Enhanced Cytosolic Ca\textsuperscript{2+} Activation Underlies a Common Defect of Central Domain Cardiac Ryanodine Receptor Mutations Linked to Arrhythmias\textsuperscript{*}\textsuperscript{S}

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Recent three-dimensional structural studies reveal that the central domain of ryanodine receptor (RyR) serves as a transducer that converts long-range conformational changes into the gating of the channel pore. Interestingly, the central domain encompasses one of the mutation hotspots (corresponding to amino acid residues 3778–4201) that contains a number of cardiac RyR (RyR2) mutations associated with catecholaminergic polymorphic ventricular tachycardia (CPVT) and atrial fibrillation (AF). However, the functional consequences of these central domain RyR2 mutations are not well understood. To gain insights into the impact of the mutation and the role of the central domain in channel function, we generated and characterized eight disease-associated RyR2 mutations in the central domain. We found that all eight central domain RyR2 mutations enhanced the Ca\textsuperscript{2+}-dependent activation of \textsuperscript{[3H]}ryanodine binding, increased cytosolic Ca\textsuperscript{2+}-induced fractional Ca\textsuperscript{2+} release, and reduced the activation and termination thresholds for spontaneous Ca\textsuperscript{2+} release in HEK293 cells. We also showed that racemic carvedilol and the non-beta-blocking carvedilol enantiomer, (R)-carvedilol, suppressed spontaneous Ca\textsuperscript{2+} oscillations in HEK293 cells expressing the central domain RyR2 mutations associated with CPVT and AF. These data indicate that the central domain is an important determinant of cytosolic Ca\textsuperscript{2+} activation of RyR2. These results also suggest that altered cytosolic Ca\textsuperscript{2+} activation of RyR2 represents a common defect of RyR2 mutations associated with CPVT and AF, which could potentially be suppressed by carvedilol or (R)-carvedilol.

Ryanodine receptors are a family of intracellular Ca\textsuperscript{2+} release channels that are essential for excitation-contraction coupling in skeletal and cardiac muscles (1–3). They also play an important role in the pathogenesis of various skeletal and cardiac muscle disorders (4, 5). Naturally occurring mutations in the skeletal muscle ryanodine receptor (RyR1)\textsuperscript{6} are linked to malignant hyperthermia and central core disease, whereas mutations in the cardiac ryanodine receptor (RyR2) are associated with different forms of cardiac arrhythmias and cardiomyopathies, such as catecholaminergic polymorphic ventricular tachycardia (CPVT), idiopathic ventricular fibrillation (IVF), atrial fibrillation (AF), and dilated cardiomyopathies (4, 5). To date, more than 300 RyR1 and 150 RyR2 disease-associated mutations have been identified. A majority of these RyR mutations are located in three regions: the N-terminal (RyR1 residues, 13–552; RyR2 residues, 44–466), central (RyR1 residues, 2101–2458; RyR2 residues, 2246–2534), and C-terminal (RyR1 residues, 4631–4990; RyR2 residues, 4497–4959) regions in RyR1 and RyR2 (4, 5). In addition to these three disease mutation hotspots, RyR2 also contains another disease mutation hotspot between residues 3778–4201 (hotspot-3). Interestingly, mutation hotspot-3 seems to be absent in RyR1 (4, 5). This difference in the distribution of disease-associated RyR1 and RyR2 mutations raises an interesting and important question about the functional impact of RyR2 mutations in hotspot-3 and the role of the domain encompassing mutation hotspot-3 in channel function.

A near-atomic resolution (3.8 Å) three-dimensional (3D) structure of RyR1 has recently been solved using cryo-electron microscopy (6). The region corresponding to mutation hotspot-3

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\textsuperscript{6}The abbreviations used are: RyR, ryanodine receptor; RyR1, skeletal ryanodine receptor; RyR2, cardiac ryanodine receptor; CPVT, catecholaminergic polymorphic ventricular tachycardia; AF, atrial fibrillation; ER, endoplasmic reticulum; SR, sarcoplasmic reticulum; CICR, Ca\textsuperscript{2+} -induced Ca\textsuperscript{2+} release; SOICR, store overload-induced Ca\textsuperscript{2+} release; EC, excitation-contraction; ICM, intracellular-like medium; ex, excitation; em, emission; KRH buffer, Krebs-Ringer-Hepes buffer.
is located within the central domain that directly interacts with the C-terminal domain, which controls the gate of the RyR channel (6). Comparison of the closed and open states of the RyR1 channel reveals that the central domain acts as a transducer that couples the conformational changes in the cytosolic assembly to the gating of the central pore of the channel (7). Furthermore, we showed previously that a point mutation E4032A in RyR1, E3987A in RyR2, and E3885A in RyR3, located in the central domain, dramatically diminished cytosolic Ca\(^{2+}\)/H\(_{11001}\) activation of the channel (8–10). These observations suggest that the central domain may mediate the Ca\(^{2+}\)/H\(_{11001}\)-dependent gating of the RyR channel, and that disease-associated RyR2 mutations in mutation hotspot-3 located within the central domain (green). The location of the Glu-3987 residue (9) critical for cytosolic Ca\(^{2+}\) activation of RyR2 is also shown.

Results

Effect of Disease-associated RyR2 Mutations Located in the Central Domain on Ca\(^{2+}\) Activation of \([^{3}H]\)Ryanodine Binding—To assess the functional impact of disease-associated RyR2 mutations located in mutation hotspot-3 (residues 3778–4201) within the central domain of the 3D structure of RyR (6) (Fig. 1), we generated a number of disease-associated RyR2 mutations in this region, including G3946A, G3946S, M3978I, H4108N, H4108Q, S4124T, T4158P, and Q4159P (16–23). We then determined the effect of these mutations on the Ca\(^{2+}\) -dependent activation of RyR2. Interestingly, the central domain contains RyR2 mutations that are associated with CPVT and AF. We showed previously that carvedilol, a clinically used beta-blocker, suppressed spontaneous activities of CPVT- and AF-associated RyR2 mutants. These observations suggest that the central domain is an important determinant of cytosolic Ca\(^{2+}\) activation of RyR2, and that altered cytosolic Ca\(^{2+}\) activation of RyR2 may enhance the susceptibility to both ventricular tachycardias and AF. Carvedilol may be beneficial for patients with RyR2 mutation-associated CPVT and AF.

FIGURE 1. Location of the RyR2 mutation hotspot-3 region in the central domain of the RyR channel. A, the open bar represents the —5,000-amino acid (5000 aa) sequence of RyR. Mutations in RyR1 associated with malignant hyperthermia (MH) and central core disease (CCD) are largely clustered in three hotspot regions (red bars): MH/CCD I, II, and III. Mutations in RyR2 associated with CPVT are mainly clustered in four hotspot regions (red bars): CPVT I, II, III, and IV. The FKBP12.6 binding sites, PKA phosphorylation sites, calmodulin (CaM) binding domain, Ca\(^{2+}\) -activation sites, and pore-forming segment are depicted as circles. The divergent and transmembrane regions are also indicated. B, three-dimensional structure of two RyR monomers (6). The mutation hotspot-3 (CPVT III), the S6 inner helix (S6), and the C-terminal domain (CTD) are highlighted. C, a close-up view showing the locations of eight disease-associated RyR2 mutations in the mutation hotspot-3 region within the central domain (green). The location of the Glu-3987 residue (9) critical for cytosolic Ca\(^{2+}\) activation of RyR2 is also shown.
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**FIGURE 2.** Central domain RyR2 mutations increase the Ca\(^{2+}\)-dependent activation of \[^{3}H\]ryanodine binding. A and B, \[^{3}H\]ryanodine binding to cell lysate prepared from HEK293 cells expressing the RyR2 WT, G3946A, G3946S, M3978I, or H4108N mutant (A) or the H4108Q, S4124T, T4158P, or Q4159P mutant (B) was carried out at various Ca\(^{2+}\) concentrations (0.2 nM to 0.1 mM) with 150 mM KCl and 5 nM \[^{3}H\]ryanodine. The amounts of \[^{3}H\]ryanodine binding (Fig. 2 and supplemental Fig. S1). For example, the H4108N and H4108Q mutations markedly decreased the EC\(_{50}\) of Ca\(^{2+}\)-dependent activation to 0.086 ± 0.004 and 0.106 ± 0.003 μM (p < 0.05), respectively. The expression levels of the RyR2 WT and central domain mutants are shown in supplemental Fig. S2. Except for the H4108Q mutant, all central domain mutants tested were expressed at a level comparable with that of the WT. Because the Ca\(^{2+}\) dependence of \[^{3}H\]ryanodine binding reflects the dependence of single RyR2 channels to activation by cytosolic Ca\(^{2+}\) (9), our \[^{3}H\]ryanodine binding data suggest that disease-associated RyR2 mutations located in the central domain of the channel enhance the sensitivity of RyR2 to cytosolic Ca\(^{2+}\) activation.

**Effect of Central Domain RyR2 Mutations on Cytosolic Ca\(^{2+}\) Regulation of Ca\(^{2+}\) Release in HEK293 Cells**—We next assessed the effect of RyR2 mutations located in the central domain on the regulation of RyR2 by cytosolic Ca\(^{2+}\) in a cellular environment. HEK293 cells expressing the RyR2 WT and eight central domain mutants were permeabilized to allow access to cytosolic Ca\(^{2+}\). Permeabilized cells were then perfused with various cytosolic Ca\(^{2+}\) concentrations (0.1–10 μM) to induce ER Ca\(^{2+}\) release. The fractional Ca\(^{2+}\) release induced by elevating cytosolic Ca\(^{2+}\) concentrations was monitored by measuring the steady state ER Ca\(^{2+}\) level using a FRET-based ER luminal Ca\(^{2+}\)-sensing protein D1ER (24–26). As shown in Fig. 3, increasing cytosolic Ca\(^{2+}\) concentrations (0.1–10 μM) reduced the steady state ER Ca\(^{2+}\) level in permeabilized HEK293 cells expressing RyR2 WT (Fig. 3, A and F). This reduction in the steady state ER Ca\(^{2+}\) level likely reflects cytosolic Ca\(^{2+}\)-induced fractional Ca\(^{2+}\) release from the ER Ca\(^{2+}\) store. The steady state ER Ca\(^{2+}\) levels in permeabilized HEK293 cells expressing central domain RyR2 mutants at various cytosolic Ca\(^{2+}\) concentrations are shown in Figs. 3 and 4. All eight RyR2 mutations located in the central domain significantly reduced the steady state ER Ca\(^{2+}\) levels in the presence of 0.1–10 μM cytosolic Ca\(^{2+}\) concentrations as compared with those in WT cells. These reduced steady state ER Ca\(^{2+}\) levels or enhanced levels of cytosolic Ca\(^{2+}\)-induced fractional Ca\(^{2+}\) release suggest that central domain RyR2 mutations increase the cytosolic Ca\(^{2+}\)-dependent activation of Ca\(^{2+}\) release. This is consistent with the observation that central domain RyR2 mutations increase the Ca\(^{2+}\)-dependent activation of \[^{3}H\]ryanodine binding (Fig. 2 and supplemental Fig. S1). Interestingly, the steady state ER Ca\(^{2+}\) level in HEK293 cells expressing central domain RyR2 mutants at 10 μM cytosolic Ca\(^{2+}\) concentration was significantly higher than that in WT cells (Figs. 3 and 4). In other words, central domain RyR2 mutant cells displayed reduced fractional Ca\(^{2+}\) release at 10 μM cytosolic Ca\(^{2+}\) concentration as compared with WT cells. This suggests that RyR2 mutations located in the central domain of the channel may alter the cytosolic Ca\(^{2+}\)-dependent inhibition of Ca\(^{2+}\) release.

**Effect of Central Domain RyR2 Mutations on the Activation and Termination Thresholds for Spontaneous Ca\(^{2+}\) Release**—Disease-associated RyR2 mutations have been shown to increase the propensity for arrhythmogenic spontaneous Ca\(^{2+}\) release during store Ca\(^{2+}\) overload, a process also known as store overload-induced Ca\(^{2+}\) release (SOICR) (27, 28). It is of interest and importance to assess whether RyR2 mutations located in the central domain of the channel also alter the properties of SOICR. To this end, we monitored the ER luminal Ca\(^{2+}\) dynamics in HEK293 cells using D1ER (24, 25). As shown in Fig. 5, elevating extracellular Ca\(^{2+}\) from 0 to 2 mM induced spontaneous ER Ca\(^{2+}\) oscillations in HEK293 cells expressing RyR2 WT (depicted as downward deflections of the FRET signal). SOICR occurred when the ER luminal Ca\(^{2+}\) content fell to another threshold level (F\(_{\text{SOICR}}\)) (Fig. 5A). The ER luminal Ca\(^{2+}\) dynamics during SOICR in HEK293 cells expressing eight RyR2 mutants located in the central domain is shown in Fig. 5, B–I. All eight central domain RyR2 mutations significantly reduced both the activation (Fig. 6A) and termination (Fig. 6B) thresholds for SOICR. However, these mutations altered the activation and termination thresholds.
olds to different extents. As a result, they differentially affected the fractional Ca$^{2+}$/H11001 release (activation threshold / termination threshold) during SOICR. Mutations H4108Q, T4158P, and Q4159P significantly reduced the fractional Ca$^{2+}$/H11001 release ($p < 0.05$), whereas mutations H4108N and S4124T slightly increased fractional release as compared with that in RyR2 WT-expressing cells ($p < 0.05$). On the other hand, mutations G3946A, G3946S, and M3978I exerted no significant impact on the fractional Ca$^{2+}$/H11001 release (Fig. 6C). It should be noted that there was no significant difference in the store capacity ($F_{\text{max}} - F_{\text{min}}$) between RyR2 WT and eight central domain RyR2 mutant cells (Fig. 6D). Furthermore, SOICR did not occur in control HEK293 cells expressing no RyR2, and SOICR was not affected by the inositol trisphosphate receptor (IP3R) inhibitor, xestospongin C (29), indicating that SOICR is mediated by RyR2. Collectively, these data indicate that disease-associated RyR2 mutations located in the central domain reduce both the SOICR activation and termination thresholds.

Racemic Carvedilol and the (R)-Carvedilol Enantiomer Suppress SOICR in HEK293 Cells Expressing Central Domain RyR2 Mutants Associated with Atrial Fibrillation—We have previously shown that racemic carvedilol, a clinically used beta-blocker, and the non-beta-blocking (R)-carvedilol enantiomer suppress spontaneous Ca$^{2+}$/H11001 oscillations (SOICR) in HEK293 cells expressing a RyR2 mutation R4496C that is associated with CPVT (11, 15). Interestingly, some of the RyR2 mutations located in the central domain, G3946A, M3978I, and Q4159P, have been associated with AF (16, 19, 23). It is unknown whether racemic carvedilol or (R)-carvedilol is able to suppress SOICR mediated by CPVT- and AF-associated RyR2 mutants. To this end, we perfused HEK293 cells expressing the CPVT- and AF-associated RyR2 mutants (G3946A, M3978I, or Q4159P) with elevated extracellular Ca$^{2+}$/H11001 from 0 to 2 mM to induce spontaneous Ca$^{2+}$/H11001 oscillations in these cells. The fractions of HEK293 cells displaying Ca$^{2+}$/H11001 oscillations were then determined before and after application of increasing concentrations (0, 1, 3, 10, and 30 μM) of racemic carvedilol or the
non-beta-blocking (R)-carvedilol. As shown in Fig. 7, racemic carvedilol or (R)-carvedilol at 10 μM markedly suppressed SOICR in HEK293 cells expressing the CPVT- and AF-associated mutants (G3946A, M3978I, and Q4159P), and completely abolished SOICR in these cells at 30 μM. Because both the beta-blocking carvedilol and non-beta-blocking (R)-carvedilol suppress SOICR, their SOICR-inhibiting effect is independent of beta-blockade. Thus, racemic carvedilol and the (R)-carvedilol enantiomer are also able to suppress SOICR mediated by CPVT- and AF-linked RyR2 mutants.

**Discussion**

The three-dimensional structure of RyR consists of a number of major domains, including the N-terminal domain, SPRY domains, phosphorylation domains, handle domain, helical domain, central domain, and channel domain (6). The channel domain, which possesses a structural fold similar to that of the superfamily of voltage-gated ion channels, is involved in the gating and ion conduction of RyR (6). However, the roles of other major domains in RyR function and regulation are poorly understood. Recent structural analysis of the closed and open states of RyR1 identified the central domain as the transducer that couples the long-range conformational changes between the cytosolic peripheral domains and the channel domain (7). Interestingly, the central domain of RyR2 encompasses one of the disease-associated mutation hotspots (hotspot-3), but the functional impact of hotspot-3 RyR2 mutations is not well defined. We have previously shown that a point mutation E3987A located in the central domain of RyR2 dramatically reduced the sensitivity of the channel to activation by cytosolic Ca\(^{2+}\) (9). This suggests that the central domain may mediate the cytosolic Ca\(^{2+}\) activation of RyR2. Consistent with this view, here we show that disease-associated RyR2 mutations located in hotspot-3 within the central domain enhance the cytosolic Ca\(^{2+}\) activation of the channel and the propensity for spontaneous Ca\(^{2+}\) release. Hence, our data provide novel insights into the molecular mechanisms of action of hotspot-3 RyR2 mutations and the functional role of the central domain.

Comparison of the distribution patterns of disease-associated RyR1 and RyR2 mutations reveals that mutation hotspot-3...
appears to be absent in RyR1 (4, 5). The reason for the lack of disease-causing RyR1 mutations in the hotspot-3 region within the central domain is unknown. It is well established that the mechanism of excitation-contraction (EC) coupling in skeletal muscle differs from that in cardiac muscle. In skeletal muscle, contraction is triggered by the release of Ca\(^{2+}\) from the sarcoplasmic reticulum through the RyR1 Ca\(^{2+}\) release channel via a mechanism known as voltage-induced Ca\(^{2+}\) release. In this process, membrane depolarization causes a conformational change in the voltage-gated Ca\(^{2+}\) channel, the voltage sensor (Cav1.1), which in turn activates the RyR1 channel through a direct physical interaction between Cav1.1 and RyR1. On the other hand, EC coupling in cardiac muscle is mediated by a mechanism known as Ca\(^{2+}\)-induced Ca\(^{2+}\) release (CICR), in
which membrane depolarization opens the voltage-dependent L-type Ca\(^{2+}\) channel (\(\text{Ca}_{\text{L},1.2}\)), leading to an influx of Ca\(^{2+}\). This small Ca\(^{2+}\) entry then activates the RyR2 Ca\(^{2+}\) release channel, resulting in a large Ca\(^{2+}\) release from the sarcoplasmic reticulum and subsequent muscle contraction. Hence, activation of RyR by cytosolic Ca\(^{2+}\) is absolutely required for EC coupling in cardiac muscle, but not in skeletal muscle (1–3). Therefore, the fractional Ca\(^{2+}\) occurs, whereas cytosolic Ca\(^{2+}\) absolutely required for EC coupling, mutations that alter the fractional Ca\(^{2+}\) release channel, such as atrial and Purkinje myocytes (31). Hence, activation of RyR2 may not be highly pathogenic. This may provide some benefits for patients with CPVT and AF associated with RyR2 mutations.

In summary, our present study demonstrates that CPVT- and AF-associated RyR2 mutations located in the central domain enhance cytosolic Ca\(^{2+}\) activation of RyR2 and spontaneous Ca\(^{2+}\) release, which can be limited by racemic carvedilol or the (\(R\))-carvedilol enantiomer. Our data suggest that the central domain of RyR2 is an important determinant of cytosolic Ca\(^{2+}\) activation of the channel, and that enhanced cytosolic Ca\(^{2+}\) activation represents a common defect of RyR2 mutations associated with CPVT and AF.

Experimental Procedures

Construction of Disease-associated RyR2 Mutations Located in the Central Domain of RyR2—The central domain RyR2 mutations (G3946A, G3946S, M3978I, H4108N, H4108Q, S4124T, T4158P, and Q4159P) were generated by the overlap extension method using PCR (32, 33). Briefly, The Sall and MluI restriction sites were introduced into the full-length mouse RyR2 cDNA at positions 11816 and 12340, respectively, without altering the amino acid sequence. The Sall-Mlu fragment containing G3946A, G3946S, M3978I, H4108N, or H4108Q was obtained by overlapping PCR and was used to replace the corresponding WT fragment in the full-length mouse RyR2 cDNA in pBluescript, which was then subcloned into pcDNA5. The MluI-BstUl fragment (at position 13237) fragment containing S4124T, T4158P, or Q4159P was obtained by overlapping PCR and was used to replace the corresponding WT fragment in the BsiWl fragment (at position 8864)-NotI (in the vector) construct of mouse RyR2 in pBluescript. This construct was

Consistent with this view, we found that central domain RyR2 mutations also reduce the threshold for activation and termination of spontaneous Ca\(^{2+}\) release. Therefore, it is possible that, by increasing the cytosolic Ca\(^{2+}\) activation of RyR2, the central domain RyR2 mutations may reduce the threshold for CICR and enhance the propensity for the initiation and propagation of spontaneous Ca\(^{2+}\) waves in atrial and Purkinje myocytes. It will be of interest to generate mouse models harboring central domain RyR2 mutations and determine whether central domain RyR2 mutations alter Ca\(^{2+}\) signaling in atrial and Purkinje cells and increase the susceptibility to CPVT and AF in mice.

Spontaneous Ca\(^{2+}\) release is believed to be the trigger for CPVT (5). Hence, suppressing spontaneous Ca\(^{2+}\) release represents a promising strategy for the treatment of CPVT. We have previously shown that carvedilol, a beta-blocker, is able to reduce the duration of openings of single RyR2 channels and suppress RyR2-mediated spontaneous Ca\(^{2+}\) release and stress-induced ventricular tachycardias in a mouse model harboring a CPVT-causing RyR2 mutation (11). We also showed that the non-beta-blocking carvedilol enantiomer, (\(R\))-carvedilol, is also able to suppress spontaneous Ca\(^{2+}\) waves and wave-evoked CPVT without the bradycardia associated with racemic carvedilol (15). Here we demonstrated that racemic carvedilol and the non-beta-blocking (\(R\))-carvedilol enantiomer also suppressed spontaneous Ca\(^{2+}\) oscillations in HEK293 cells expressing the CPVT- and AF-associated RyR2 mutations. These observations suggest that carvedilol or (\(R\))-carvedilol may provide some benefits for patients with CPVT and AF associated with RyR2 mutations.

FIGURE 6. Central domain RyR2 mutations reduce the thresholds for SOICR activation and termination. A and B, to minimize the influence by CFP/YFP cross-talk, we used relative FRET measurements for calculating the activation threshold (A) and termination threshold (B). The activation threshold was determined by the equation \(\left(\frac{F_{\text{SOICR}} - F_{\text{min}}}{F_{\text{max}} - F_{\text{min}}}\right) \times 100\%\), and the termination threshold was determined by the equation \(\left(\frac{F_{\text{term}} - F_{\text{min}}}{F_{\text{max}} - F_{\text{min}}}\right) \times 100\%. F_{\text{SOICR}}\) indicates the FRET level at which SOICR occurs, whereas \(F_{\text{term}}\) represents the FRET level at which SOICR terminates. C, the fractional Ca\(^{2+}\) release was calculated by subtracting the termination threshold from the activation threshold. The maximum FRET signal \(F_{\text{max}}\) is defined as the FRET level after tetracaine treatment. The minimum FRET signal \(F_{\text{min}}\) is defined as the FRET level after caffeine treatment. D, the store capacity was calculated by subtracting \(F_{\text{min}}\) from \(F_{\text{max}}\). Data shown are mean ± S.E. (\(n = 3–5\)), \(p < 0.05\) versus WT; NS, not significant.)
then subcloned into the full-length mouse RyR2 cDNA in the pcDNA5. All mutations were confirmed by DNA sequencing.

**Generation of Stable, Inducible Cell Lines Expressing WT and Central Domain Mutants of RyR2**—Stable, inducible HEK293 cell lines expressing RyR2 WT and central domain mutants were generated using the Flp-In T-REx Core Kit from Invitrogen. Briefly, Flp-In T-REx HEK293 cells were co-transfected with the inducible expression vector pcDNA5/FRT/TO containing the mutant cDNAs and the pOG44 vector encoding the Flp recombinase in 1:5 ratios using the Ca2+/H11001 phosphate precipitation method. The transfected cells were washed with PBS (137 mM NaCl, 8 mM Na2HPO4, 1.5 mM KH2PO4, and 2.7 mM KCl, pH 7.4) 24 h after transfection followed by a change into fresh medium for 24 h. The cells were then washed with PBS, harvested, and plated onto new dishes. After the cells had attached (~4 h), the growth medium was replaced with a selection medium containing 200 g/ml hygromycin (Invitrogen). The selection medium was changed every 3–4 days until the desired number of cells was grown. The hygromycin-resistant cells were pooled, aliquoted (1 ml), and stored at 80 °C. These positive cells are believed to be isogenic, because the integration of RyR2 cDNA is mediated by the Flp recombinase at a single FRT (flippase recognition target) site.

**Western Blotting**—HEK293 cells grown for 24 h after transfection with RyR2 WT and central domain mutant cDNAs were washed with PBS plus 2.5 mM EDTA and harvested in the same solution by centrifugation for 8 min at 700 g in an IEC Centra-CL2 centrifuge. The cells were then washed with PBS without EDTA and centrifuged again at 700 g for 8 min. The PBS-washed cells were solubilized in a lysis buffer containing 25 mM Tris, 50 mM Hepes (pH 7.4), 137 mM NaCl, 1% CHAPS, 0.5% soy bean phosphatidylcholine, 2.5 mM DTT, and a protease inhibitor mix (1 mM benzamidine, 2 g/ml leupeptin, 2 g/ml pepstatin A, 2 g/ml aprotinin, and 0.5 mM PMSF). This mixture was incubated on ice for 1 h. Cell lysate was obtained by centrifuging twice at 16,000 g in a microcentrifuge at 4 °C for 30 min to remove unsolubilized materials. The RyR2 WT and mutant proteins were subjected to SDS-PAGE (6% gel) and transferred onto nitrocellulose membranes at 90 V for 1.5 h at 4 °C in the presence of 0.01% SDS (35). The nitrocellulose membranes containing the transferred proteins were blocked for 30 min with PBS containing 0.5% Tween 20 and 5% (w/v) nonfat dried skimmed milk powder. The blocked membrane was incubated with the anti-RyR antibody (34C) (Thermo Scientific, MA3-925, lot number PG200294) (1:1000 dilution) and then incubated with the secondary anti-(mouse IgG (heavy and light chains)) antibody conjugated to horseradish peroxidase (HRP) (Thermo Scientific, 34070, lot number PG200294) (1:4000 dilution). The membranes were washed with PBS-Tween 20 (0.05% Tween 20) and incubated with a chemiluminescence substrate (Pierce, Rockford, IL) according to the manufacturer’s instructions. The chemiluminescence signal was detected on a Kodak X-OMAT film and the intensity was quantified using the ImageJ software (National Institutes of Health, Bethesda, MD).
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light) antibodies conjugated to horseradish peroxidase (1:20,000 dilution). After washing for 5 min three times, the bound antibodies were detected using an enhanced chemiluminescence kit from Pierce. The intensity of each band was determined from its intensity profile obtained by ImageQuant LAS 4000 (GE Healthcare Life Sciences) and analyzed by using the ImageJ software (36).

[^H]Ryanodine Binding—HEK293 cells were grown to 95% confluence in a 75-cm\(^2\) flask, dissociated with PBS, and plated in 100-mm tissue culture dishes at ~10% confluence for 18–20 h before transfection with RyR2 WT and central domain mutant cDNAs. After transfection for 24 h, the cells were harvested and lysed in 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 \(\mu\)g/ml leupeptin, 2 \(\mu\)g/ml pepstatin A, 2 \(\mu\)g/ml apronitin, and 0.5 mM PMSF) on ice for 60 min. Cell lysate was obtained after removing the unsolubilized materials by centrifugation twice in a microcentrifuge at 4 °C for 30 min each. Equilibrium[^H]ryanodine binding to cell lysates was performed as described previously (9) with some modifications.[^H]Ryanodine binding was carried out in a total volume of 300 \(\mu\)l of binding solution containing 30 \(\mu\)l of cell lysate, 150 mM KCl, 25 mM Tris, 50 mM Hepes (pH 7.4), and 5 mM[^H]ryanodine and CaCl\(_2\) to set free [Ca\(^{2+}\)]\(_{\text{er}}\) from pCa 9.89 to pCa 4 and a protease inhibitor mix at 37 °C for 20 min. The Ca\(^{2+}\)/EGTA ratio was calculated using the computer program of Fabiato and Fabiato (37). 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/B filters presoaked with 1% polyethyleneimine. The filters were washed three times, and the radioactivity associated with the filters was determined by liquid scintillation counting. Nonspecific binding was determined by measuring[^H]ryanodine binding in the presence of 50 \(\mu\)M unlabeled ryanodine. All binding assays were done in duplicate.

**Single Cell Luminal Ca\(^{2+}\) Imaging**—Luminal Ca\(^{2+}\) levels in HEK293 cells expressing RyR2 WT or central domain mutants were measured using single-cell Ca\(^{2+}\) imaging and the fluorescent Ca\(^{2+}\) indicator dye Fura-2 AM as described previously (27, 28). Briefly, cells grown on glass coverslips for 8–18 h after induction (as indicated) by 1 \(\mu\)g/ml tetracycline (Sigma) were loaded with 5 \(\mu\)M Fura-2 AM in KRH buffer plus 0.02% Pluronic F-127 and 0.1 mg/ml BSA for 20 min at room temperature (23 °C). The coverslips were then mounted in a perfusion chamber (Warner Instruments) on an inverted microscope (Nikon TE2000-S). The cells were then perfused continuously with KRH buffer containing increasing extracellular Ca\(^{2+}\) concentrations (0, 1.0, and 2.0 mM). The cells were then perfused continuously with 2 mM Ca\(^{2+}\) in KRH buffer containing increasing concentrations of carvedilol (0, 1, 3, 10, and 30 \(\mu\)M). Caffeine (10 \(\mu\)M) was applied at the end of each experiment to confirm the expression of active RyR2 channels. Time-lapse images (0.25 frame/s) were captured and analyzed with Compix SimplePCI 6 software. Fluorescence intensities were measured from regions of interest centered on individual cells. Only cells that responded to caffeine were analyzed. The filters used for Fura-2 imaging were \(\lambda_{\text{ex}} = 340 \pm 26 \text{ nm}\) and \(387 \pm 11 \text{ nm}\), and \(\lambda_{\text{em}} = 510 \pm 84 \text{ nm}\) with a dichroic mirror (410 nm).

**Statistical Analysis**—All values shown are mean ± S.E. unless indicated otherwise. To test for differences between groups, we used Student’s t test (two-tailed) or one-way analysis of variance with post hoc test. A p value <0.05 was considered to be statistically significant.


**References**

Enhanced RyR2 Ca\(^{2+}\) Activation and Cardiac Arrhythmias