Defective ryanodine receptor N-terminus inter-subunit interaction is a common mechanism in neuromuscular and cardiac disorders

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The ryanodine receptor (RyR) is a homotetrameric channel mediating sarcoplasmic reticulum Ca²⁺ release required for skeletal and cardiac muscle contraction. Mutations in RyR1 and RyR2 lead to life-threatening malignant hyperthermia episodes and ventricular tachycardia, respectively. In this brief report, we use chemical cross-linking to demonstrate that pathogenic RyR1 R163C and RyR2 R169Q mutations reduce N-terminus domain (NTD) tetramerization. Introduction of positively-charged residues (Q168R, M399R) in the NTD-NTD inter-subunit interface normalizes RyR2-R169Q NTD tetramerization. These results indicate that perturbation of NTD-NTD inter-subunit interactions is an underlying molecular mechanism in both RyR1 and RyR2 pathophysiology. Importantly, our data provide proof of concept that stabilization of this critical RyR1/2 structure-function parameter offers clear therapeutic potential.

KEYWORDS
amino-terminus, catecholaminergic polymorphic ventricular tachycardia, malignant hyperthermia, inter-subunit interaction, tetramerization, ryanodine receptor (RyR)

Introduction

Myocyte contraction is effected by a transient rise in the intracellular Ca²⁺ concentration mediated by the ryanodine receptor (RyR), the Ca²⁺ release channel of the sarcoplasmic reticulum. It is triggered by an action potential that depolarizes the plasma membrane to activate the voltage-gated Ca²⁺ channel, also known as dihydropyridine receptor (DHPR). In adult mammalian heart, the ensuing small Ca²⁺ influx activates the RyR to result in a much larger Ca²⁺ release from the sarcoplasmic reticulum Ca²⁺ store (Bers, 2002). This process, termed Ca²⁺-induced Ca²⁺ release, is also operational in skeletal muscle, but its contribution to contraction of adult mammalian muscles appears negligible (Rios, 2018). Unlike the heart, skeletal muscle is able to contract in the absence of extracellular Ca²⁺ influx due to physical coupling between
DHPR and RyR, where the action potential-evoked conformation change in DHPR is mechanically transmitted to open the RyR (Hernandez-Ochoa and Schneider, 2018). The RyR, a homotetramer of ~2.2 MDa, is the largest ion channel known to exist (Zissimopoulos and Lai, 2007). There are three mammalian isoforms, RyR1, the predominant type in skeletal muscle, RyR2, the exclusive type in cardiac muscle, and RyR3, which is expressed at low levels in skeletal and smooth muscles. The three RyR isoforms have ~70% peptide sequence homology and very similar geometry. Abnormal SR Ca\(^{2+}\) release due to missense mutations in RyR1 and RyR2 results in neuromuscular (e.g., malignant hyperthermia, MH) and cardiac disease (e.g., catecholaminergic polymorphic ventricular tachycardia, CPVT), respectively (Hernandez-Ochoa et al., 2016; Fowler and Zissimopoulos, 2022). Mutations are found throughout the RyR peptide sequence, but tend to concentrate on fivestructural domains, namely, the N-terminus domain (NTD), bridging solenoid B, core solenoid, transmembrane domain and C-terminus domain (Fowler and Zissimopoulos, 2022). Neighboring NTDs from the four RyR subunits interact with each other to promote channel closure (Tung et al., 2010; Zissimopoulos et al., 2013; Zissimopoulos et al., 2014). Notably, pathogenic RyR1 and RyR2 mutations impair NTD-NTD inter-subunit interactions and result in hyperactive and leaky channels (Iyer et al., 2020; Seidel et al., 2021; Seidel et al., 2015; Xiong et al., 2018; Zheng and Liu, 2017).

The aim of this study was two-fold. First, to empirically assess whether defective RyR1 NTD tetramerization is involved in MH. Second, to explore targeted amino acid modification for potential to restore RyR2 NTD tetramerization in CPVT.

Materials and methods

Materials

The human embryonic kidney (HEK) 293 cell line was obtained from ATCC® (CRL-1573), mammalian cell culture reagents from Thermo Scientific, electrophoresis equipment and reagents from Bio-Rad, enhanced chemiluminescence detection kit from Thermo Scientific, mouse anti-c-Myc (9E10) from Santa Cruz Biotechnology, goat anti-mouse IgG conjugated with horseradish peroxidase from Abcam, DNA restriction endonucleases from New England Biolabs, Pfu DNA polymerase from Promega, site-directed mutagenesis kit (QuikChange II XL) from Agilent Technologies, oligonucleotides and all other reagents from Sigma.

Plasmid construction

The plasmids encoding for wild-type rabbit RyR1 and human RyR2 N-terminal constructs tagged with the cMyc epitope at the N-terminus have been described previously (Zissimopoulos et al., 2014; Zissimopoulos et al., 2013). Desired missense mutations were generated using the site-directed mutagenesis QuikChange II XL kit and appropriate primers as recommended by the supplier. All plasmid constructs were verified by direct DNA sequencing.

Results

MH mutation R163C disrupts RyR1 NTD tetramerization

We have previously shown that the CPVT R176Q mutation disrupts NTD tetramerization to produce a hyperactive channel (Seidel et al., 2021). The equivalent residue in human RyR1, R163, is frequently mutated in individuals susceptible to MH. Two different substitutions have been reported, R163C and R163L (Table 1). We chose to study the R163C variant because its gain of function characteristics have been studied extensively in vivo and in vitro (Tong et al., 1997; Avila and Dirksen, 2001; Yang et al., 2003; Yang et al., 2006; Feng et al., 2011; Chen et al., 2018; Iyer et al., 2020).

R164C, the corresponding mutation in the rabbit isoform, was introduced by site-directed mutagenesis in rabbit RyR1 N-terminal residues 1–915 tagged with the cMyc peptide epitope (NT\(^{\text{WT}}\)RyR1-R164C\(^{\text{WT}}\)). NT\(^{\text{WT}}\)RyR1-WT and NT\(^{\text{WT}}\)RyR1-R164C\(^{\text{WT}}\) were expressed
in mammalian HEK293 cells with an apparent molecular weight of ~100 kDa as a monomer (Figure 1A). Chemical cross-linking was carried out using glutaraldehyde, a homo-bi-functional reagent with two aldehyde groups that react with the free amino group in the side chain of lysine residues to create stable covalent bonds. Glutaraldehyde does not induce tetramerization but merely cross-links pre-existing tetramers which could then be preserved despite protein denaturation during SDS-PAGE. Cross-linking of NTRyR1−WT and subsequent Western blotting using Ab cMyc revealed the existence of the tetrameric species (Figure 1A), as previously reported (Zissimopoulos et al., 2014). Cross-linking of NTRyR1-R164C also demonstrated the presence of the tetramer, which increased in a time-dependent manner, however its relative abundance was low. Collective data (n = 7) following densitometry analysis indicated that the R164C mutation results in significant reduction (by 51% at 60 min) of the tetramer compared to WT (Figure 1B).

**Rescue of defective RyR2 NTD tetramerization in CPVT**

We have previously shown that RyR2 NTD tetramerization is mediated by the interaction between the β8-β9 (amino acids

| RyR1/2 mutation | Disease | First report | Defective N-terminal inter-subunit interaction | References |
|-----------------|---------|--------------|-----------------------------------------------|------------|
| Q155K           | MH      | Robinson et al., Hum Mutat, 2006 | Cryo-electron microscopy | Iyer et al., Sci Adv, 2020 |
| R156K           | MH      | Galli et al., Hum Mutat, 2006 | Chemical cross-linking | Present study |
| R156W           | Myopathy | Amburgey et al., Orphanet J Rare Dis, 2013 | Molecular dynamics simulations | Xiong et al., J Mol Cell Cardiol, 2018 |
| G159E           | Myopathy | Witting et al., Neurol Genet, 2017 | Chemical cross-linking | Present study |
| E160G           | CCD     | Shepherd et al., J Med Genet, 2004 | Molecular dynamics simulations | Xiong et al., J Mol Cell Cardiol, 2018 |
| R163C           | CCD & MH | Quane et al., Nat Genet, 1993 | Chemical cross-linking | Present study |
| R163L           | MH      | Monnier et al., Hum Mutat, 2005 | Molecular dynamics simulations | Xiong et al., J Mol Cell Cardiol, 2018 |
| G165R           | MH      | Monnier et al., Hum Mutat, 2005 | Chemical cross-linking | Present study |
| D166N           | MH      | Rueffert et al., Acta Anaesthesiol Scand, 2002 | Molecular dynamics simulations | Xiong et al., J Mol Cell Cardiol, 2018 |
| D166G           | MH      | Robinson et al., Hum Mutat, 2006 | Chemical cross-linking | Present study |
| H382N           | MH      | Broman et al., Br J Anaesth, 2009 | Molecular dynamics simulations | Seidel et al., Cardiovasc Res, 2021 |
| P164S           | CPVT    | Choi et al., Circulation, 2004 | Molecular dynamics simulations | Xiong et al., J Mol Cell Cardiol, 2018 |
| A165D           | CPVT    | Xiong et al., J Mol Cell Cardiol, 2018 | Chemical cross-linking | Present study |
| S166C           | Long QT syndrome | Shigemizu et al., PLoS One, 2015 | Molecular dynamics simulations | Xiong et al., J Mol Cell Cardiol, 2018 |
| R169L           | CPVT    | Ohno et al., PLoS One, 2015 | Chemical cross-linking | Present study |
| R169Q           | CPVT    | Hsueh et al., Int J Cardiol, 2006 | Molecular dynamics simulations | Xiong et al., J Mol Cell Cardiol, 2018 |
| G172E           | CPVT    | Shimamoto et al., Heart, 2022 | Molecular dynamics simulations | Xiong et al., J Mol Cell Cardiol, 2018 |
| E173G           | CPVT    | Shimamoto et al., Heart, 2022 | Chemical cross-linking | Present study |
| R176Q           | ARVD2& CPVT | Tiso et al., Hum Mol Genet, 2001 | Molecular dynamics simulations | Seidel et al., Cardiovasc Res, 2021 |
| G178A           | CPVT    | Ohno et al., PLoS One, 2015 | Chemical cross-linking | Present study |
| D179N           | CPVT    | Kawata et al., Circ J, 2016 | Molecular dynamics simulations | Xiong et al., J Mol Cell Cardiol, 2018 |
| D400H           | SUD     | Tester et al., Mayo Clin Proc, 2012 | Chemical cross-linking | Present study |
| D401G           | SUD     | Ststatt et al., Int J Legal Med, 2016 | Molecular dynamics simulations | Seidel et al., Cardiovasc Res, 2021 |
165–179) and β23–β24 loops (amino acids 395–402) (Seidel et al., 2021) (Figure 2A). The experiments described above with RyR1 R163C as well as those with the equivalent residue in RyR2, R176Q (Seidel et al., 2021), demonstrate the involvement of NTD self-association and specifically, of the β8–β9 loop in RyR1/2 pathophysiology. To extend our findings to other β8–β9 loop CPVT mutations (Table 1), we assessed the impact of R169Q on RyR2 NTD tetramerization.

R169Q was introduced by site-directed mutagenesis in human RyR2 N-terminal residues 1–906 tagged with the cMyc peptide epitope (NTR169Q). NTWT and NTR169Q were expressed in mammalian HEK293 cells, subjected to chemical cross-linking using glutaraldehyde, and analyzed by Western blotting using AbcMyc. Both NTWT and NTR169Q were capable of tetramer formation but to quantitatively different extents. Collective data (n ≥ 7) following densitometry analysis indicated that the R169Q mutation results in significant reduction (by 49% at 60 min) of the tetramer compared to WT (Figure 2B; Table 2).

We next sought to genetically manipulate the RyR2 peptide sequence in order to normalize NTD tetramerization that was impaired by the R169Q mutation. Candidate amino acids are the ones that form the inter-subunit interface between the β8–β9 and β23–β24 loops, as indicated by the recent high-resolution electron cryo-microscopy structures of RyR1/2 in the closed and open configurations (des Georges et al., 2016; Peng et al., 2016). Specifically, the following pairs of closely apposed residues can be targeted: Q168-D400, D179-M399, D180-H398, D180-M399. We therefore hypothesized that the following two manipulations may reinforce NTβ169Q tetramerization (Figure 2B): 1. Substitution of Q168 for arginine, a basic amino acid with a long side chain, which may increase the electrostatic interaction with the negatively-charged side chain of D400, 2. Substitution of M399 for arginine, which may increase the electrostatic interaction with both acidic residues D179 and D180.

Initially, we tested whether Q168R and M399R affect RyR2 NTD tetramerization. As shown in Figure 2C, NTR169Q+Q168R and NTR169Q+M399R displayed higher tetramer formation (77% and 84% of WT at 60 min, respectively) compared to NTR169Q (51% of WT at 60 min). Although NTR169Q+Q168R and NTR169Q+M399R tetramer formation did not reach statistical significance relative to NTβ169Q, it was also not significantly different to NTWT (Table 2). These results indicate that the engineered
Q168R and M399R substitutions partially restore NTD tetramerization to RyR2-R169Q.

Discussion

Numerous RyR1 and RyR2 mutations associated with MH and CPVT, respectively, have been functionally characterized with the vast majority resulting in gain-of-function channels (Hernandez-Ochoa et al., 2016; Fowler and Zissimopoulos, 2022). However, how pathogenic RyR mutations function at the molecular or structural level, i.e., what is the defective RyR regulatory mechanism(s), is poorly understood. Here, we demonstrate that perturbed NTD tetramerization is a common molecular mechanism operating in RyR1 and RyR2 genetic disease.

RyR1 R163C is one of the most studied MH mutations in heterologous (HEK293) and homologous expression systems (dyspedic myotubes) as well as in heterozygous knockin mice. Results obtained from single channel recordings, [3H] ryanodine binding and Ca2+ imaging indicate enhanced basal activity, increased sensitivity to Ca2+ activation, increased sensitivity to pharmacological activators, and elevated resting cytosolic Ca2+ concentration (Tong et al., 1997; Avila and Dirksen, 2001; Yang et al., 2003; Yang et al., 2006; Feng et al., 2011; Chen et al., 2018; Iyer et al., 2020). Here, we used chemical cross-linking to demonstrate that R163C impairs RyR1 NTD tetramerization (Figure 1). Our biochemical findings are in agreement with the 3D structure of RyR1-R163C, which was recently solved at near-atomic resolution (Iyer et al., 2020). R163C was found to cause a shift and rotation in the NTD that resulted in increased NTD-NTD inter-subunit distance. This stabilized the NTD interaction with the core solenoid, which in turn altered the high-affinity Ca2+-binding site. Thus, RyR1-R163C is inherently hyperactive and leaky primarily due to defective N-terminal inter-subunit interactions.
RyR2 R169Q is a gain-of-function CPVT mutation as indicated by higher propensity for spontaneous Ca\(^{2+}\) oscillations in HEK293 cells and increased Ca\(^{2+}\)-dependent \(^{3}H\)ryanodine binding (Nozaki et al., 2020). Our chemical cross-linking analysis demonstrates that R169Q impairs RyR2 NTD tetramerization (Figure 2; Table 2), similar to our previous observation with the R176Q mutation (Seidel et al., 2021). The mutations studied here do not involve the loss or introduction of lysine residues and therefore there is no change in the number or location of amino acids amenable to glutaraldehyde reaction, however, we cannot rule out the possibility that these mutations indirectly alter the ability of glutaraldehyde to cross-link lysine residues in adjacent RyR NTD monomers by affecting the local conformation. We should also note that we studied pathogenic mutations in a homozygous scenario, whereas MH and CPVT1 are autosomal dominant diseases. Our results are consistent with previous molecular dynamics simulations indicating that R169Q, as well as A165D and R176Q, increase the NTD-NTD inter-subunit distance (Xiong et al., 2018). Hence, the present study adds to the mounting evidence from biochemical, structural, and molecular dynamics analyses that RyR1 and RyR2 pathogenic mutations located within the \(\beta_{8}-\beta_{9}\) loop disrupt NTD tetramerization (Table 1) (Iyer et al., 2020; Seidel et al., 2021; Xiong et al., 2018).

The RyR N-terminus has previously been implicated in neuromuscular and cardiac disease because of disrupted interaction with the central domain (Ikemoto and Yamamoto, 2002), also known as "helical domain-1" (Peng et al., 2016; Yan et al., 2015) and "bridging solenoid (BSol)" (des Georges et al., 2016). However, RyR1-R163C and RyR2-R169Q are unlikely to affect this structure-function parameter because the NTD interface with the BSol (RyR1 residues 2146–2712, RyR2 residues 2111–2679) involves residues other than those of the \(\beta_{8}-\beta_{9}\) loop (des Georges et al., 2016; Peng et al., 2016; Yan et al., 2015). Indeed, no differences in the NTD-BSol interface were reported in the RyR1-R163C structure compared to WT (Iyer et al., 2020). On the other hand, altered NTD-BSol interface was recently reported for RyR1-R615C, which induced a distinct pathological conformation in RyR1 to facilitate channel opening (Woll et al., 2021). Thus, N-terminal mutations may affect different RyR inter-domain associations, but all the available evidence suggests that mutations within the \(\beta_{8}-\beta_{9}\) loop (e.g., RyR1-R163C, RyR2-R169Q) alter N-terminal inter-subunit interactions.

Importantly, diminished NTD tetramerization due to the CPVT R169Q mutation can be reinforced by introducing positively-charged amino acids at the NTD-NTD interface (Figure 2). R169Q likely results in diminished NTD tetramerization because of the loss of a salt bridge with D400 on the neighboring subunit (Peng et al., 2016; Xiong et al., 2018). On the other hand, our introduced structural mutations Q168R and M399R may create new salt bridges with D400 and D179/D180, respectively, which appear to compensate for the loss of R169-D400 electrostatic interaction. These observations add further weight to the role of the \(\beta_{8}-\beta_{9}\) and \(\beta_{23}-\beta_{24}\) loops in mediating efficient N-terminal inter-subunit interactions. Notably, they act as proof of concept for stabilizing N-terminal inter-subunit interactions in RyR2 disease. While such genetic manipulation is an unlikely therapeutic tool, the development of small chemical compounds that target the RyR2 NTD-NTD interface holds great promise as potential therapy in CPVT.

**Data availability statement**

The raw data supporting the conclusion of this article will be made available by the authors, without undue reservation.

**Author contributions**

SZ conceived the study designed the experiments and wrote the paper; YZ, CRdM, and AB performed and analyzed the experimental data; FAL contributed reagents and materials; SZ and FAL edited the manuscript.

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**Conflict of interest**

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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