu-4937, Tyr-4149, Gln-4936, and Ser-3984 are also important for Ca\(^{2+}\) activation of RyR2, whereas residues Tyr-4149 and Gln-4936 are important for stabilizing the closed state of RyR2 in the near absence of Ca\(^{2+}\), (~0.1 nm) (Fig. 4, B and C and Table 1). We also performed caffeine-induced Ca\(^{2+}\) release assays in HEK293 cells transfectected with these mutations to determine their effect on the activation of RyR2 by...
caffeine. Consistent with their effects on Ca\(^{2+}\)-dependent \([3H]\)ryanodine binding to RyR2, mutations T3929A, Q3932A, E4146A, T4934A, and E4937A suppressed, whereas mutations S3984A, Y4149S, and Q4936A increased caffeine activation of RyR2.

Mutations Q3933A and N3989A had no significant effect on caffeine activation (Fig. 4, D and I–L, Table 2, and Fig. 5).

**Effect of mutating residues with negatively charged or oxygen-containing side chains in the central domain on Mg\(^{2+}\) inhibition of RyR2**

We next determined whether mutating residues with negatively charged and oxygen-containing side chains in the central domain affects the inhibition of RyR2 by Mg\(^{2+}\). To minimize the influence of Ca\(^{2+}\) on Mg\(^{2+}\)-dependent inhibition of RyR2, we activated RyR2 by ATP (3 mM) plus caffeine (3 mM) in the near absence of Ca\(^{2+}\) (~0.1 mM) and determined the effect of Mg\(^{2+}\) (2 mM) on \([3H]\)ryanodine binding to each of the ATP/caffeine activated mutants. Under these conditions, Mg\(^{2+}\) exerted >80% inhibition on the RyR2 WT channel (Fig. 6A). However, Mg\(^{2+}\)-dependent inhibition of \([3H]\)ryanodine binding to mutants Y4149S, E4937A, E3848A, Q4936A, and S3984A was markedly reduced compared with that of \([3H]\)ryanodine binding to RyR2 WT (Fig. 6A). On the other hand, mutations E3922A, Q3933A, T4934A, N3989A, Q3932A, and T3929A had no significant effect on the inhibition of \([3H]\)ryanodine binding to Mg\(^{2+}\). Interestingly, mutation T4931A slightly increased Mg\(^{2+}\)-dependent inhibition of \([3H]\)ryanodine binding (Fig. 6A). The effect of mutations H3850A and Q3925A on Mg\(^{2+}\) inhibition of RyR2 cannot be determined, because they displayed little or no \([3H]\)ryanodine binding under these conditions. Taken together, these data indicate that residue Glu-3848, located in the Ca\(^{2+}\) activation site, and residues Ser-3984, Tyr-4149, Gln-4936, and Glu-4937, located near the Ca\(^{2+}\) activation site, are important for Mg\(^{2+}\)-dependent inhibition of RyR2 (Fig. 6B).

**Disease-associated mutations in the central domain affect Ca\(^{2+}\) and caffeine activation and Mg\(^{2+}\) inhibition of RyR2**

The central domain harbors a large number of disease-causing RyR2 mutations (Fig. 7A). RyR2 mutations K3997E (39, 44), F4020L (45), R4157Q (39, 46), T4196A (47), and Q4201R (48) were identified in individuals presenting with syncope, CPVT,
and/or sudden unexplained death. The L4188P mutation was found in a 15-year-old girl with a history of seizure-like episodes often triggered by anxiety (49). The N4097S mutation was found in a sudden unexplained death individual through molecular autopsy (50). The M3999V mutation was found in the CPVT post-mortem panel in ClinVar (National Center for Biotechnology Information. ClinVar; VCV000201320.2). To understand the effect of these disease-associated RyR2 mutations on channel function, we generated each of these mutations. Ca$^{2+}$-dependent $[^3H]$ryanodine binding was carried out to determine their effect on Ca$^{2+}$ activation and Mg$^{2+}$ inhibition of RyR2. All of these mutations significantly enhanced the sensitivity of RyR2 to Ca$^{2+}$ activation (Fig. 7, B and E–G and Table 1). Furthermore, M3999V, L4188P, T4196A, and Q4201R mutations, but not K3997E, F4020L, N4097S, and R4157Q mutations, increased the basal activity of RyR2 (Fig. 7, A and C–G and Table 1). This is consistent with the effect of other disease-associated RyR2 mutations located in the central domain (51). We also determined their effect on the activation of RyR2 by caffeine. Consistent with their effects on Ca$^{2+}$-dependent $[^3H]$ryanodine binding to RyR2, mutations M3999V, F4020L, N4097S, R4157Q, L4188P, T4196A, and Q4201R significantly enhanced caffeine activation of RyR2. Interestingly, the K3997E mutation had no significant effect on caffeine activation of RyR2, although it slightly reduced the EC$_{50}$ of Ca$^{2+}$ activation of $[^3H]$ryanodine binding to RyR2 (Fig. 7, D and H–J, Table 2, and Fig. 8). The effect of these disease-associated mutations on Mg$^{2+}$-dependent inhibition of RyR2 is shown in Fig. 9. Mutations M3999V, F4020L, T4196A, L4188P, Q4201R, N4097S, and R4157Q significantly reduced the Mg$^{2+}$-dependent inhibition of RyR2, whereas mutation K3997E had no effect on the inhibition of RyR2 by Mg$^{2+}$. These results suggest that enhanced Ca$^{2+}$ and caffeine activation and reduced Mg$^{2+}$ inhibition are common defects of central domain disease-associated RyR2 mutations.

Figure 5. Effects of RyR2 mutations of residues with negatively charged or oxygen-containing side chains on caffeine-induced Ca$^{2+}$ release in HEK293 cells. HEK293 cells were transfected with RyR2 WT or mutants of residues with negatively charged or oxygen-containing side chains. The fluorescence intensity of the Fluo-3-loaded transfected cells was monitored continuously before and after each caffeine addition.

Figure 6. Effects of Mg$^{2+}$ on $[^3H]$ryanodine binding to RyR2 WT and mutants. A, percentage of Mg$^{2+}$ inhibition of $[^3H]$ryanodine binding. $[^3H]$ryanodine binding to cell lysates prepared from HEK293 cells expressing the RyR2 WT and mutants was carried out at 0.1 nM [Ca$^{2+}$] and 2 mM Mg$^{2+}$. ATP (3 mM) and caffeine (3 mM) were included to stimulate $[^3H]$ryanodine binding. B, 3D locations of mutations that reduce Mg$^{2+}$ inhibition. Data points shown are mean ± S.D. (error bars) from three separate experiments. Dashed red line indicates the percentage of Mg$^{2+}$ inhibition in WT. *, $p < 0.05$; **, $p < 0.01$. 

RyR2 central domain in activation, regulation, and stability
Discussion

The overall 3D structure of RyR consists of a large cytoplasmic assembly and a channel domain, which are connected by the central domain. Thus, the central domain is believed to be the primary transducer that integrates structural changes in the cytoplasmic assembly to the gating of the channel pore (29–36). Furthermore, recent structural studies mapped the Ca\(^{2+}\)-, ATP-, and caffeine-binding sites within or near the central domain (34–36). This suggests that the central domain also serves as a signaling hub that controls the activity of RyR. Indeed, mutations of the putative Ca\(^{2+}\)-activation site within the central domain abolished or markedly diminished Ca\(^{2+}\) activation of RyR2 (37). Consistent with these studies, we also found that mutating the Ca\(^{2+}\)-coordinating residues (Glu-3848, Glu-3922, and Gln-3925) of RyR2 to alanine dramatically reduced Ca\(^{2+}\) activation of RyR2. The double mutation E3848A/E3922A nearly completely abolished Ca\(^{2+}\) activation of RyR2. Mutating the other two residues involved in Ca\(^{2+}\) coordination (His-3850 and Thr-4931) has a relatively lower effect on Ca\(^{2+}\) activation of RyR2. Surprisingly, mutation E3848A also markedly enhanced the basal activity of RyR2 in the near absence of Ca\(^{2+}\). This suggests that the E3848A mutation may destabilize the closed state of RyR2, resulting in spontaneous channel opening in the absence of activating Ca\(^{2+}\), but the underlying mechanism is unknown. It is possible that in the absence of Ca\(^{2+}\), the negatively charged side chains of the Ca\(^{2+}\)-coordinating residues would tend to move away from each other because of electrostatic repulsion, which may contribute to the stabilization of the closed state of the channel. Removal of the negative charge from Glu-3848 (in the E3848A mutant) would permit the E3848A mutant reside to move closer to other Ca\(^{2+}\)-coordinating residues, which may mimic the Ca\(^{2+}\)-bound Glu-3848 state and thus destabilize the closed state. Further studies are needed to test this hypothesis. Interestingly, the enhanced basal activity in the E3848A mutant channel was inhibited by increasing concentrations of Ca\(^{2+}\) (~1 μM–1 mM). This suggests the presence of a putative Ca\(^{2+}\)-dependent inactivation site(s) that is different from the Glu-3848/Glu-3922–based high-affinity Ca\(^{2+}\) activation site. However, the identity, molecular mechanism, and physiological relevance of this putative Ca\(^{2+}\)-dependent inactivation site(s) have yet to be investigated. Taken together, these
observations suggest that residue Glu-3848 not only controls Ca$^{2+}$ activation but also determines the basal activity of the channel in the near absence of Ca$^{2+}$. The close proximity of the Ca$^{2+}$-, ATP-, and caffeine-binding sites within the central domain suggests that these ligands may interact with one another and interdependently modulate the activity of RyR2. Indeed, we found that ATP/caffeine increased both the basal activity and the Ca$^{2+}$ sensitivity of the RyR2 WT and T4931A mutant channels. On the other hand, ATP/caffeine increased mainly the Ca$^{2+}$-independent basal activity but had a relatively lower effect on the threshold or sensitivity of Ca$^{2+}$ activation (by high concentrations of Ca$^{2+}$) of [3H]ryanodine binding to the E3848A and E3922A mutants (Fig. 3, C, D, and H). This suggests that the action of ATP/caffeine in the Ca$^{2+}$-dependent activation of RyR2 depends on Glu-3848 and Glu-3922 but that the action of ATP/caffeine in the Ca$^{2+}$-independent basal activity of RyR2 does not. In contrast, ATP/caffeine increased predominantly the Ca$^{2+}$ sensitivity, but not the basal activity, of the H3850A and Q3925A mutants. This suggests that the H3850A and Q3925A mutations strongly stabilized the closed state of the channel in the near absence of Ca$^{2+}$, opposite to the effect of the E3848A mutation. Collectively, these data indicate that ATP and caffeine affect both the Ca$^{2+}$-dependent activation and Ca$^{2+}$-independent basal activity of RyR2 and that, vice versa, the Ca$^{2+}$ activation site also has a major role in determining the actions of ATP/caffeine. These results also suggest that the Ca$^{2+}$ activation site not only plays a critical role in Ca$^{2+}$ activation but is also involved in controlling the stability of the closed state of RyR2.

It is of note that adjacent to the Ca$^{2+}$ activation site, there is a cluster of residues with negatively charged or oxygen-containing side chains. The exact roles of these residues in RyR2 function are unknown. We previously reported that mutating one of these residues (E3987A) in RyR2 and the corresponding residue in RyR3 (E3885A) markedly reduced the Ca$^{2+}$ activation of the channel (25, 26). Structural analysis suggested that although the Glu-3987 residue is not directly involved in Ca$^{2+}$ coordination, it may play a role in the formation of the Ca$^{2+}$ binding pocket by stabilizing the interface between the central domain and the CTD (34–36). Our current work showed that mutations T3929A, Q3932A, E4146A, T4934A, and E4937A near residue Glu-3987 also significantly decreased, whereas S3984A, Y4149S, and Q4936A significantly increased Ca$^{2+}$ activation of RyR2, but their effect on Ca$^{2+}$ activation is much lower compared with that of the Ca$^{2+}$ coordination site mutations. Thus, these residues are unlikely to be directly involved in Ca$^{2+}$ binding.

Although the residues with negatively charged or oxygen-containing side chains adjacent to the Ca$^{2+}$ activation site may not play a major role in Ca$^{2+}$ activation of RyR2, they may be
involved in regulation of the channel by other cations, such as Mg$^{2+}$. It is well established that Mg$^{2+}$ inhibits the RyR channel (20, 23, 24, 38, 52), but the molecular basis of Mg$^{2+}$-dependent inhibition of RyR is unknown. It has been proposed that Mg$^{2+}$ inactivates RyR by binding to the Ca$^{2+}$ activation site (20, 23, 24, 38, 52). Interestingly, the E3848A mutant that markedly reduced the Ca$^{2+}$ activation site remained sensitive to both Ca$^{2+}$ and Mg$^{2+}$ inhibition. This suggests that Mg$^{2+}$ may bind to another site, in addition to the Ca$^{2+}$ activation site, to inactivate the RyR2 channel. To test this idea, we systematically assessed the effect of mutating the residues with negatively charged or oxygen-containing side chains located in the central domain on Mg$^{2+}$ inhibition of RyR2. We found that mutations Y4149S, E4937A, E3848A, Q4936A, and S3984A significantly reduced Mg$^{2+}$-dependent inhibition of RyR2. Interestingly, residues Tyr-4149, Glu-4937, Gln-4936, and Ser-3984 are located in close proximity and could potentially form a binding pocket (Fig. 6A). These results suggest that the central domain may also be involved in Mg$^{2+}$ inhibition and Ca$^{2+}$ activation of RyR2. However, it is possible that these mutations could alter Mg$^{2+}$-dependent inhibition of RyR2 via an allosteric mechanism. High-resolution structural analysis will be needed to determine whether these residues are involved in Mg$^{2+}$ binding.

We have previously shown that disease-associated RyR2 mutations located in the central domain increase the Ca$^{2+}$ activation of RyR2 (51). We have now characterized additional RyR2 central domain mutations that are associated with CPVT and sudden death. Consistent with our previous work, we found that CPVT-associated mutations M3999V, F4020L, N4097S, R4157Q, L4188P, T4196A, and Q4201R (except for K3997E) significantly enhanced Ca$^{2+}$ and caffeine activation and reduced Mg$^{2+}$-dependent inhibition of RyR2. However, the exact molecular mechanisms by which these disease-associated RyR2 mutations alter Ca$^{2+}$ and caffeine activation and Mg$^{2+}$ inhibition of RyR2 have yet to be defined. A close examination of the locations of these mutations in the 3D structure of RyR2 revealed some clues. Among the central domain mutations characterized, the M3999V mutation exerted the strongest effect on RyR2 function by markedly increasing Ca$^{2+}$ and caffeine activation and the basal activity of the channel. We speculate that this Met-3999 residue may potentially form hydrophobic interactions with Leu-3986, Leu-3982, Trp-3941, and Leu-4105, which may stabilize the adjacent Ca$^{2+}$ binding pocket (Fig. 10A). Mutating Met-3999 to valine (M3999V) may strengthen these hydrophobic interactions, which may favor Ca$^{2+}$ activation. The K3997E mutation that is located in the same helix as M3999V may alter the interaction among Lys-3997, Asn-3992, and Met-4109 and the Ca$^{2+}$ activation of RyR2 in a similar manner but to a different extent (Fig. 10B). We further speculate that the Phe-4020 residue may potentially interact with Arg-4086 via cation-π interactions (53) and with Phe-4016 and Leu-4023 via hydrophobic interactions (Fig. 10C). Mutation F4020L could disrupt the cation-π interactions, which may allosterically alter the confirmation of the central domain and the confirmation of the Ca$^{2+}$ activation site. We also hypothesize that the N4097S mutation may alter potential interactions among Asn-4097, Lys-3976, and Val-3979 and thus the confirmation of the adjacent CTD and Ca$^{2+}$ activation site (Fig. 10D). Mutations R4157Q, L4188P, T4196A, and Q4201R are all located in the U-motif (Fig. 10E). This U-motif is the part of the central domain that grasps the CTD, forming a direct pathway transducing conformational changes from the central domain to the channel gate through the CTD and the

Figure 10. 3D locations of disease-associated mutations and neighboring residues in the central domain of RyR2. A, 3D location of mutation M3999V and potentially interacting residues. B, 3D location of mutation K3997E and potentially interacting residues. C, 3D location of mutation F4020L and potentially interacting residues. D, 3D location of mutation N4097S and potentially interacting residues. E, 3D locations of mutations R4157Q, L4188P, T4196A, and Q4201R within the U-motif. F, 3D location of mutation R4157Q and potentially interacting residues. G, 3D location of mutation L4188P and potentially interacting residues. H, 3D locations of mutations T4196A and Q4201R and potentially interacting residues. Disease-associated mutations are colored in magenta and their potentially interacting residues in yellow.
activation of RyR2 by cytosolic Ca\textsuperscript{2+} propensity for CPVT and sudden death are unknown. Because by which RyR2 mutations in the central domain increase the inhibition of RyR2 represent a common defect of RyR2 mutations located in the central domain. However, the exact mechanisms through which these mutations increase the propensity for the generation and propagation of arrhythmias and thus CPVT and sudden death.

Our current and previous studies (51) consistently demonstrate that enhanced Ca\textsuperscript{2+} activation and/or reduced Mg\textsuperscript{2+} inhibition of RyR2 represent a common defect of RyR2 mutations located in the central domain. However, the exact mechanisms by which RyR2 mutations in the central domain increase the propensity for CPVT and sudden death are unknown. Because activation of RyR2 by cytosolic Ca\textsuperscript{2+} is a critical component of the CICR mechanism that controls cardiac muscle contraction, enhanced RyR2 sensitivity to cytosolic Ca\textsuperscript{2+} activation or reduced sensitivity to Mg\textsuperscript{2+} inhibition would result in increased CICR sensitivity. An increased CICR sensitivity would enhance the propensity for the generation and propagation of arrhythmogenic spontaneous Ca\textsuperscript{2+} waves. Thus, it is possible that by increasing Ca\textsuperscript{2+} activation or decreasing Mg\textsuperscript{2+} inhibition of RyR2, the RyR2 central domain mutations may enhance CICR sensitivity and thus the propensity for spontaneous Ca\textsuperscript{2+} wave-evoked delayed afterdepolarizations, and triggered activity, and thus CPVT and sudden death.

The effect of the central domain mutations on caffeine activation was assessed and compared with their effect on the Ca\textsuperscript{2+} activation of RyR2. Notably, mutations that suppressed Ca\textsuperscript{2+} activation also suppressed caffeine activation, whereas mutations that enhanced Ca\textsuperscript{2+} activation also enhanced caffeine activation of RyR2. On the other hand, the effect of the central domain mutations on the Ca\textsuperscript{2+}-independent basal activity of RyR2 has no correlation with that on caffeine activation. Because the caffeine-binding site differs from the Ca\textsuperscript{2+} activation site, some mutations may affect caffeine activation but not Ca\textsuperscript{2+} activation of RyR2. Indeed, we found that the T4931A mutation markedly suppressed caffeine activation of RyR2 but had little or no effect on Ca\textsuperscript{2+} activation of [\textsuperscript{3}H]ryanodine binding. This indicates that although there is a close relationship between Ca\textsuperscript{2+} and caffeine activation, caffeine activation does not always reflect Ca\textsuperscript{2+} activation of RyR2.

In summary, our present study demonstrates that the central domain is a pivotal signaling hub that not only controls Ca\textsuperscript{2+} activation and basal activity of RyR2 but also determines Mg\textsuperscript{2+} inhibition of RyR2. The central domain also controls the modulation of the channel by ATP and caffeine and the stability of the closed state of RyR2 in the near absence of Ca\textsuperscript{2+}. We also reveal that ATP and caffeine can enhance RyR2 channel activity in a Ca\textsuperscript{2+}-dependent and Ca\textsuperscript{2+}-independent manner. Our work also provides novel insights into the mechanism of RyR2 central domain mutations linked to cardiac arrhythmias and sudden death.

**Experimental procedures**

**Materials**

[\textsuperscript{3}H]ryanodine was purchased from PerkinElmer. Ryanodine was purchased from Abcam. Caffeine was obtained from Sigma-Aldrich. ATP was obtained from EMD Millipore.

**Construction of RyR2 mutations**

The central domain RyR2 mutations, E3848A, E3922A, H3850A, Q3925A, T4931A, T3929A, Q3932A, Q3933A, S3984A, N3989A, E4146A, Y4149S, T4934A, Q4936A, E4937A, M3999V, F4020L, R4157Q, L4188P, T4196A, K3997E, N4097S, and Q4201R, were generated by the overlap extension method using PCR (25, 54). Briefly, the NruI-NotI (in the vector) fragment containing the mutation was obtained by overlapping PCR and used to replace the corresponding WT fragment in the full-length mouse RyR2 cDNA in pcDNA5. All mutations were confirmed by DNA sequencing.

**Preparation of HEK293 cell lysates**

HEK293 cells were transfected with RyR2 WT or central domain mutant cDNAs using the calcium phosphate precipitation method as described previously (25, 55). Twenty-four hours after transfection, the cells were harvested and resuspended in the lysis buffer containing 25 mM Tris, 50 mM HEPES, pH 7.4, 137 mM NaCl, 1% CHAPS, 0.5% egg phosphatidylcholine, 2.5 mM DTT, and a protease inhibitor mix (1 mM benzamidine, 2 μg/ml leupeptin, 2 μg/ml pepstatin A, 2 μg/ml aprotinin, and 0.5 mM PMSF) on ice for 60 min. Cell lysates were obtained after removing insolubilized materials by centrifugation.

**[\textsuperscript{3}H]ryanodine binding assay**

Equilibrium [\textsuperscript{3}H]ryanodine binding to cell lysates was performed as described previously (25, 55) with some modifications. Cell lysates were incubated with 5 nM [\textsuperscript{3}H]ryanodine at 37 °C for 2 h in 300 μl of a binding solution containing 500 mM KCl, 25 mM Tris, and 50 mM HEPES, pH 7.4. For testing the effects of RyR2 modulators on channel function, Ca\textsuperscript{2+} (0.1 mM–100 mM), Mg\textsuperscript{2+} (2 mM), ATP (3 mM), and caffeine (3 mM) were added to the incubation solution. Free [Ca\textsuperscript{2+}] was adjusted by EGTA and CaCl\textsubscript{2} solutions using the computer program of Fabiato and Fabiato (56). Free [Mg\textsuperscript{2+}] was adjusted by EGTA and MgCl\textsubscript{2} solutions according to the Maxchelator program. At the completion of incubation, the samples were diluted with 5 ml of ice-cold washing buffer containing 25 mM Tris, pH 8.0, and 250 mM KCl and filtered through Whatman GF/B filters presoaked with 1% polyethyleneimine. The filters were washed immediately with 2 × 5 ml of the same buffer. The amount of [\textsuperscript{3}H]ryanodine retained in the filters was determined by liquid scintillation counting. Specifically bound [\textsuperscript{3}H]ryanodine was calculated by subtracting nonspecific binding that was determined in the presence of 50 μM unlabeled ryanodine. All
binding assays were performed in duplicate. [3H]ryanodine binding data were fitted with the Hill equation using the Prism 8 (GraphPad Software), from which EC_{50} values for Ca^{2+} activation were calculated.

**Caffeine-induced Ca^{2+} release in HEK293 cells**

The free cytosolic Ca^{2+} concentration in transfected HEK293 cells was measured using the fluorescence Ca^{2+} indicator dye Fluo-3 AM (Molecular Probes). HEK293 cells grown on 100-mm tissue culture dishes for 18–20 h after subculture were transfected with 12–16 μg of RyR2 WT or mutant cDNAs. Cells grown for 18–20 h after transfection were washed four times with PBS and incubated in KRH (Krebs–Ringer–Hepes, 125 mM NaCl, 5 mM KCl, 1.2 mM KH_{2}PO_{4}, 6 mM glucose, 1.2 mM MgCl_{2}, 2 mM CaCl_{2}, and 25 mM HEPES, pH 7.4) buffer without MgCl_{2} and CaCl_{2} at room temperature for 40 min and at 37 °C for 40 min. After being detached from culture dishes by pipetting, the cells were collected by centrifugation at 1,000 rpm for 2 min in a Beckman TH-4 rotor. Cell pellets were loaded with 5 μM Fluo-3 AM in high glucose DMEM at room temperature for 30 min, followed by washing with KRH buffer plus 2 mM CaCl_{2} and 1.2 mM MgCl_{2} (KRH+ buffer) three times and resuspended in 150 μl of KRH+ buffer plus 0.1 mg/ml BSA and 250 μM sulfinpyrazone. The Fluo-3 AM-loaded cells were added to 2 ml (final volume) of KRH+ buffer in a cuvette. The fluorescence intensity of Fluo-3 AM at 530 nm was measured before and after repeated cumulative additions of various concentrations of caffeine (0.025–5 mM) in an SLM Aminco series 2 luminescence spectrometer with 480 nm excitation at 25 °C (SLM Instruments). The peak levels of each caffeine-induced Ca^{2+} release were determined and normalized to the highest level (100%) of caffeine-induced Ca^{2+} release for each experiment. The normalized data of the ascending part of the caffeine dose response curve were fitted with the Hill equation to calculate the apparent EC_{50} value of caffeine activation for each construct using the curve fitting module of Prism 8 (GraphPad Software).

**Statistical analysis**

All data shown are means ± S.D. One-way analysis of variance (ANOVA) followed by Dunnett’s multiple comparisons test or Student’s t test (two-tailed) was performed using GraphPad Prism version 8 to assess the difference between mean values. A p value <0.05 was considered statistically significant.

**Data availability**

All data are contained within this article.

**Author contributions**—W. G., B. S., R. W., and S. W. C. conceptualization; W. G., J. P. E., and R. W. data curation; W. G. and B. S. formal analysis; W. G., B. S., and S. W. C. investigation; W. G., B. S., and R. W. methodology; W. G., R. W., and S. W. C. writing-original draft; B. S., J. P. E., and S. W. C. writing-review and editing; J. P. E. and S. W. C. project administration; S. W. C. resources; S. W. C. supervision; S. W. C. funding acquisition.

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**Conflict of interest**—The authors declare that they have no conflicts of interest with the contents of this article.

**Abbreviations**—The abbreviations used are: RyR2, cardiac ryanodine receptor; CICR, Ca^{2+}-induced Ca^{2+} release; CTD, C-terminal domain; CPVT, catecholaminergic polymorphic ventricular tachycardia; KRH, Krebs–Ringer–Hepes; ANOVA, analysis of variance.

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