Abnormal Termination of Ca\textsuperscript{2+} Release is a Common Defect of RyR2 Mutations Associated With Cardiomyopathies

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Rationale: Naturally occurring mutations in the cardiac ryanodine receptor (RyR2) have been associated with both cardiac arrhythmias and cardiomyopathies. It is clear that delayed afterdepolarization resulting from abnormal activation of sarcoplasmic reticulum Ca\textsuperscript{2+} release is the primary cause of RyR2-associated cardiac arrhythmias. However, the mechanism underlying RyR2-associated cardiomyopathies is completely unknown.

Objective: In the present study, we investigate the role of the NH\textsubscript{2}-terminal region of RyR2 in and the impact of these results provide the first evidence that the NH\textsubscript{2}-terminal region of RyR2 is an important determinant of Ca\textsuperscript{2+} release termination and increased fractional Ca\textsuperscript{2+} release. The RyR2 A1107M mutation associated with hypertrophic cardiomyopathy had the opposite action (ie, increased the threshold for Ca\textsuperscript{2+} release termination and reduced fractional release).

Conclusions: These results provide the first evidence that the NH\textsubscript{2}-terminal region of RyR2 is an important determinant of Ca\textsuperscript{2+} release termination, and that abnormal fractional Ca\textsuperscript{2+} release attributable to aberrant termination of Ca\textsuperscript{2+} release is a common defect in RyR2-associated cardiomyopathies. (Circ Res. 2012;110:00-00.)

Key Words: Ca\textsuperscript{2+} release termination | cardiac arrhythmias | cardiomyopathies | ryanodine receptor mutations | sarcoplasmic reticulum

The cardiac ryanodine receptor (RyR2) is the major Ca\textsuperscript{2+} release channel of the sarcoplasmic reticulum (SR) and plays an essential role in excitation–contraction coupling and SR Ca\textsuperscript{2+} homeostasis.\textsuperscript{1} Abnormal SR Ca\textsuperscript{2+} handling attributable to defective RyR2 function is a well-known cause of ventricular tachyarrhythmias and sudden death.\textsuperscript{2,3} Naturally occurring RyR2 mutations have been linked to catecholaminergic polymorphic ventricular tachycardia (CPVT) and catecholaminergic idiopathic ventricular fibrillation.\textsuperscript{4–6} More than 150 disease-associated RyR2 mutations have been identified to date.\textsuperscript{5,7} Most of these RyR2 mutations are associated with stress-induced ventricular tachyarrhythmias and sudden death in structurally normal hearts. However, some of them are associated with cardiomyopathies as well as cardiac arrhythmias.\textsuperscript{6,7} For example, an in-frame deletion of 35 amino acid residues (Asn57-Gly91) in the NH\textsubscript{2}-terminal region, corresponding to exon-3 of the RYR2 gene, was identified in several unrelated families. This deletion is associated with an expanding spectrum of phenotypes that include sinoatrial nodal dysfunction, atrial fibrillation, AV block, decreased left ventricular function, increased trabeculation, CPVT, and dilated cardiomyopathy (DCM).\textsuperscript{7–9} Furthermore, a number of RyR2 NH\textsubscript{2}-terminal point mutations, including A77V, R176Q/ T2504M, R420W, and L433P, are associated with arrhythmogenic right ventricular displasia type 2 (ARVD2).\textsuperscript{6,7,10–14} RyR2 mutations also may be associated with hypertrophic cardiomyopathy (HCM).\textsuperscript{15} A definitive link between RyR2 mutations and a specific type of cardiomyopathy has not been firmly estab-
lished because of the small number of RyR2 mutation carriers and their variable clinical phenotypes. However, an increasing body of evidence suggests that defective RyR2 function can lead not only to cardiac arrhythmias but also to cardiomyopathies.

An important question is how the various RyR2 mutations produce all the different phenotypes. One possibility is that different RyR2 mutations may alter different aspects of SR Ca\(^{2+}\) release. The initiation/activation of SR Ca\(^{2+}\) release is normally well-controlled by membrane depolarization via a mechanism known as Ca\(^{2+}\)-induced Ca\(^{2+}\) release.\(^{1}\) Under conditions of SR Ca\(^{2+}\) overload, however, SR Ca\(^{2+}\) release in the form of Ca\(^{2+}\) waves can occur spontaneously in the absence of membrane depolarization.\(^{1,16-21}\) These spontaneous Ca\(^{2+}\) waves, also termed store overload-induced Ca\(^{2+}\) release (SOICR),\(^{22,23}\) may result in delayed afterdepolarizations (DADs) and triggered arrhythmias.\(^{2,3,16-21}\) We have recently demonstrated that a number of RyR2 mutations associated with CPVT reduce the threshold for SOICR.\(^{22,23}\) A reduced SOICR threshold will increase the propensity for DADs and, thus, triggered arrhythmias. Therefore, abnormal activation of Ca\(^{2+}\) release attributable to inappropriate opening of RyR2 represents a common mechanism for RyR2-associated CPVT.\(^{6}\)

The mechanism for RyR2-associated cardiomyopathies remains unknown. Recent studies demonstrate that SR Ca\(^{2+}\) stores are only partially depleted during local or depolarization-induced global Ca\(^{2+}\) transients,\(^{24-26}\) indicating that SR Ca\(^{2+}\) release terminates well before the store is empty. The process that terminates Ca\(^{2+}\) release is thought to be critical for maintaining stable excitation–contraction coupling and controlling the cytosolic Ca\(^{2+}\) transient.\(^{27-30}\) Abnormal cytosolic Ca\(^{2+}\) transients have been proposed to trigger the cardiac remodeling\(^{11,32}\) associated with cardiomyopathies.\(^{33-35}\) It is therefore possible that RyR2 mutations associated with cardiomyopathies may alter the process of Ca\(^{2+}\) release termination. To test this possibility, we assessed the impact on Ca\(^{2+}\) release of a number of NH\(_2\)-terminal RyR2 mutations that are associated with cardiomyopathies. We report that the NH\(_2\)-terminal region of RyR2 is an important determinant of Ca\(^{2+}\) release termination. Further, we show that altered Ca\(^{2+}\) release termination is a common defect of RyR2 mutations associated with cardiomyopathies.

### Methods

#### Site-Directed Mutagenesis and DNA Transfection

All RyR2 mutations were generated using the overlap extension method as previously described.\(^{23,36}\) HL-1 cardiac cells were transfected with the GFP-tagged RyR2 wild-type (WT) or the GFP-tagged RyR2 mutant, Del-Exon-3, using the Nucleofector kit (Amaza) according to the manufacturer’s instructions.

#### Generation of Stable Inducible HEK293 Cell Lines Expressing RyR2 WT and Mutants

Stable inducible HEK293 cell lines expressing RyR2 WT or mutants were generated using the Flp-In T-REx Core kit (Invitrogen Life Technologies) according to the manufacturer’s instructions.

#### Single-Cell Ca\(^{2+}\) Imaging

Luminal Ca\(^{2+}\) transients in HEK293 cells expressing RyR2 WT or mutant channels were measured using the Ca\(^{2+}\)-sensitive fluorescence resonance energy transfer (FRET)-based cameleon protein D1ER.\(^{37,38}\) Stable inducible HEK293 cells were transfected with the D1ER cDNA 24 hours before the induction of RyR2 expression. Fluorescent images were captured using an inverted microscope (Nikon TE2000-S) with S-Fluor 20×0.75 objective. The amount of FRET was determined from the ratio of the emissions at 535 and 470 nm (excitation at 430 nm). Cytosolic Ca\(^{2+}\) transients in these cells were monitored using the fluorescent Ca\(^{2+}\) indicator dye Fura-2 acetoxymethyl ester as previously described.\(^{22,23}\) Time-lapse images (0.5 frame/s) were captured and analyzed with the Compix Simple PCI 6 software (Compix). Fluorescence intensities were measured from regions of interest centered on individual cells.

Detailed Methods are provided in the Online Supplement.

### Results

#### The NH\(_2\)-Terminal Region of RyR2 Is an Important Determinant of Ca\(^{2+}\) Release Termination

There are more than 20 disease-causing mutations in the NH\(_1\)-terminal region of RyR2.\(^{6,7}\) The functional role of this region and the consequences of these mutations are unclear. To gain insights into these unknowns, we used a deletion approach in which we removed the first 305 NH\(_2\)-terminal residues of RyR2 (Del-305) (Supplemental Figure 1). Ca\(^{2+}\) release assays and immunoblotting analysis revealed that the Del-305 mutant was expressed in HEK293 cells and remained functional (Supplemental Figure IB, IC). Thus, deletion of the first 305 NH\(_2\)-terminal residues of RyR2 did not abolish its expression or function.

We used a FRET-based endoplasmic reticulum (ER) luminal Ca\(^{2+}\)-sensing protein D1ER\(^\text{\textregistered}\) to monitor the ER luminal Ca\(^{2+}\) dynamics and to determine whether the NH\(_2\)-terminal deletion alters SOICR. As shown in Figure 1, elevating extracellular Ca\(^{2+}\) from 0 to 2 mmol/L induced SOICR in HEK293 cells expressing RyR2 WT (observed as the downward deflections of the FRET signal, Figure 1A). SOICR occurred when the ER Ca\(^{2+}\) increased to a threshold level (the SOICR activation threshold, F\(_{\text{SOICR}}\)) and terminated when the ER Ca\(^{2+}\) declined to another threshold level (the SOICR termination threshold, F\(_{\text{term}}\); Figure 1A). This SOICR in HEK293 cells expressing RyR2 WT terminated at a threshold of 57% store capacity, which is similar to that (60%) observed in cardiomyocytes.\(^{26}\) Note that SOICR is mediated by RyR2, not by any endogenous inositol-1,4,5-trisphosphate receptors (IP3Rs) that may be present, because xestospongin C, an inhibitor of IP3Rs,
had no effect on SOICR in HEK293 cells expressing RyR2 WT (Supplemental Figure II).

SOICR was also observed in HEK293 cells expressing the RyR2 Del-305 mutant (Figure 1B). The SOICR in the Del-305 mutant cells exhibited a marked reduction in the termination threshold (35% vs 57% in WT; \( P < 0.01 \)) and a slightly lowered activation threshold (90% vs 94% in WT; \( P < 0.01 \); Figure 1C, 1D). As a result, the fractional Ca\(^{2+}\) release during SOICR (activation threshold \(\div\) termination threshold) is significantly greater in the Del-305 cells (55%) than in the WT cells (36%; \( P < 0.01 \); Figure 1E). There were no significant differences in the maximum FRET signal (\( F_{\text{max}} \)) obtained after tetracaine treatment, the minimum FRET signal (\( F_{\text{min}} \)) obtained after 20 mmol/L caffeine application, or the store capacity (\( F_{\text{max}} - F_{\text{min}} \)) between the WT and Del-305 mutant cells (Figure 1F–H). Note that the D1ER probe was not saturated in HEK293 cells (data not shown), similar to that reported previously.\(^{39}\) These studies show that the NH\(_2\)-terminal region of RyR2 has an important role in the termination of Ca\(^{2+}\) release.

**Deletion of Exon-3 in RyR2 Reduces the Threshold for Ca\(^{2+}\) Release Termination**

We next determined whether the cardiomyopathy-associated deletion of exon-3 (Del-exon-3) within the NH\(_2\)-terminal region (Supplemental Figure IIIA) alters Ca\(^{2+}\) release termination. Supplemental Figure III shows that the Del-exon-3 mutant formed functional RyR2s in HEK293 cells (Supple-
mental Figure IIIB) and was expressed at a level similar to that of RyR2 WT (Supplemental Figure IIIC). The ER luminal Ca\(^{2+}\) dynamics in these Del-exon-3 cells was assessed. SOICR in the Del-exon-3 cells had a slightly lower activation threshold (88% vs 94% in WT; \(P<0.01\)) and a markedly reduced termination threshold (39% vs 57% in WT; \(P<0.01\); Figure 2A–D). The fractional Ca\(^{2+}\) release in the Del-exon-3 cells (49%) was also significantly greater than in the WT cells (36%; \(P<0.01\); Figure 2E). There were no significant differences in \(F_{\text{max}}, F_{\text{min}},\) or store capacity between the WT and Del-Exon-3 cells (Figure 2F–H). These results demonstrate that the cardiomyopathy-associated exon-3 deletion in RyR2 reduces the threshold for Ca\(^{2+}\) release termination and increases fractional release in HEK293 cells.

**Exon-3 Deletion Enhances the Propensity for SOICR and the Magnitude of Ca\(^{2+}\) Transients**

A reduced termination threshold would increase the magnitude of Ca\(^{2+}\) release into the cytosol. To directly test this, we monitored the cytosolic Ca\(^{2+}\) transient using the cytosolic Ca\(^{2+}\) dye, Fura-2 acetoxymethyl ester. In both the WT (Figure 3A) and Del-Exon-3 (Figure 3B) cells, increasing extracellular Ca\(^{2+}\) induced SOICR observed as oscillatory cytosolic Ca\(^{2+}\) transients. Importantly, the Del-305 deletion also enhanced the propensity and amplitude of SOICR (Supplemental Figure IV). Note that the store Ca\(^{2+}\) contents in the RyR2 WT
Del-exon-3 (98% of WT) and Del-305 (104% of WT) cells were not significantly different. To determine whether the Del-exon-3 deletion alters SOICR in cardiac cells, we transfected the HL-1 cardiac cells (a mouse atrial cell line) with the GFP-tagged RyR2 WT or GFP-tagged Del-exon-3 mutant. Cytosolic Ca\textsuperscript{2+} transients (Figure 4) indicative of SOICR are shown in Figure 4. The Del-exon-3 deletion significantly increased the occurrence (Figure 4C) and amplitude (193% of WT; \(P < 0.01\)) but not the frequency (133% of WT; \(P = 0.06\); Figure 4D) of SOICR in HL-1 cardiac cells. There was no significant difference in the store Ca\textsuperscript{2+} contents between the WT (100%) and Del-exon-3 (104% of WT; \(P = 0.74\)) transfected HL-1 cardiac cells. Overall, these results suggest that the cardiomyopathy-associated exon-3 deletion promotes SOICR by reducing its activation threshold and increases the amplitude of Ca\textsuperscript{2+} release (fractional release) by reducing its termination threshold.

RyR2 NH\textsubscript{2}-Terminal Mutations Associated With ARVD2 Reduce the Threshold for Ca\textsuperscript{2+} Release Termination

A number of point mutations in the NH\textsubscript{2}-terminal region of RyR2 are associated with ARVD2 cardiomyopathy.\textsuperscript{6,7,10–14} To determine whether these NH\textsubscript{2}-terminal ARVD2-associated RyR2 mutations also affect Ca\textsuperscript{2+} release termination, we generated HEK293 cell lines that express the RyR2 wild-type (WT) and the RyR2 exon-3 deletion mutant (Del-Exon-3) were loaded with 5 \(\mu\)mol/L Fura-2 acetoxymethyl ester (Fura-2 am) in KRH buffer. The cells were then perfused continuously with KRH buffer containing increasing levels of extracellular Ca\textsuperscript{2+} (0–2 mmol/L) to induce SOICR. Fura-2 ratios of representative RyR2 WT (A) and Del-exon-3 (B) cells were recorded using single cell Ca\textsuperscript{2+} imaging. The percentages of RyR2 WT (337) and Del-exon-3 (443) cells that display Ca\textsuperscript{2+} oscillations at various extracellular Ca\textsuperscript{2+} concentrations. The amplitude (D) and frequency (E) of SOICR in RyR2 WT and Del-exon-3 cells were determined by measuring the averaged peak amplitude and frequency of Ca\textsuperscript{2+} oscillations at 2 mmol/L extracellular Ca\textsuperscript{2+} and was normalized to that in the RyR2 WT cells (100%). Data shown are mean ± standard error of the mean (SEM; \(n = 8–9\)). *\(P < 0.05\), †\(P < 0.01\) vs WT.

The Mouse RyR2 A1107M Mutation Increases the Threshold for Ca\textsuperscript{2+} Release Termination

The human RyR2 T1107M mutation is associated with HCM.\textsuperscript{15} We generated a stable inducible HEK293 cell line expressing the mouse RyR2 A1107M mutation, which corresponds to the human HCM-associated RyR2 T1107M mutation.
Interestingly, the A1107M mutation significantly increased the termination threshold (61% vs 57% in WT; P<0.05), but it did not significantly affect the activation threshold (Figure 6A–C). This resulted in a significant reduction in the fractional Ca\(^{2+}\) release (32% vs 36% in WT; P<0.01; Figure 6D). There were no significant differences in F\(_{\text{max}}\), F\(_{\text{min}}\), and store capacity between the WT and A1107M mutant cells (Figure 6E–G). The expression levels of the RyR2 WT and A1107M mutant were similar (Figure 6H).

Therefore, in contrast to the RyR2 mutations associated with DCM and ARVD2, the A1107M mutation associated with HCM increases the threshold for Ca\(^{2+}\) release termination and decreases the fractional Ca\(^{2+}\) release.

**Discussion**

Mutations in RyR2 are associated with stress-induced ventricular tachyarrhythmias and cardiomyopathies. Extensive investigations over the past decade have demonstrated that spontaneous Ca\(^{2+}\) wave (SOICR)-evoked DADs are the major cause of RyR2-associated CPVT.\(^{4–6}\) We and others have shown that CPVT RyR2 mutations reduce the threshold for SOICR.\(^{22,23,40,42–45}\) This reduced SOICR threshold increases the likelihood of spontaneous SR Ca\(^{2+}\) release during SR Ca\(^{2+}\) overload and, thus, the likelihood of SOICR-evoked DADs and triggered arrhythmias.\(^{3,16–21}\) In contrast, it is unclear how RyR2 mutations lead to cardiomyopathies. We show here that the DCM-associated RyR2 exon-3 deletion and several ARVD2-associated RyR2 NH\(_{2}\)-terminal mutations (A77V, R176Q/T2504M, R420W, and L433P) all reduce the threshold for Ca\(^{2+}\) release termination, whereas an HCM-associated RyR2 mutation (A1107M) increases the termination threshold. These data demonstrate for the first time to our knowledge that RyR2 mutations associated with cardiomyopathies alter the termination threshold of Ca\(^{2+}\) release.

The exact mechanism by which SR Ca\(^{2+}\) release is terminated remains unclear. Localized SR luminal Ca\(^{2+}\)-dependent inactivation of RyR2 is likely to be involved.\(^{24,26,29,46,47}\) Elegant studies by Zima et al\(^{26}\) showed that spontaneous (diastolic) and stimulated (systolic) SR Ca\(^{2+}\) release in cardiomyocytes terminate at the same SR luminal Ca\(^{2+}\) threshold. This implies that systolic and diastolic Ca\(^{2+}\) release is terminated by a similar process. We demonstrate here that cardiomyopathy-associated RyR2 mutations alter the termination threshold of SOICR or spontaneous Ca\(^{2+}\) release. Based on the finding of Zima et al,\(^{26}\) these RyR2 mutations are likely to alter the termination threshold of...
The sensitivity of myofilaments to Ca\textsuperscript{2+} proteins. Most disease-associated sarcomeric mutations alter these are generally associated with mutations in sarcomeric (A77V, R176Q/T2504M, R420W, or L433P), or the RyR2 E189D and R4496C mutations associated with catecholaminergic mutations (A77V, R176Q/T2504M, R420W, or L433P), or the RyR2 E189D and R4496C mutations associated with catecholaminergic polymorphic ventricular tachycardia (CPVT) only. The activation threshold (A), termination threshold (B), and fractional Ca\textsuperscript{2+} release (C) in RyR2 WT (250) and mutant (87–110) cells were determined as described in Figure 1. Data shown are mean±standard error of the mean (SEM; n=5–13.∗P<0.01 vs WT. D, Immunoblotting of RyR2 WT and RyR2 mutants from the same amount of cell lysates using the anti-RyR antibody (34c; n=3).

Abnormal systolic Ca\textsuperscript{2+} release also could result from altered Ca\textsuperscript{2+} release termination. A reduced termination threshold would delay the termination of SR Ca\textsuperscript{2+} release and thus increase the cytosolic Ca\textsuperscript{2+} transient. An increased termination threshold would cause premature termination of Ca\textsuperscript{2+} release and consequently would limit the size of the cytosolic Ca\textsuperscript{2+} transient. Here, we show that a DCM-associated RyR2 mutation reduces the termination threshold and increases the fractional Ca\textsuperscript{2+} release, whereas an HCM-associated RyR2 mutation increases the termination threshold and decreases fractional release. These changes in fractional release may alter cytosolic Ca\textsuperscript{2+} transients. These effects are similar to those of sarcomeric mutations associated with DCM and HCM. It is important to note that the fractional Ca\textsuperscript{2+} release is determined by both the termination and activation thresholds. We show that CPVT-only RyR2 mutations (E189D and R4496C) reduce the activation and termination thresholds to a similar extent, resulting in no significant changes in the fractional Ca\textsuperscript{2+} release. Therefore, our data suggest a link between abnormal fractional Ca\textsuperscript{2+} release resulting from aberrant Ca\textsuperscript{2+} release termination and RyR2-associated cardiomyopathies.

Aberrant Ca\textsuperscript{2+} release termination may also contribute to CPVT. It has been estimated that a diastolic Ca\textsuperscript{2+} wave (SOICR) that liberates 50% to 70% SR Ca\textsuperscript{2+} content is required to produce DADs of sufficient amplitude to induce triggered arrhythmias. A reduced termination threshold would increase the fractional Ca\textsuperscript{2+} release and, thus, the amplitude of Ca\textsuperscript{2+} waves during SR Ca\textsuperscript{2+} overload. Larger Ca\textsuperscript{2+} waves would, in turn, produce more robust DADs and enhance the propensity for triggered activities. Thus, the reduced termination threshold for SOICR of the DCM-associated and ARVD2-associated RyR2 mutations combined with their lowered SOICR activation threshold may explain why these mutations also enhance the susceptibility to CPVT.

The recently solved three-dimensional structures of the NH\textsubscript{2}-terminal region of RyR2\textsuperscript{52–55} have provided some new insights into how mutations in the NH\textsubscript{2}-terminal region of RyR2 alter Ca\textsuperscript{2+} release termination. The NH\textsubscript{2}-terminal region of RyR contains three domains that interact with each other to form a cytoplasmic vestibule at the center of the channel. Many disease-causing RyR mutations are located in interfaces between these three domains or between these domains and other parts of the channel. Interestingly, the myofilaments represent a major pool of intracellular Ca\textsuperscript{2+} binding sites, changes in the Ca\textsuperscript{2+} sensitivity of myofilaments can alter their response to Ca\textsuperscript{2+} release, which can, in turn, change the amplitude and dynamics of the cytosolic Ca\textsuperscript{2+} transient (but not the total release of Ca\textsuperscript{2+}).\textsuperscript{35,48,49} HCM-associated sarcomeric mutations tend to increase the myofilament Ca\textsuperscript{2+} sensitivity and thus reduce cytosolic Ca\textsuperscript{2+} transients. However, DCM-associated sarcomeric mutations tend to decrease the myofilament Ca\textsuperscript{2+} sensitivity and thus increase cytosolic Ca\textsuperscript{2+} transients.\textsuperscript{33,35,48,49} The abnormal cytosolic Ca\textsuperscript{2+} transient resulting from altered myofilament Ca\textsuperscript{2+} sensitivity is thought to trigger the cardiac remodeling (via Ca\textsuperscript{2+}/calmodulin-dependent signaling pathways, the calcineurin/NFAT pathways, or apoptotic signaling) that can lead to HCM or DCM.\textsuperscript{31–35,50}

Abnormal systolic Ca\textsuperscript{2+} transients also are generally associated with mutations in sarcomeric proteins. Most disease-associated sarcomeric mutations alter the sensitivity of myofilaments to Ca\textsuperscript{2+}.\textsuperscript{33,35,48,49} Because the

Figure 5. Arrhythmogenic right ventricular displasia type 2 (ARVD2)-associated ryanodine receptor (RyR2) NH\textsubscript{2}-terminal mutations decrease the termination threshold and increase the fractional Ca\textsuperscript{2+} release. DTER fluorescence resonance energy transfer (FRET) imaging was performed in single HEK293 cells expressing the RyR2 wild-type (WT), ARVD2-associated RyR2 mutations (A77V, R176Q/T2504M, R420W, or L433P), or the RyR2 E189D and R4496C mutations associated with catecholaminergic polymorphic ventricular tachycardia (CPVT) only. The activation threshold (A), termination threshold (B), and fractional Ca\textsuperscript{2+} release (C) in RyR2 WT (250) and mutant (87–110) cells were determined as described in Figure 1. Data shown are mean±standard error of the mean (SEM; n=5–13.∗P<0.01 vs WT. D, Immunoblotting of RyR2 WT and RyR2 mutants from the same amount of cell lysates using the anti-RyR antibody (34c; n=3).

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cytoplasmic vestibule formed by the NH₂-terminal domains undergoes conformational changes during channel gating. It has been proposed that the NH₂-terminal domains are allosterically coupled to the transmembrane pore forming domains of the channel. Therefore, mutations in the NH₂-terminal region may affect the gating of the channel and, thus, Ca²⁺/H¹ release by altering the allosteric coupling between the NH₂-terminal domains and the channel pore forming domains. These structural studies also suggest that in addition to the NH₂-terminal domains, other regions of RyR2 are likely involved in Ca²⁺ release termination.

The importance of Ca²⁺ release termination in cardiac physiology and pathophysiology has become increasingly clear. Besides the link between cardiomyopathies and abnormal Ca²⁺ release termination established here, reduced Ca²⁺ release termination threshold also has been shown in heart failure. This suggests that, like activation of Ca²⁺ release, termination of Ca²⁺ release is also an important target of regulation. Aberrant Ca²⁺ release termination may be a common defect associated with cardiomyopathies and other cardiac abnormalities.

Conclusions
Our studies in HEK293 cells demonstrate that cardiomyopathy-associated RyR2 mutations alter the termination of Ca²⁺ release. However, HEK293 cells lack many cardiac-specific proteins, and thus the Ca²⁺ release termination defects of cardiomyopathy-associated RyR2 mutations have yet to be confirmed in cardiac cells. At the moment, such studies would require the development of mouse models harboring cardiomyopathy-associated RyR2 mutations. These animal models are presently unavailable. Our studies also do not define the molecular mechanisms underlying the activation/termination thresholds or how these might be regulated by various proteins (eg, calsequestrin) and factors (eg, cytosolic or luminal Ca²⁺). Further comprehensive and detailed investigations will be required to address these important issues.

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None.

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The NH2-terminal region of RyR2 contains an important determinant of SR Ca2+ release self-terminates, and this termination process critically controls normal cytosolic Ca2+ signaling. What New Information Does This Article Contribute?

- The process of SR Ca2+ release termination is controlled by intrinsic properties of the RyR2 channel.
- The NH2-terminal region of RyR2 contains an important determinant of SR Ca2+ release termination.
- Abnormal Ca2+ release termination is a common defect of RyR2 mutations associated with cardiomyopathies.

RyR2 mutations have been linked to both stress-induced cardiac arrhythmias and cardiomyopathies. It has become clear that spontaneous Ca2+ waves resulting from abnormal activation of RyR2 are the major cause of cardiac arrhythmias. However, the causal mechanism of RyR2-associated cardiomyopathies is completely unknown. We examined the impact of a number of cardiomyopathy-associated RyR2 mutations and NH2-terminal deletions on the termination of Ca2+ release. Our results show that the deletion of exon 3 in the cardiac ryanodine receptor is rescued by beta strand switching. Structure. 2011;19:790–798.


Novelty and Significance

- Naturally occurring mutations in the cardiac ryanodine receptor (RyR2) are associated with stress-induced ventricular tacharyrhythmias (VTs) and cardiomyopathies.
- RyR2 mutations associated with VTs cause abnormal activation of RyR2 and, thus, aberrant sarcoplasmic reticulum (SR) Ca2+ release.
- SR Ca2+ release self-terminates, and this termination process critically controls normal cytosolic Ca2+ signaling.

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- The NH2-terminal region of RyR2 contains an important determinant of SR Ca2+ release termination.
- Abnormal Ca2+ release termination is a common defect of RyR2 mutations associated with cardiomyopathies.

RyR2 mutations have been linked to both stress-induced cardiac arrhythmias and cardiomyopathies. It has become clear that spontaneous Ca2+ waves resulting from abnormal activation of RyR2 are the major cause of cardiac arrhythmias. However, the causal mechanism of RyR2-associated cardiomyopathies is completely unknown. We examined the impact of a number of cardiomyopathy-associated RyR2 mutations and NH2-terminal deletions on the termination of Ca2+ release. Our results show that the deletion of exon 3 in the cardiac ryanodine receptor is rescued by beta strand switching.
Abnormal Termination of Ca\(^{2+}\) Release is a Common Defect of RyR2 Mutations Associated With Cardiomyopathies
Yijun Tang, Xixi Tian, Ruiwu Wang, Michael Fill and S.R. Wayne Chen

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SUPPLEMENTAL MATERIAL

Abnormal Termination of Ca$^{2+}$ Release is a Common Defect of RyR2 Mutations Associated with Cardiomyopathies

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Short Title: Ca$^{2+}$ Release Termination and Cardiomyopathies

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SUPPLEMENTAL METHODS:

Site-directed mutagenesis  The RyR2 deletion mutant Del-305 (deletion of the first 305 NH2-terminal residues) was constructed as described previously. Briefly, an NheI-AflIII adaptor was generated by annealing two primers, 5’-CTAGCAGCGCGAGCCATGGGCTGATTAC-3’ (forward) and 5’-TTAAGTAATCAGCCATGGCTCCGCGCTG-3’ (reverse). The full-length mouse RyR2 cDNA was digested with NheI and AflIII. The NheI (vector)-AflIII (915) fragment was discarded, and the remaining fragment was ligated with the NheI-AflIII adaptor to form the NH2-terminal deletion mutant Del-D305. The RyR2 deletion mutant Del-Exon-3 and the RyR2 point mutations, A77V, R176Q/T2504M, R420W, L433P and A1107M, were constructed using the overlap extension method. A fragment containing Del-Exon-3, A77V, R176Q, R420W, or L433P was produced by PCR and subcloned into the full-length mouse RyR2 WT or T2504M cDNA using NheI (vector) and AflIII (915) or AflII (915) and ClaI (2350). A fragment containing the point mutation A1107M was generated by PCR and subcloned into RyR2 cDNA using ClaI (2350) and KpnI (3823, 7553). The missing KpnI (3823)-KpnI (7553) fragment was then ligated back to form a full length RyR2-A1107M construct. The mutations and sequences of all PCR-amplified regions were confirmed by DNA sequencing. GFP-tagged RyR2 WT was generated as described previously. The same GFP insertion was made in the GFP-tagged Del-Exon-3 mutant. Briefly, the DNA encoding GFP flanked by glycine-rich linkers and an AscI site was obtained by PCR. The AscI site was introduced into the divergent region 1 of RyR2 WT and the Del-Exon-3 mutant after Asp-4365 by overlap-extension PCR. An AscI fragment containing GFP and the glycine-linkers was then subcloned into the full-length RyR2 WT or Del-Exon-3 mutant via the AscI site. The sequences of all PCR fragments were verified by DNA sequencing.

Caffeine-induced Ca2+ release measurements  Free cytosolic Ca2+ concentration in transfected HEK293 cells was measured using the fluorescence Ca2+ indicator dye Fluo-3 AM as described previously. Briefly, 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 cDNA. The cells grown for 18-20 hr after transfection were washed four times with PBS and incubated in KRH buffer without MgCl2 and CaCl2 (KRH buffer: 125 mM NaCl, 5 mM KCl, 1.2 mM KH2PO4, 6 mM glucose, 1.2 mM MgCl2, 2 mM CaCl2, and 25 mM Hepes, pH 7.4) at room temperature for
40 min. and at 37°C for 40 min. After being detached from culture dishes by pipetting, cells were collected by centrifugation at 1,000 rpm for 2 min. in a Beckman TH-4 rotor. Cell pellets were washed twice with KRH buffer and loaded with 10 µM Fluo 3-AM in KRH buffer plus 0.1 mg/ml BSA and 250 µM sulfinpyrazone at room temperature for 60 min., followed by washing with KRH buffer three times and resuspended in 150 µl KRH buffer plus 0.1 mg/ml BSA and 250 µM sulfinpyrazone. The Fluo 3 loaded cells were added to 2 ml (final volume) KRH buffer in a cuvette. Fluorescence intensity of Fluo-3 at 530 nm was measured in an SLM-Aminco series 2 luminescence spectrometer with 480 nm excitation at 25 °C (SLM Instruments, Urbana, IL).

**Generation of stable, inducible HEK293 cell lines** Stable, inducible HEK293 cell lines expressing RyR2 WT and mutants were generated using the Flp-In T-REx Core Kit from Invitrogen. Briefly, the full-length cDNA encoding the RyR2 WT or mutant channel was subcloned into the inducible expression vector, pcDNA5/FRT/TO. Flp-In T-REx-293 cells were then co-transfected with the inducible expression vector, pcDNA5/FRT/TO containing the RyR2 WT or mutant cDNA and the pOG44 vector encoding the Flp recombinase in 1:5 ratios using the Ca²⁺ phosphate precipitation method. Transfected cells were washed with PBS (137 mM NaCl, 8 mM Na₂HPO₄, 1.5 mM KH₂PO₄, 2.7 mM KCl) 1 day after transfection and allowed to grow for 1 more day in fresh medium. The cells were then washed again with PBS, harvested, and plated onto new dishes. After the cells had attached (~ 4 hrs), the growth medium was replaced with a selection medium containing 200 µg/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, 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 site.

**Western blotting** HEK293 cell lines grown for 24 hrs after induction were washed with PBS (137 mM NaCl, 8 mM Na₂HPO₄, 1.5 mM KH₂PO₄, 2.7 mM KCl) plus 2.5 mM EDTA and harvested in the same solution by centrifugation for 8 min at 700 x g in an IEC Centra-CL2 centrifuge. The cells were then washed with PBS without EDTA and centrifuged again at 700 x 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% 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS), 0.5% soybean 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/0.5 mM PMSF). This mixture was incubated on ice for 1 hr. Cell lysate was obtained by centrifuging twice at 16,000 x g in a microcentrifuge at 4°C for 30 min to remove unsolubilized materials. The RyR2 WT and mutant proteins were subjected to 6% SDS-PAGE and transferred to nitrocellulose membranes at 45 V for 18–20 h at 4 °C in the presence of 0.01% SDS. The nitrocellulose membranes containing the transferred proteins were blocked for 30 min with PBS containing 0.5% Tween-20 and 5% skimmed-milk powder. The blocked membrane was incubated with anti-RyR2 antibodies (34c) (1:1000) and then incubated with the secondary anti-mouse IgG (H&L) antibodies conjugated to horseradish peroxidase (1:20000). After washing for 15 min, three times, the bound antibodies were detected using an enhanced chemiluminescence kit from Pierce.

**Single-cell Ca²⁺ imaging (luminal Ca²⁺) of HEK293 cells**

Luminal Ca²⁺ transients in HEK293 cells expressing WT or mutant channels were measured using single-cell Ca²⁺ imaging and the Ca²⁺ sensitive fluorescence resonance energy transfer (FRET) based cameleon protein D1ER. Stable, inducible HEK293 cells expressing WT or mutant channels were used with the addition of transfection, using the Ca²⁺ phosphate precipitation method, with D1ER cDNA 24 hours before induction of RyR2 expression. The cells were perfused continuously with Krebs-Ringer- Hepes (KRH) buffer (125 mM NaCl, 5 mM KCl, 1.2 mM KH₂PO₄, 6 mM glucose, 1.2 mM MgCl₂, 25 mM Hepes, pH 7.4) containing various concentrations of CaCl₂ (0-2 mM) and tetracaine (1 mM) or caffeine (20 mM) at room temperature. Images were captured with Compix Inc. Simple PCI 6 software at 470 nm and 535 nm emission, with excitation at 430 nm, every 2 seconds using an inverted microscope (Nikon TE2000-S) equipped with a S-Fluor 20x/0.75 objective. The amount of FRET was determined from the ratio of the light emission at 535 and 470 nm.

**Single-cell Ca²⁺ imaging (cytosolic Ca²⁺) of HEK293 cells**

Intracellular cytosolic Ca²⁺ changes in stable, inducible HEK293 cells expressing RyR2 WT or mutant channels were monitored using single-cell Ca²⁺ imaging and the fluorescent Ca²⁺ indicator dye Fura-2 acetoxymethyl ester (Fura-2 AM) as described previously. Briefly, cells grown on glass coverslips for 18-22 hours after induction by 1 µg/ml tetracycline were loaded with 5 µM Fura
2/AM in KRH (Krebs-Ringer-Hepes) buffer (125 mM NaCl, 5 mM KCl, 1.2 mM KH$_2$PO$_4$, 6 mM glucose, 1.2 mM MgCl$_2$ and 25 mM Heps, pH 7.4) 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, Hamden, CT, U.S.A.) on an inverted microscope (Nikon TE2000-S). The cells were continuously perfused with KRH buffer containing increasing extracellular Ca$^{2+}$ concentrations (0-2.0 mM). Caffeine (10 mM) was applied at the end of each experiment to confirm the expression of active RyR2 channels. Time-lapse images (0.5 frame/s) were captured and analyzed with the Compix Simple PCI 6 software (Compix Inc., Sewickley, PA, USA). Fluorescence intensities were measured from regions of interest centered on individual cells. Only cells that responded to caffeine were analyzed.

**Culture, transfection, and single cell Ca$^{2+}$ imaging of mouse HL-1 cardiac cells**

HL-1 cardiac cells were kindly provided by Dr. William C. Claycomb from the Louisiana State University Health Sciences Center. Cells were thawed and grown in a 75cm$^2$ tissue culture flask coated with 0.02% (wt/vol) gelatin. The cells were grown in the Claycomb media (JRH Biosciences) supplemented with 10% (volume/volume) fetal bovine serum, penicillin/streptomycin (100U/ml/100ug/ml), 2mM L-glutamine and 0.1mM norepinephrine. For transfection, HL-1 cells (~ 3x10$^6$ cells) were washed with PBS and collected in a 13ml-Falcon tube. The cells were then centrifuged at 700 x g in an IEC Centra-CL2 centrifuge for 2 min and the supernatant was removed. The cell pellet was then gently mixed with 10 µg of RyR2 WT or Del-Exon-3 cDNA in the Cell Line Nucleofection Solution V in a total volume of 100 µl. The mixture of cells and DNA was subjected to Nucleofection by using the Amaxa apparatus with the A033 program. Transfected cells were then plated onto a 6-well plate containing 12-mm glass coverslips coated with gelatin and fibronectin and grown for 24-28 h. Intracellular Ca$^{2+}$ transients in transfected HL-1 cells were measured by using single-cell Ca$^{2+}$ imaging and the fluorescence Ca$^{2+}$ indicator dye Fura-2 acetoxymethyl ester$^{12}$. Briefly, cells grown on glass coverslips for 24-28 h after transfection were loaded with 5 µM Fura-2 acetoxymethyl ester in a modified Krebs-Ringer-Hepes buffer (125mM NaCl/5mM KCl/6mM glucose/1.2mM MgCl$_2$/25mM Heps (pH 7.4)) (without KH$_2$PO$_4$) plus 0.02% Pluronic F-127 (Molecular Probes) and 0.1 mg/ml BSA for 20 min at room temperature. The coverslips were then mounted in a perfusion chamber (Warner Instruments, Hamden, CT) on an inverted microscope (Nikon...
TE2000-S). The cells were continuously perfused with Krebs-Ringer-Hepes buffer containing different concentrations of CaCl$_2$ (0 to 10 mM) at room temperature (23°C). Time-lapse images (0.5 frames s$^{-1}$) were captured through a Fluor-X20 objective and a Chroma filter set using the Simple PCI System. Data were analyzed with the Compix Simple PCI 6 software (Compix Inc., Sewickley, PA, USA). All chemicals were obtained from Sigma (St. Louis, MO) unless otherwise specified.

**Statistical analysis**  All values shown are mean ± SEM unless indicated otherwise. To test for differences between groups, we used Student's $t$ test (2-tailed) or one-way ANOVA with post hoc test. A $P$ value <0.05 was considered to be statistically significant.

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SUPPLEMENTAL DATA:

Online Figure Legends

Online Fig. I. Construction and functional expression of an NH₂-terminally truncated RyR2 in HEK293 cells

(A) Schematic liner structure of the NH₂-terminal region of RyR2, depicting its 3 structural domains (A, B, and C)\textsuperscript{13}. The first 305 NH₂-terminal residues (i.e. the entire domain A plus part of the domain B) were removed to generate the RyR2-Del-305 mutant. (B) HEK293 cells were transfected with RyR2 WT or the RyR2 deletion mutant, Del-305. Fluorescence intensity of the Fluo-3-loaded transfected cells was monitored continuously before and after the additions of caffeine or ryanodine. Note that the addition of ryanodine (100 \( \mu \)M) following the addition of caffeine (0.25 mM) caused a slow release of Ca\(^{2+}\). This is likely to be due to the binding of ryanodine to a small population of RyR2 channels that have been activated by the low concentration of caffeine (0.25 mM) and consequently an increase in the open probability of these channels. Subsequent addition of 2.5 mM caffeine activated the remaining ryanodine-unmodified RyR2 channels resulting in a large Ca\(^{2+}\) release. In the continuous presence of ryanodine, the caffeine-activated channels would be modified by ryanodine into a fully activated state, leading to a depletion of intracellular Ca\(^{2+}\) store. The released Ca\(^{2+}\) would be extruded from the cytosol into the extracellular space, resulting in a transient Ca\(^{2+}\) release. Subsequent additions of caffeine yielded little or no Ca\(^{2+}\) release. This is because the ryanodine-modified channels are already in the fully activated state with little or no intracellular Ca\(^{2+}\) store. Importantly, the pattern of caffeine- and ryanodine-response of the Del-305 mutant is indistinguishable from that of the RyR2 WT. The sharp decreases in fluorescence intensity immediately after the addition of caffeine were due to quenching of the Fluo-3 fluorescence by caffeine. Traces shown are from representative experiments. Similar results were obtained from 3-4 separate experiments. (C) Immuno-blotting of RyR2-WT and the Del-305 mutant from the same amount of cell lysates with the anti-RyR antibody (34c) (n=3).

Online Fig. II. Effect of xestospongin C on SOICR in HEK293 cells

Stable, inducible HEK293 cells expressing RyR2-WT were transfected with the D1ER cDNA 48 h before single cell FRET imaging. The expression of RyR2-WT was induced 24 h before imaging. The cells
were perfused with KRH buffer containing increasing levels of extracellular Ca\(^{2+}\) (0-2 mM) to induce SOICR. This was followed by the addition of 1 µM xestospongin C, 1.0 mM tetracaine, and then 20 mM caffeine. (A) D1ER FRET signals from a representative RyR2-WT expressing cell. The activation threshold (B) and termination threshold (C) of SOICR before (-XesC) and after (+XesC) the addition of xestospongin C to RyR2-WT cells (50 cells) were determined using the equations shown in panel A. \(F_{SOICR}\) indicates the FRET level at which SOICR occurs. \(F_{termi}\) depicts the FRET level at which SOICR terminates. The maximum FRET signal \(F_{max}\) is defined as the FRET level after tetracaine treatment. The minimum FRET signal \(F_{min}\) is defined as the FRET level after caffeine treatment. Data shown are mean ± SEM (n = 4).

**Online Fig. III. Construction and functional expression of the RyR2 exon-3 deletion mutant**

(A) Schematic linear structure of the NH\(_2\)-terminal region of RyR2 WT and the RyR2 exon-3 deletion mutant (Del-Exon-3). The amino acid sequence of exon-3 and its secondary structures are also shown. (B) HEK293 cells were transfected with Del-Exon-3. Fluorescence intensity of the Fluo-3-loaded transfected cells was monitored continuously before and after the additions of caffeine or ryanodine. Note that the pattern of caffeine- and ryanodine-response of the Del-Exon-3 mutant is indistinguishable from that of the RyR2 WT (see Online Fig. I). The trace shown is from a representative experiment. Similar results were obtained from 3 separate experiments. (C) Immuno-blotting of RyR2-WT and the Del-Exon-3 mutant from the same amount of cell lysates with the anti-RyR antibody (34c) (n=3).

**Online Fig. IV. Deletion of the first 305 NH\(_2\)-terminal residues of RyR2 enhances the propensity for SOICR and the amplitude of Ca\(^{2+}\) release** Stable, inducible HEK293 cells expressing RyR2 WT and the NH\(_2\)-terminal deletion mutant (Del-305) were loaded with 5 µM Fura-2 AM in KRH buffer. The cells were then perfused continuously with KRH buffer containing increasing levels of extracellular Ca\(^{2+}\) (0 - 2 mM) to induce SOICR. Fura-2 ratios of representative RyR2-WT (A) and Del-305 (B) cells were recorded using single cell Ca\(^{2+}\) imaging. (C) The percentages of RyR2 WT (318) and Del-305 (410) cells that display Ca\(^{2+}\) oscillations at various extracellular Ca\(^{2+}\) concentrations. (D, E) The amplitude (D) and frequency (E) of SOICR in RyR2 WT and Del-305 expressing HEK293 cells were determined by measuring the averaged peak amplitude and frequency of Ca\(^{2+}\) oscillations at 2 mM extracellular
Ca^{2+}, and normalized to that in the RyR2 WT cells (100%). Data shown are mean ± SEM (n = 7) (#P < 0.05; *P < 0.01 vs WT).

**Online Fig. V. Effect of RyR2 NH2-terminal mutations on SOICR** FRET imaging was performed in single HEK293 cells expressing the RyR2 WT and RyR2 mutants (A77V, R176Q/T2504M, R420W, L433P, E189D, and R4496C). The maximum FRET signal (F_{max}) (A), the minimum FRET signal (F_{min}) (B), and the store capacity (C) in RyR2 WT (250) and RyR2 mutants (87-110) cells were determined as described in Online Figure legend I. Data shown are mean ± SEM (n=5-13).
Online Fig. I

A  N-terminal domains

RyR2-WT
\[ \begin{array}{cccc}
\text{NH}_2 & A & B & C \\
1 & 1 & 217 & 409 & 543 \text{ aa} & \text{COOH} \\
\end{array} \]

Del-305
\[ \begin{array}{c}
1 & 305 \\
\end{array} \]

B  RyR2-WT

\[ \text{C'} \quad \text{R} \quad \text{C} \quad \text{C} \quad \text{C} \quad \text{C} \]

\[ \Delta F/F_0 \]

50 s

\[ \text{Del-305} \]

\[ \text{C'} \quad \text{R} \quad \text{C} \quad \text{C} \quad \text{C} \quad \text{C} \]

\[ \Delta F/F_0 \]

50 s

C' = 0.25 mM caffeine; R = 100 \mu M ryanodine; C = 2.5 mM caffeine

C  Immuno-blotting

anti-RyR(34c)
A  RyR2-WT

<table>
<thead>
<tr>
<th>[Ca^{2+}]_o</th>
<th>0 mM</th>
<th>1 mM</th>
<th>2 mM</th>
<th>1.0 μM xestospongin C</th>
<th>2 mM Ca</th>
<th>1.0 mM</th>
<th>20 mM caffeine</th>
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<td>Caffeine</td>
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</table>

Activation Threshold = \( \frac{(F_{SOICR} - F_{min})}{(F_{max} - F_{min})} \times 100\% \)

Termination Threshold = \( \frac{(F_{termi} - F_{min})}{(F_{max} - F_{min})} \times 100\% \)

B  

C  

Online Fig. II
Online Fig. III

A  N-terminal domains

RyR2-WT

\[
\begin{array}{c}
\text{NH}_2 \\
1 \quad 217 \quad 409 \quad 543 \text{ aa} \\
\text{COOH}
\end{array}
\]

Del-Exon-3

Exon-3

\[
\text{NVPPDLSICTFVEQSLSVRALQEMLANTVEKSEG}
\]

310 \quad \beta 4 \quad \alpha 1

B  Del-Exon-3

\[
\text{C'} = 0.25 \text{ mM caffeine; R} = 100 \text{ } \mu \text{M ryanodine; C} = 2.5 \text{ mM caffeine}
\]

C  Immuno-blotting

anti-RyR(34c)


RYR2-WT  Del-Exon-3
Online Fig. IV

A

[Ca^{2+}]_0 (mM)

0  0.1  0.2  0.3  0.5  1  2  10 mM

[Ca^{2+}]

2.5

2.1

HEK293 Cells Expressing RyR2-WT

Fura-2 ratio

0.5

0.9

1.3

1.7

2.1

2.5

Time (second)

0

200

400

600

800

1000

1200

1400

B

[Ca^{2+}]_0 (mM)

0  0.1  0.2  0.3  0.5  1  2  10 mM

[Ca^{2+}]

2.5

2.1

HEK293 Cells Expressing Del-305

Fura-2 ratio

0.5

0.9

1.3

1.7

2.1

2.5

Time (second)

0

200

400

600

800

1000

1200

1400

C

Oscillating cells (%)

0  20  40  60  80

[Ca^{2+}]_0 (mM)

0  0.4  0.8  1.2  1.6  2.0

Del-305

RyR2-WT

* * * * *

D

SOICR amplitude (%)

0  40  80  120

RyR2-WT  Del-305

* *

E

SOICR frequency (%)

0  20  40  60  80  100

RyR2-WT  Del-305

P = 0.20
Online Fig. V

A

\[ F_{\text{max}} (%) \]

- RyR2-WT
- A77V
- R176Q/T250A
- R420W
- L433P
- E189D
- R4496C

B

\[ F_{\text{min}} (%) \]

- RyR2-WT
- A77V
- R176Q/T250A
- R420W
- L433P
- E189D
- R4496C

C

\[ \text{Store capacity} (%) \]

- RyR2-WT
- A77V
- R176Q/T250A
- R420W
- L433P
- E189D
- R4496C