Roles of the NH$_2$-terminal Domains of Cardiac Ryanodine Receptor in Ca$^{2+}$ Release Activation and Termination*

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The NH$_2$-terminal region (residues 1–543) of the cardiac ryanodine receptor (RyR2) harbors a large number of mutations associated with cardiac arrhythmias and cardiomyopathies. Functional studies have revealed that the NH$_2$-terminal region is involved in the activation and termination of Ca$^{2+}$ release. The three-dimensional structure of the NH$_2$-terminal region has recently been solved. It is composed of three domains (A, B, and C). However, the roles of these individual domains in Ca$^{2+}$ release activation and termination are largely unknown. To understand the functional significance of each of these NH$_2$-terminal domains, we systematically deleted these domains and assessed their impact on caffeine- or Ca$^{2+}$-induced Ca$^{2+}$ release and store overload-induced Ca$^{2+}$ release (SOICR) in HEK293 cells. We found that all deletion mutants were capable of forming caffeine- and ryanodine-sensitive functional channels, indicating that the NH$_2$-terminal region is not essential for channel gating. Ca$^{2+}$ release measurements revealed that deleting domain A markedly reduced the threshold for SOICR termination but had no effect on caffeine or Ca$^{2+}$ activation or the threshold for SOICR activation, whereas deleting domain B substantially enhanced caffeine and Ca$^{2+}$ activation and lowered the threshold for SOICR activation and termination. Conversely, deleting domain C suppressed caffeine activation, abolished Ca$^{2+}$ activation and SOICR, and diminished protein expression. These results suggest that domain A is involved in channel termination, domain B is involved in channel suppression, and domain C is critical for channel activation and expression. Our data shed new insights into the structure-function relationship of the NH$_2$-terminal domains of RyR2 and the action of NH$_2$-terminal disease mutations.

The cardiac ryanodine receptor (RyR2)$^4$ is an essential player in excitation-contraction coupling in the heart. It governs the release of Ca$^{2+}$ from the sarcoplasmic reticulum that drives muscle contraction (1, 2). This RyR2-mediated sarcoplasmic reticulum Ca$^{2+}$ release also plays a critical role in the control of heart rhythm (1, 2). Consistent with its fundamental role in cardiac function, naturally occurring mutations in RyR2 are associated with cardiac arrhythmias and cardiomyopathies (2–5). Interestingly, most of the disease-associated RyR2 mutations are clustered in three hot spots in the linear sequence of the channel: the NH$_2$-terminal, central, and COOH-terminal regions (5, 6). Although the functional impact of disease-linked RyR2 mutations has been extensively studied, the molecular basis of actions of these disease mutations is largely unknown. This is in part due to the lack of understanding of the structure-function relationship in the RyR2 channel.

The recently solved crystal structures of the NH$_2$-terminal region of RyR have provided novel insights into the structural basis of disease mechanisms associated with the NH$_2$-terminal mutations (7–14). The three-dimensional structure of the NH$_2$-terminal region of RyR contains three domains: domain A (residues 1–217), domain B (residues 218–409), and domain C (residues 410–543) (9). This NH$_2$-terminal region harbors more than 50 disease mutations. Interestingly, almost all of the disease-causing mutations in this region are located at domain interfaces (9). Docking the NH$_2$-terminal structure into low resolution cryo-electron maps of the RyR1 structure places these NH$_2$-terminal domains at the top of the cytoplasmic assembly, forming a ring structure around the 4-fold axis of the RyR chan-

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‡4 The abbreviations used are: RyR2, cardiac ryanodine receptor; RyR, ryanodine receptor; IP$_6$, inositol 1,4,5-trisphosphate; IP$_7$R, inositol 1,4,5-trisphosphate receptor; SOICR, store overload-induced Ca$^{2+}$ release; SD, suppressor domain; IBC, IP$_7$ binding core; KRH, Krebs-Ringer-Hepes; ER, endoplasmic reticulum; CFP, cyan fluorescent protein; Del, deletion.
nel (9). This central ring structure is connected to the channel pore-forming domain via inner branches (15). Furthermore, this central region has been shown to undergo large conformational changes upon channel activation (15). Based on these observations, it has been hypothesized that disease mutations in the NH2-terminal region destabilize domain interfaces, which in turn alters conformational changes in the NH2-terminal region that are important for channel gating (7, 9–12, 14). Consistent with this hypothesis, NH2-terminal disease mutations have been shown to enhance the activation of the RyR2 channel (16–19). We have recently shown that a naturally occurring deletion of exon 3, corresponding to residues Asn57–Gly91 within domain A in the NH2-terminal region, markedly reduces the threshold at which Ca2+ release terminates (18). However, it is unclear how mutations in the NH2-terminal region of RyR2 alter the activation and/or termination of Ca2+ release.

The structure of the NH2-terminal region of RyR is remarkably similar to that of the inositol 1,4,5-trisphosphate receptor (IP3R) despite considerable differences in their amino acid sequences (9, 20). The IP3R NH2-terminal region is also composed of three domains: the suppressor domain (SD) (residues 1–223), IP3 binding core−β (IBC−β) (residues 224–436), and IBC−α (residues 437–604), corresponding to domains A, B, and C of RyR, respectively (20–23). Functional studies revealed that domains IBC−α and IBC−β form the IP3 binding pocket, whereas the SD inhibits IP3 binding (20–22, 24, 25). Given the structural similarities between the NH2-terminal domains of IP3R and RyR, it is possible that individual NH2-terminal domains of RyR2 may also play a distinct role in channel function. To test this possibility, in the present study, we deleted individual NH2-terminal domains of RyR2 and assessed the impact of these deletions on the activation and termination of Ca2+ release. We found that deletion of domain A markedly delayed the termination of Ca2+ release, whereas deletion of domain B significantly enhanced the activation of Ca2+ release. Deletion of domain C drastically reduced the expression of the channel protein. Our data suggest that individual NH2-terminal domains of RyR2 are involved in distinct roles in channel function.

**EXPERIMENTAL PROCEDURES**

**Construction of NH2-terminal Deletion Mutants of RyR2**

The NH2-terminal deletions in mouse RyR2 were generated by the overlap extension method using PCR (26, 27). Briefly, an Nhel/AflII fragment containing deletion-B (Del-B), Del-C, Del-AB, or Del-ABC was obtained by overlapping PCR and used to replace the corresponding wild type (WT) fragment in the full-length RyR2 cDNA in pcDNA3, which was then subcloned into pcDNA5. An Nhel/AflIII fragment containing Del-A was obtained by overlapping PCR and used to replace the corresponding WT fragment. The sequences of all deletions were confirmed by DNA sequencing.

**Generation of Stable, Inducible Cell Lines Expressing WT and Deletion Mutants of RyR2**

Stable, inducible HEK293 cell lines expressing RyR2 Del-A, Del-B, Del-C, Del-AB, and Del-ABC 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 (flippase recognition target)/TO containing the mutant cDNAs and the pOG44 vector encoding the Flp recombinase in 1:5 ratios using the calcium phosphate precipitation method. The transfected cells were washed with phosphate-buffered saline (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 again with PBS, harvested, and plated on new dishes. After the cells had attached (~4 h), 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 (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 site.

**Caffeine-induced Ca2+ Release in HEK293 Cells**

The free cytosolic Ca2+ concentration in transfected HEK293 cells was measured using the fluorescence Ca2+ 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 WT or deletion mutant RyR2 cDNAs. Cells grown for 18–20 h after transfection were washed four times with PBS and incubated in Krebs-Ringer-Hepes (KRH) buffer 1 (125 mM NaCl, 5 mM KCl, 1.2 mM KH2PO4, 6 mM glucose, and 25 mM HEPES, pH 7.4 with NaOH) without MgCl2 and CaCl2 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 loaded with 10 μM Fluo-3 AM in high glucose Dulbecco's modified Eagle's medium at room temperature for 60 min followed by washing with KRH buffer 1 plus 2 mM CaCl2 and 1.2 mM MgCl2 (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 additions or single additions of various concentrations of caffeine (0.025–5 mM) in an SLM-Amino series 2 luminescence spectrometer with 480-nm excitation at 25 °C (SLM Instruments). For ryanodine sensitivity studies, the RyR2 WT or mutant channels were first sensitized by a relatively low concentration of caffeine (0.1 or 0.25 mM). The caffeine-sensitized channels were then treated with ryanodine (25 μM). The ryanodine-treated channels were further activated by multiple additions of a relatively high concentration of caffeine (1 mM). The peak levels of each caffeine-induced Ca2+ release were determined and normalized to the highest level (100%) of caffeine-induced Ca2+ release for each experiment.

**Single Cell Ca2+ Imaging**

**Cytosolic Ca2+ Measurements—Cytosolic Ca2+ levels in stable, inducible HEK293 cells expressing RyR2 WT or mutants were monitored using single cell Ca2+ imaging and the fluores-
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cent Ca²⁺ indicator dye Fura-2 AM as described previously (16, 28). Briefly, cells grown on glass coverslips for 8–18 h after induction (as indicated) by 1 µg/ml tetracycline (Sigma) were loaded with 5 µM Fura-2 AM in KRH buffer 2 (125 mM NaCl, 5 mM KCl, 6 mM glucose, 1.2 mM MgCl₂, and 25 mM HEPES, pH 7.4 with NaOH) 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) and an inverted microscope (Nikon TE2000-S). The cells were perfused continuously with KRH buffer 2 containing increasing extracellular Ca²⁺ concentrations (0, 0.1, 0.2, 0.3, 0.5, 1.0, and 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 (0.25 frame/s) were captured and analyzed with Compix Simple PCI 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 λ_em = 510 ± 84 nm with a dichroic mirror (410 nm).

Luminal Ca²⁺ Measurements—Luminal Ca²⁺ levels in HEK293 cells expressing RyR2 WT or mutants were measured using single cell Ca²⁺ imaging and the fluorescence resonance energy transfer (FRET)-based endoplasmic reticulum (ER) luminal Ca²⁺-sensitive chameleon protein D1ER as described previously (29, 30). The cells were grown to 95% confluence in a 75-cm² flask, passaged with PBS, and plated in 100-mm-diameter tissue culture dishes at ~10% confluence 18–20 h before transfection with D1ER cDNA using the calcium phosphate precipitation method. After transfection for 24 h, the growth medium was then changed to an induction medium containing 1 µg/ml tetracycline. In intact cell studies, after induction for ~22 h, the cells were perfused continuously with KRH buffer 2 containing various concentrations of CaCl₂ (0, 1, and 2 mM) and tetracaine (1 mM) for estimating the store capacity or caffeine (20 mM) for estimating the minimum store level by depleting the ER Ca²⁺ stores at room temperature (23 °C). In permeabilized cells studies, the cells were first permeabilized by 50 µg/ml saponin (31) in incomplete intracellular-like medium (125 mM KCl, 19 mM NaCl, and 10 mM HEPES, pH 7.4 with KOH) at room temperature (23 °C) for 3–4 min. The cells were then switched to complete intracellular-like medium (incomplete intracellular-like medium plus 2 mM ATP, 2 mM MgCl₂, 0.05 mM EGTA, and 100 mM free Ca²⁺, pH 7.4 with KOH) for 5–6 min to remove saponin. The permeabilized cells were then perfused with various concentrations of Ca²⁺ (0.1, 0.2, 0.4, 1, and 10 µM) followed by tetracaine (1 mM) for estimating the store capacity and caffeine (10 mM) for estimating the minimum store level by depleting the ER Ca²⁺ stores. Images were captured with Compix Simple PCI 6 software every 2 s using an inverted microscope (Nikon TE2000-S) equipped with an S-Fluor 20×/0.75 objective. The filters used for D1ER imaging were λ_ex = 436 ± 20 nm for CFP, λ_ex = 500 ± 20 nm for YFP, λ_em = 465 ± 30 nm for CFP, and λ_em = 535 ± 30 nm for YFP with a dichroic mirror (500 nm). The amount of FRET was determined from the ratio of the light emission at 535 and 465 nm.

Western Blotting

HEK293 cell lines grown for certain periods of time after induction 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 mixture (1 mM benzamidine, 2 µg/ml leupeptin, 2 µg/ml peptatin A, 2 µg/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) (32) and transferred onto nitrocellulose membranes at 90 V for 1.5 h at 4 °C in the presence of 0.01% SDS (33). 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) (1:1,000 dilution) and then incubated with the secondary anti-mouse IgG (heavy and 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 using ImageQuant LAS 4000 (GE Healthcare), analyzed using ImageJ software, and normalized to that of β-actin.

Statistical Analysis

All values shown are mean ± S.E. unless indicated otherwise. To test for differences between two groups, we used unpaired Student’s t tests (two-tailed). A p value <0.05 was considered to be statistically significant.

RESULTS

Construction and Expression of RyR2 NH₂-terminal Deletion Mutants—To understand the role of individual NH₂-terminal domains (A, B, and C) in RyR2 function, we used a deletion approach in which NH₂-terminal domain A (residues 1–217), B (residues 218–409), C (residues 410–543), AB (residues 1–409), or ABC (residues 1–543) was deleted in the full-length RyR2 (Fig. 1A). The boundary of each domain was selected based on the three-dimensional structure of the NH₂-terminal region (residues 1–543) of RyR (9, 13). These deletion constructs were generated by site-directed mutagenesis and transiently expressed in HEK293 cells. Immunoblotting analysis revealed that the expression level of Del-A was reduced, whereas the expression level of Del-B was increased compared with that of RyR2 WT. The expression levels of Del-AB and WT were comparable. Conversely, the expression level of Del-C or Del-ABC was markedly reduced compared with that of WT (Fig. 1, B and C). Thus, deletion of domain C considerably impaired the expression of the RyR2 protein.
The NH$_2$-terminal Deletion Mutants of RyR2 Form Caffeine- and Ryanodine-sensitive Functional Ca$^{2+}$ Release Channels—

We first determined whether these NH$_2$-terminal deletion mutants are functional. HEK293 cells were transfected with RyR2 WT or Del-A, Del-B, Del-C, Del-AB, or Del-ABC mutants. The transfected HEK293 cells were loaded with the fluorescent Ca$^{2+}$/H$^{11001}$ indicator dye Fluo-3, AM, and the cytosolic Ca$^{2+}$/H$^{11001}$ level was monitored by using a luminescence spectrometer before and after the addition of caffeine or ryanodine. The RyR2 WT or mutant channels were first sensitized by a relatively low concentration of caffeine (0.1 or 0.25 mM). The caffeine-sensitized channels were then treated with ryanodine (25/9262 M). The ryanodine-treated channels were further activated by multiple additions of a relatively high concentration of caffeine (1 mM). As shown in Fig. 2, the ryanodine-untreated (-ryanodine) HEK293 cells expressing RyR2 WT responded to repeated stimulations by submaximal concentrations of caffeine, each resulting in partial Ca$^{2+}$/H$^{11001}$ release (Fig. 2A, top panel). In contrast, WT-expressing HEK293 cells treated with ryanodine (25/9262 M) (+ryanodine) only responded to the first subsequent stimulation by caffeine (Fig. 2A, bottom panel). It is known that ryanodine only binds to the open RyR channel and that the binding of ryanodine converts the channel to a mainly fully open state (34, 35). Thus, in the presence of ryanodine, the caffeine-activated channels would be modified by ryanodine into a fully activated state, leading to a depletion of the intracellular Ca$^{2+}$/H$^{11001}$ store. Therefore, subsequent additions of caffeine yielded little or no Ca$^{2+}$/H$^{11001}$ release in ryanodine-treated cells. However, in the absence of ryanodine, a submaximal concentration of caffeine induced only partial Ca$^{2+}$/H$^{11001}$ release, a phenomenon known as quantal Ca$^{2+}$/H$^{11001}$ release (36–38). Importantly, similar to cells expressing RyR2 WT, HEK293 cells expressing Del-A, Del-B, Del-C, Del-AB, or Del-ABC all exhibited quantal Ca$^{2+}$/H$^{11001}$ release induced by submaximal concentrations of caffeine in the absence of ryanodine (Fig. 2, B–F, top panels). Ryanodine pretreatment rendered all these deletion mutant cells unresponsive to repeated caffeine stimulations (Fig. 2, B–F, bottom panels). These observations indicate that all these NH$_2$-terminal deletion mutants are able to form caffeine- and ryanodine-sensitive functional Ca$^{2+}$/H$^{11001}$ release channels. It is noted that there were immediate drops in the fluorescence level after additions of caffeine. This is due to fluorescence quenching by caffeine (39, 40).

Effect of NH$_2$-terminal Deletions on the Sensitivity of Caffeine Activation of RyR2—We next assessed whether NH$_2$-terminal deletions affect the sensitivity of the RyR2 channel to caffeine...
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activation. To this end, we determined the response of each of these deletion mutants to activation by increasing concentrations of caffeine. As shown in Fig. 3, the level of Ca²⁺ release in HEK293 cells transfected with RyR2 WT increased progressively with each consecutive addition of caffeine (from 0.05 to 1.0 mM) and then decreased with further additions of caffeine (2.5 and 5 mM) likely due to the depletion of the intracellular Ca²⁺ stores by the prior additions of caffeine (0.025–1.0 mM) (Fig. 3A). The response to caffeine activation of HEK293 cells expressing the Del-A (Fig. 4B) and Del-AB (Fig. 4E) mutants exhibited a similar fraction of cells that displayed spontaneous Ca²⁺ oscillations as compared with WT cells (Fig. 4G and H). Conversely, Del-B caused a significant leftward shift in caffeine response (Fig. 3C and G), whereas Del-C (Fig. 3D) and Del-ABC (Fig. 3F) resulted in a significant rightward shift (Fig. 3G). Del-AB slightly inhibited the caffeine response (Fig. 3E and G). Collectively, these data indicate that Del-A has no significant effect on the activation of RyR2 by caffeine and Del-B enhances it, whereas Del-C reduces it.

NH₂-terminal Deletions of RyR2 Alter the Propensity for SOICR—Disease-causing mutations in the NH₂-terminal region of RyR2 have been shown to increase the propensity for arrhythmogenic spontaneous Ca²⁺ release during store Ca²⁺ overload, a process also known as store overload-induced Ca²⁺ release (SOICR). It is of interest to assess whether deletion of individual NH₂-terminal domains of RyR2 alters the propensity for SOICR. To this end, we generated stable, inducible HEK293 cell lines expressing the RyR2 WT and Del-A, Del-B, Del-C, Del-AB, and Del-ABC mutants. These HEK293 cells were perfused with elevating extracellular Ca²⁺ (0–2.0 mM) to induce spontaneous Ca²⁺ oscillations as described previously (16, 28). The resultant SOICR was then monitored by using a fluorescence Ca²⁺ indicator, Fura-2 AM, and single cell Ca²⁺ imaging. As shown in Fig. 4, HEK293 cells expressing the Del-A (Fig. 4B) and Del-AB (Fig. 4E) mutants exhibited a similar fraction of cells that displayed spontaneous Ca²⁺ oscillations as compared with WT cells (Fig. 4G and H). In contrast, the Del-B (Fig. 4C) mutant-expressing cells exhibited an increased fraction of oscillating cells (p < 0.01) as compared with WT (Fig. 4G). Conversely, HEK293 cells expressing Del-C (Fig. 4D) and Del-ABC (Fig. 4F) showed a caffeine response but no SOICR at all (Fig. 4H). It is important to note that the enhanced SOICR

FIGURE 2. The NH₂-terminal deletion mutants of RyR2 form caffeine- and ryanodine-sensitive Ca²⁺ release channels. HEK293 cells were transfected with RyR2 WT (A), Del-A (B), Del-B (C), Del-C (D), Del-AB (E), or Del-ABC (F) cDNA. Fluorescence intensity of Fluo-3-loaded cells was measured continuously after addition of 0.1 or 0.25 mM caffeine, DMSO, or 25 μM ryanodine followed by three doses of 1 mM caffeine (caff) (n = 3–4). Arrows indicate the presence of Ca²⁺ release in ryanodine-untreated cells and the absence of Ca²⁺ release in ryanodine-treated cells. Note that the immediate drops in fluorescence after the addition of caffeine were due to fluorescence quenching by caffeine.
Del-C or Del-ABC is unlikely due to the reduced expression level of these mutants as SOICR still occurred in WT-expressing HEK293 cells when the expression of the WT protein was reduced to a level similar to or less than that of Del-C or Del-ABC (Fig. 5, B and C). Thus, these results demonstrate that Del-A has no major impact on SOICR and Del-B enhances the propensity for SOICR, whereas Del-C abolishes SOICR.

Effect of NH2-terminal Deletions on the SOICR Activation and Termination Thresholds—To assess the impact of NH2-terminal deletions on the activation and termination threshold for SOICR, we monitored the ER luminal Ca2+ dynamics in HEK293 cells using a FRET-based ER luminal Ca2+-sensing protein, D1ER (29, 30). As shown in Fig. 6, elevating extracellular Ca2+ from 0 to 2 mM induced spontaneous ER Ca2+ oscillations in RyR2 WT-expressing HEK293 cells (depicted as downward deflections of the FRET signal). SOICR occurred when the ER luminal Ca2+ content increased to a threshold level (Fsoicr) and terminated when the ER luminal Ca2+ content fell to another threshold level (Fterm) (Fig. 6A). The ER luminal Ca2+ dynamics in Del-A-, Del-B-, and Del-AB-expressing cells during SOICR is shown in Fig. 6, B, C, and D. The Del-A and Del-AB mutations markedly reduced the SOICR termination threshold (34.7 ± 2.3% in Del-A and 38.0 ± 2.9% in Del-AB versus 59.4 ± 1.0% in WT) (p < 0.01) but had no significant effect on the SOICR activation threshold (93.2 ± 0.4% in Del-A and 92.6 ± 0.7% in Del-AB versus 93.1 ± 0.5% in WT). As a result, the fractional Ca2+ release during SOICR (activation threshold – termination threshold) in Del-A or Del-AB mutant cells (58.5 ± 2.5% in Del-A and 54.7 ± 3.6% in Del-AB) was significantly increased compared with that of the WT cells (33.7 ± 0.9%) (p < 0.01) (Fig. 6, E, F, and G). Conversely, the Del-B mutation substantially decreased the SOICR activation threshold (80.0 ± 1.0 versus 93.1 ± 0.5% in WT) (p < 0.01), which is in agreement with its increased SOICR propensity (Fig. 4). The Del-B mutation also significantly reduced the SOICR termination threshold (41.6 ± 1.4 versus 59.4 ± 1.0% in WT) (p < 0.01). The fractional Ca2+ release in Del-B mutant cells (38.4 ± 0.5%) was also significantly different from that of WT cells (33.7 ± 0.9%) (p < 0.01) (Fig. 6, E, F, and G). It should be noted that there was no significant difference in the store capacity (Fmax – Fmin) between RyR2 WT and deletion mutant cells (Fig. 6H). Consistent with their lack of SOICR activity (Fig. 4), no ER luminal Ca2+ oscillations were observed in HEK293 cells expressing Del-C or Del-ABC (not shown). Furthermore, SOICR did not occur in control HEK293 cells expressing no RyR2, and SOICR was not affected by the IP3R inhibitor xestospongin C (18), indicating that SOICR is mediated by RyR2. Collectively, these data indicate that deletion of domain A only affects the termination threshold for SOICR, whereas deletion of domain B alters both the SOICR activation and termination thresholds.

Effect of NH2-terminal Deletions on the Cytosolic Ca2+ Regulation of Ca2+ Release—To determine the impact of NH2-terminal deletions on the regulation of Ca2+ release by cytosolic Ca2+, we measured the steady state ER Ca2+ level in permeabilized HEK293 cells (31) expressing the RyR2 WT or deletion mutants in the presence of increasing cytosolic Ca2+ concentrations (0.1–10 μM). The steady state ER Ca2+ level likely

FIGURE 3. Effect of NH2-terminal deletions on the sensitivity of caffeine activation of RyR2. HEK293 cells were transfected with RyR2 WT (A), Del-A (B), Del-B (C), Del-C (D), Del-AB (E), or Del-ABC (F). Fluorescence intensity of the Fluo-3-loaded transfected cells before and after additions of increasing concentrations of caffeine (0.025–5 mM) was monitored continuously. G, Ca2+ release-cumulative caffeine concentration relationships in HEK293 cells transfected with RyR2 WT and NH2-terminal deletion mutants. The amplitude of each caffeine peak was normalized to that of the maximum peak for each experiment. Data shown are mean ± S.E., and error bars represent S.E. (n = 7) (∗, p < 0.05 versus WT).
reflects the equilibrium between ER Ca\(^{2+}\) release and Ca\(^{2+}\) uptake. As shown in Fig. 7, elevating cytosolic Ca\(^{2+}\) reduced the steady state ER Ca\(^{2+}\) level in permeabilized HEK293 cells expressing RyR2 WT in a concentration-dependent manner most likely due to increased Ca\(^{2+}\) release as a result of enhanced cytosolic Ca\(^{2+}\) activation of the RyR2 channel (Fig. 7, A and G). HEK293 cells expressing Del-A showed a response to cytosolic Ca\(^{2+}\) similar to that seen with the WT cells (Fig. 7, B and G). Conversely, HEK293 cells expressing Del-B showed a very different response to cytosolic Ca\(^{2+}\) (Fig. 7, C and G). The steady state ER Ca\(^{2+}\) level at resting cytosolic Ca\(^{2+}\) (100 nM) in Del-B cells was markedly reduced as compared with that in WT cells (42.2 ± 0.03% in Del-B versus 73.3 ± 0.01% in WT) (p < 0.001). This suggests that Del-B may enhance cytosolic Ca\(^{2+}\) activation of RyR2. Increasing cytosolic Ca\(^{2+}\) from 100 to 200 nM reduced the steady state ER Ca\(^{2+}\) level in Del-B cells similarly to that seen in WT cells. However, different from that seen in WT cells, further elevation in cytosolic Ca\(^{2+}\) concentration to 400 nM, 1 µM, and 10 µM increased the steady state ER Ca\(^{2+}\) level in Del-B cells (Fig. 7, C and G). These observations suggest that Del-B may also enhance cytosolic Ca\(^{2+}\)-dependent inactivation of RyR2. Cells expressing Del-AB exhibited reduced steady state ER Ca\(^{2+}\) level at 200 nM cytosolic Ca\(^{2+}\) as compared with that in WT cells (Fig. 7, D and G), suggesting that Del-AB is able to sensitize RyR2 to cytosolic Ca\(^{2+}\) activation. However, Del-AB cells displayed increased steady state ER Ca\(^{2+}\) levels at 1 and 10 µM cytosolic Ca\(^{2+}\) as compared with those in WT cells (Fig. 7, D and G), suggesting that Del-AB may also sensitize RyR2 to cytosolic Ca\(^{2+}\)-dependent inactivation. The steady state ER Ca\(^{2+}\) level in HEK293 cells expressing Del-C or Del-ABC did not respond to increasing cytosolic Ca\(^{2+}\) concentrations (100 nM–100 µM) and was only slightly reduced upon caffeine addition (Fig. 7, E and F). These data indicate that Del-C and Del-ABC diminish the cytosolic Ca\(^{2+}\) response and impair caffeine activation of RyR2. Taken together, our results suggest that the NH\(_2\)-terminal domains play an important role in cytosolic Ca\(^{2+}\) activation and inactivation of RyR2.

**DISCUSSION**

The NH\(_2\)-terminal region of RyR2 is a hot spot of naturally occurring mutations associated with cardiac arrhythmias and cardiomyopathies (5, 6). We have recently shown that disease-
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causing RyR2 mutations in the NH2-terminal region alter the activation and/or termination of Ca2+ release (18). However, how the NH2-terminal region regulates the activation and termination of Ca2+ release and how mutations in this region impair these processes are unclear. The NH2-terminal region of RyR2 encompasses three well defined domains: domain A (residues 1–217), domain B (residues 218–409), and domain C (residues 410–543) (9, 13). In the present study, we assessed the role of these individual domains in Ca2+ release activation and termination. Our data indicate that domain A is an important determinant of Ca2+ release termination, whereas domains B and C play a critical role in Ca2+ release activation. These results provide novel insights into the structure-function relationship of the NH2-terminal domains of RyR2 and the understanding of disease mechanisms.

The NH2-terminal domains (A, B, and C) of RyR have been mapped to the central region around the 4-fold symmetry axis of the channel. There are extensive domain-domain interactions in the NH2-terminal region. Domains A and B through intra- and intersubunit interactions form a central ring structure at the top of the cytoplasmic assembly (9). This ring structure is connected to the transmembrane domain of the channel via some central electron-dense columns and to the peripheral “clamp” region via domain C (9, 15). To gain insights into the functional significance of these domain-domain interactions, we determined the role of each NH2-terminal domain in channel function. We found that removing domain A (Del-A) markedly reduced the threshold for Ca2+ release termination, suggesting that domain A is involved in the termination of Ca2+ release. Hence, it is possible that mutations that alter interactions with domain A may affect Ca2+ release termination. We have recently shown that cardiomyopathy-associated RyR2 mutations A77V and R176Q and exon 3 deletion markedly reduce the termination threshold for Ca2+ release (9). Interestingly, these mutations are located in the domain interface between domain A and the central electron-dense columns (also known as interface 4) (9, 15), suggesting that interface 4 may be involved in Ca2+ release termination.

The intra- and intersubunit interactions between domains A and B are believed to be important for stabilizing the closed state of the channel. Disease mutations located in interfaces between domains A and B would weaken these interactions, thus facilitating channel opening (7–14). Del-A would be expected to remove both intra- and intersubunit interactions between domains A and B, leading to destabilization of the closed state and channel activation. Surprisingly, Del-A did not significantly affect channel activation. The sensitivity to activation by caffeine or Ca2+2+, the propensity for SOICR, or the SOICR activation threshold of the Del-A mutant were not significantly different from those of the WT. Conversely, deleting domain B (Del-B) significantly enhanced the sensitivity of RyR2 to caffeine, increased cytosolic Ca2+ activation and the propensity for SOICR, and reduced the threshold for SOICR activation. These observations suggest that disease mutations located in interfaces between domains A and B may enhance channel activity by affecting the function of domain B. It should be noted that Del-B also reduced the threshold for Ca2+ release termination, implying that domain B may also be involved in Ca2+ release termination directly or indirectly via interaction with domain A. Furthermore, Del-B also altered the cytosolic Ca2+-dependent inactivation of RyR2. Thus, domain B plays an important role in stabilizing the closed state of the RyR2 channel.

Del-A or Del-B resulted in gain of function either by delaying Ca2+ release termination or by sensitizing Ca2+ release activation. In contrast, deleting domain C (Del-C) suppressed caffeine activation of RyR2 and completely abolished cytosolic Ca2+ activation and SOICR. Furthermore, unlike Del-A or Del-B, Del-C drastically reduced the protein expression of RyR2. It should be noted that reducing the expression level of WT similar to or less than that of Del-C did not abolish SOICR in WT-expressing cells. Thus, the lack of SOICR in Del-C-expressing cells is unlikely due solely to their reduced expression level. These observations suggest that domain C is required for channel activation and expression.

Docking the crystal structure of the NH2-terminal domains of RyR1 in the open and closed states of the cryo-EM structure of RyR1 revealed that the opening of the channel is associated with large conformational changes in the NH2-terminal domains (14, 15). These have been confirmed in recent FRET-based studies using conformational probes inserted into the NH2-terminal domains (41). During the transition from the closed to the open state, the triangle-like structure formed by domains A, B, and C within the same subunit appear to be tilted upward and outward around a hinge located near domain C. As such, domains A and B rotated ~7–8 Å, whereas domain C rotated ~4 Å (14). Hence, part of domain C may act as a hinge and play an important structural role in mediating and controlling the movement of domains A and B during channel gating. Therefore, deleting domain C may affect the structure/folding
of this region, which may contribute to the markedly reduced expression level of the Del-C or Del-ABC mutant protein.

We also determined the impact of deleting the first two NH$_2$-terminal domains (Del-AB) or all three domains (Del-ABC) on Ca$^{2+}$ release. Del-AB substantially reduced the termination threshold for Ca$^{2+}$ release, which is consistent with the impact of Del-A or Del-B on Ca$^{2+}$ release termination. Del-AB also enhanced cytosolic Ca$^{2+}$-dependent activation and inactivation of RyR2 similarly to Del-B. However, unlike Del-B, Del-AB did not significantly affect the activation of SOICR. One would expect that Del-AB would have the combined effect of Del-A and Del-B, but this is not the case. The reason for this seemingly contradictory data is unclear. It is possible that the stimulating effect of Del-B on Ca$^{2+}$ release may require the presence of domain A. Del-ABC markedly inhibited caffeine activation, reduced protein expression, and completely abolished cytosolic Ca$^{2+}$ activation of RyR2 and SOICR, which are similar to the effects of Del-C, suggesting that Del-C has a dominant impact on channel function. These results also demonstrate that the NH$_2$-terminal region is not essential for the gating of the RyR2 channel, although it plays an important role in regulating it.

Crystal structures of the NH$_2$-terminal region of the IP$_3$R have also been solved recently. The overall structure of the NH$_2$-terminal region of IP$_3$R is very similar to that of RyR (20–22). As with RyR, IP$_3$R contains three NH$_2$-terminal domains: the SD, IBC-β, and IBC-α, corresponding to domains A, B, and C in RyR, respectively. The functional role of individual NH$_2$-terminal domains of IP$_3$R has been well studied. IBC-β and IBC-α are involved in IP$_3$ binding, whereas the SD is believed to clamp domains IBC-β and IBC-α in a conformation with reduced affinity for IP$_3$, thus acting as a suppressor of IP$_3$ binding (20–25). Interestingly, it has recently been shown that the IP$_3$R SD and domain A of RyR are functionally interchangeable (20). An RyR-IP$_3$R chimeric channel in which the SD in the full-length IP$_3$R was replaced with domain A of RyR was still gated by IP$_3$. These observations suggest that the SD of IP$_3$R and domain A of RyR may share similar functional roles. However, it is important to know that deletion of the SD in IP$_3$R completely abolished IP$_3$-induced Ca$^{2+}$ release (25), whereas Del-A or even the deletion of the entire NH$_2$-terminal region (Del-ABC) retained caffeine-induced Ca$^{2+}$ release. Thus, the respective NH$_2$-terminal region plays a very different role in IP$_3$-de-
pendent gating of IP₃R and the caffeine-induced activation of RyR. These observations also suggest that the mechanism of IP₃-induced opening of IP₃R differs from that of caffeine-induced opening of RyR.

In summary, our data show that domain A is important for Ca²⁺ release termination but not for Ca²⁺ release activation. Conversely, domain B is involved in stabilizing the closed state of the channel, which is important for both activation and termination of Ca²⁺ release, whereas domain C is important for channel activation. RyR2 lacking domain AB remains functional, indicating that it is not essential for channel gating. Thus, domain AB plays a regulatory role in channel gating. These results provide new insights into the function of the NH₂-terminal domains and the disease mechanism of mutations associated with the NH₂-terminal region.

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