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Dantrolene rescues aberrant N-terminus intersubunit interactions in mutant pro-arrhythmic cardiac ryanodine receptors

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Aims The ryanodine receptor (RyR2) is an intracellular Ca^{2+} release channel essential for cardiac excitation–contraction coupling. Abnormal RyR2 channel function results in the generation of arrhythmias and sudden cardiac death. The present study was undertaken to investigate the mechanistic basis of RyR2 dysfunction in inherited arrhythmogenic cardiac disease.

Methods and results We present several lines of complementary evidence, indicating that the arrhythmia-associated L433P mutation disrupts RyR2 N-terminus self-association. A combination of yeast two-hybrid, co-immunoprecipitation, and chemical cross-linking assays collectively demonstrate that a RyR2 N-terminal fragment carrying the L433P mutation displays substantially reduced self-interaction compared with wild type. Moreover, sucrose density gradient centrifugation reveals that the L433P mutation impairs tetramerization of the full-length channel. [^{3}H]Ryanodine-binding assays demonstrate that disrupted N-terminal intersubunit interactions within $RyR2^{L433P}$ confer an altered sensitivity to Ca^{2+} activation. Calcium imaging of $RyR2^{L433P}$-expressing cells reveals substantially prolonged Ca^{2+} transients and reduced Ca^{2+} store content indicating defective channel closure. Importantly, dantrolene treatment reverses the L433P mutation-induced impairment and restores channel function.

Conclusion The N-terminus domain constitutes an important structural determinant for the functional oligomerization of RyR2. Our findings are consistent with defective N-terminus self-association as a molecular mechanism underlying RyR2 channel deregulation in inherited arrhythmogenic cardiac disease. Significantly, the therapeutic action of dantrolene may occur via the restoration of normal RyR2 N-terminal intersubunit interactions.

Keywords arrhythmias • dantrolene • excitation–contraction coupling • oligomerization • ryanodine receptor

1. Introduction

The cardiac ryanodine receptor (RyR2) is a key component of excitation–contraction coupling, mediating rapid Ca^{2+} release from the sarcoplasmic reticulum (SR). RyR2 channels are closed in the muscle relaxation phase, whereas inappropriate SR Ca^{2+} release causes deleterious effects on cardiac function. Indeed, RyR2 dysfunction leading to diastolic SR Ca^{2+} leak has been associated with both genetic and acquired cardiac disorders.[1–4] Mutations in the RyR2 gene, inherited in an autosomal dominant fashion, have been linked to two diseases displaying overlapping clinical phenotypes: catecholaminergic polymorphic ventricular tachycardia (CPVT1) and arrhythmogenic right ventricular dysplasia (ARVD2). Both disorders are associated with episodes of potentially lethal arrhythmias triggered by emotional or physical stress and exhibit a mortality rate of ~30%. CPVT1/ARVD2 exhibits incomplete penetrance, that is, carriers of the same mutation display differences in the severity of symptoms and some remain completely asymptomatic indicating the involvement of other factors or compensatory mechanisms. The CPVT1 phenotype is manifested in the absence of structural abnormalities, whereas ARVD2 is associated with progressive degeneration and fibrofatty replacement of the right ventricular myocardium.[5,6] The two disorders may represent a variable phenotype of the same disease, because the same mutation has been described as causative of both CPVT1 and ARVD2 in different patients.[6–8]

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To date, around 150 arrhythmia-associated RyR2 mutations have been reported, the vast majority of which are missense (http://www.fsm.it/cardmoc/, 13 November 2014). RyR2 mutations tend to cluster in three distinct regions: the N-terminus, a small central domain, and a large C-terminal region. Most of the mutations studied induce gain-of-function changes in RyR2, reflected by an increased sensitivity to activation by physiological and pharmacological agonists including Ca^{2+}, caffeine, and cAMP-mobilizing agents.[9–15] Reduction and loss-of-function RyR2 mutations causative of CPVT1/ARVD2 have also been described.[13,16] This is reminiscent of central core disease, where most RyR1 mutations are gain-of-function, but a few loss-of-function, mutants have also been reported.[17,18] Different molecular mechanism(s) underlying RyR2 channel deregulation have been proposed, including abnormal binding of FKBP12.6,[12,15] reduced threshold for store overload-induced Ca^{2+} release,[10,19] and defective inter-domain interactions.[14,20] Although there are conflicting reports for a given mutation that could be down to methodological differences, it is possible that there is no single unifying hypothesis, with each RyR2 mutation differentially affecting the diverse channel regulation mechanisms.

RyRs are the largest ion channels known, composed of four identical subunits of ~5000 amino acids. The C-terminal part contains the transmembrane domains that form a Ca^{2+}-conducting pore and is also believed to comprise the primary tetramerization determinant.[21–25] The large cytoplasmic N-terminal portion is required for regulation of the pore and serves as a scaffold for accessory proteins, ions, and other modulators.[26] Communication of the stimulatory/inhibitory cytoplasmic signals to the channel pore is conveyed by long-range conformation changes and interactions between structural and functional domains. Recently, we identified a novel N-terminus intersubunit interaction within RyR2, which is conserved in mammalian RyR isoforms and the related inositol trisphosphate receptor.[27,28] We further presented functional evidence, suggesting that RyR2 N-terminus tetramerization is involved in the stabilization of the closed state of the channel.[28] Given that the N-terminus is one of the three mutation hot spots, we speculated that arrhythmia-linked mutations may have an effect on N-terminus self-association and RyR2 channel function. In this study, we find that a single missense mutation (L433P) has a profound effect on tetramerization of both the isolated N-terminus domain and the full-length protein. Functional assays further demonstrate that the $RyR2^{L433P}$ channel is unstable, and it also displays altered Ca^{2+} dependence and compromised channel closure. Remarkably, dantrolene, a drug used to treat the RyR1-mediated neuromuscular disorder malignant hyperthermia (MH), has the ability to reverse the mutation-induced phenotype.

2. Methods

A detailed description is available in Supplementary Material online on the *Cardiovascular Research* website. Unless otherwise stated, cumulative data were derived from at least three independent experiments.

2.1 Yeast two-hybrid, co-immunoprecipitation, and chemical cross-linking assays

Yeast two-hybrid (Y2H), co-immunoprecipitation (co-IP), and chemical cross-linking assays were carried out as described in Zissimopoulos *et al.*[27,28] For the experiments performed to test the effect of dantrolene, cell homogenates were incubated with 1 μM dantrolene for 1 h at 37°C prior to cross-linking. The optical density of relevant bands was determined by densitometry using a GS-700 scanner (Bio-Rad) and the Quantity One software (Bio-Rad). Statistical analysis was carried out using the GraphPad Prism software; one-way ANOVA with Bonferroni *post hoc* test for ambient conditions, Kruskal–Wallis test with Dunn's *post hoc* test was used for group comparison with unequal variance ($P < 0.05$ in Bartlett's test) for reducing conditions.

2.2 Sucrose density gradient centrifugation

Sucrose density gradient centrifugation was carried out as previously described.[28] For the experiments performed to test the effect of dantrolene, all buffers were supplemented with dantrolene at 1 μM. Densitometry analysis was performed and the amount of RyR2 in each fraction was normalized against the amount of input protein in the microsomes.

2.3 [^{3}H]ryanodine binding

[^{3}H]ryanodine-binding assays were performed as previously described.[28] For the experiments to test the effect of dantrolene, samples were pre-incubated in the presence of 1 μM dantrolene for 1 h at room temperature. Sigmoidal dose–response curve fitting (four-parameter logistic equation with bottom and top constrained to 0 and 100, respectively) and statistical analysis were performed using the GraphPad Prism software.

2.4 Calcium imaging

HEK293 cells expressing mutant or wild-type (WT) RyR2 were loaded with Fluo-3 AM (10 μM) for 1 h at 30°C and immersed in Krebs–Ringer–Henseleit solution (120 mM NaCl, 25 mM HEPES, 5.5 mM glucose, 4.8 mM KCl, 1.3 mM $CaCl_2$, 1.2 mM KH_2PO_4, 1.2 mM $MgCl_2$, and pH 7.4) to initiate waves. For the experiments to test the effect of dantrolene, cells were cultured in the presence of 1 μM of the drug. Cells were imaged for 3 min at high resolution (10 frames/s) using a Leica RS5 confocal laser scanning microscope, and Ca^{2+} handling parameters were quantified using the LAS-AF Lite software (Leica Microsystems). For transient amplitude, duration, and store load, statistical analysis was performed using one-way ANOVA with Bonferroni *post hoc* test. The frequency of calcium transients (number of events per recording) was analysed using the non-parametric (Kruskal–Wallis) test with the Dunn *post hoc* test.

3. Results

3.1 The L433P mutation disrupts RyR2 N-terminus self-association

To test the effect of the L433P mutation on RyR2 N-terminus self-association, we employed the Y2H system reporting on protein–protein interactions in a living cell. The RyR2 N-terminus domain (1–906 amino acids) was expressed in yeast as a fusion with GAL4 AD (AD4L constructs) or with DNA-BD (BT4L constructs). The interaction of the WT fragment with itself ($BT4L^{WT} + AD4L^{WT}$) was very strong, as previously reported.[28] Quantitative liquid β-galactosidase assays indicated that the presence of the L433P mutation severely compromises RyR2 N-terminus self-interaction *in situ* (*Figure 1A*). In the heterozygote scenario ($BT4L^{L433P} + AD4L^{WT}$ or $BT4L^{WT} + AD4L^{L433P}$), the interaction between the mutant and WT fragments was reduced by ~85% compared with the self-interaction of the WT fragments ($BT4L^{WT} + AD4L^{WT}$).

The interaction between mutant and WT RyR2 N-terminal fragments was further assessed by co-IP assays following co-expression in mammalian HEK293 cells. WT RyR2 N-terminus tagged with the HA epitope ($AD4L^{WT}$, residues 1–906) was immunoprecipitated with Ab^{HA} from CHAPS-solubilized cell lysates under reducing conditions, and the presence of co-precipitated BT4L (residues 1–906 tagged with the cMyc epitope) was analysed by western blotting using Ab^{cMyc}. *Figure 1B*

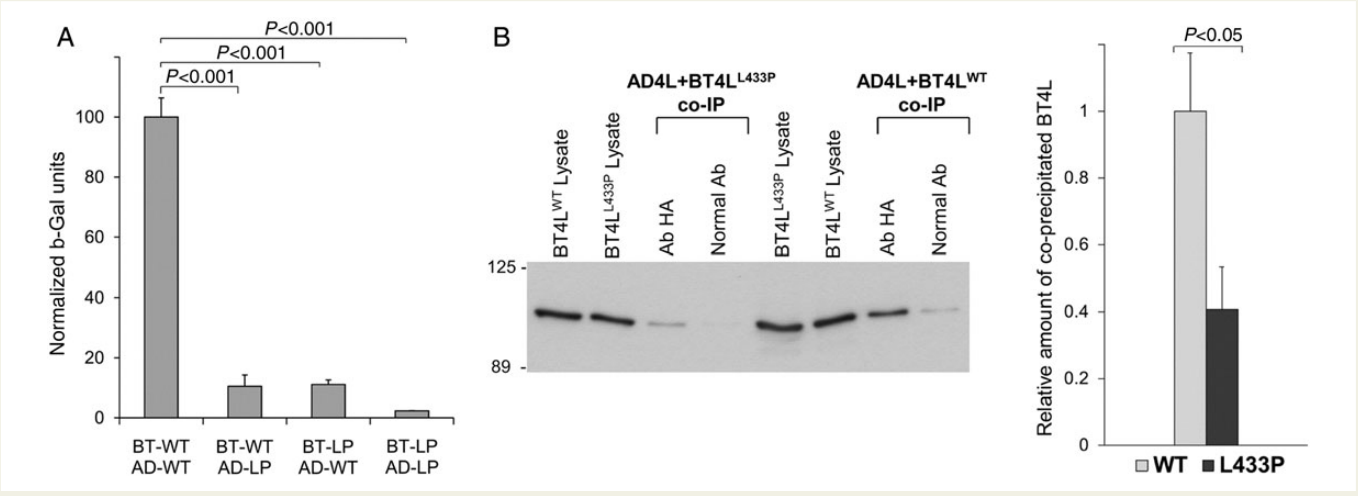

Figure 1 The L433P mutation impairs RyR2 N-terminus self-interaction *in situ* and *in vitro*. (*A*) *In situ* Y2H assays: protein interactions were assessed in yeast co-expressing AD4L with BT4L constructs (RyR2 residues 1–906 tagged with GAL4 AD or DNA-BD, respectively) using quantitative liquid β-galactosidase assays ($n = 5$). Data are given as mean value ± SEM; statistical analysis was carried out using unpaired, two-tailed Student's *t*-test. (*B*) *In vitro* co-IP assays from HEK293 cells co-expressing AD4L with BT4L (RyR2 residues 1–906 tagged with HA or cMyc epitope, respectively). Left panel, $AD4L^{WT}$ was immunoprecipitated with Ab^{HA} from CHAPS-solubilized cell lysates and the presence of co-precipitated $BT4L^{WT}$/$BT4L^{L433P}$ was analysed by western blotting using Ab^{cMyc}. 'Normal Ab' refers to non-immune rabbit IgG and serves as negative control; cell lysate, 1/50th of the volume processed in IP samples, was included in the gel to assess protein expression. Right panel, cumulative data ($n = 5$) following densitometry analysis; data are given as mean value ± SEM; statistical analysis was carried out using paired, two-tailed Student's *t*-test.

shows that cMyc-tagged $BT4L^{WT}$ is efficiently co-immunoprecipitated by $AD4L^{WT}$ and Ab^{HA}, whereas the non-specific binding is negligible in the negative control with non-immune antibody. $AD4L^{WT}$ also results in co-IP of $BT4L^{L433P}$ (*Figure 1B*); however, the amount of the recovered mutant fragment is substantially lower than that of its WT counterpart. Quantification using densitometry analysis ($n = 5$) of the amount of $BT4L^{L433P}$ or $BT4L^{WT}$ recovered in the $AD4L^{WT}$ immunoprecipitate indicates that the presence of the L433P substitution in the RyR2 N-terminus reduces its ability to interact with the WT fragment by ~60% (*Figure 1B*).

3.2 Dantrolene enhances $RyR2^{L433P}$ N-terminus tetramerization

The effect of the L433P mutation on RyR2 N-terminus oligomerization was tested by chemical cross-linking assays.[28] $BT4L^{L433P}$ and $BT4L^{WT}$ were expressed in mammalian HEK293 cells; cell homogenates were reacted with glutaraldehyde and analysed by western blotting using Ab^{cMyc}. For the WT fragment, we observed time-dependent tetramer formation under both ambient and reducing conditions (*Figure 2A* and *D*), in agreement with our previous report.[28] Chemical cross-linking of the $BT4L^{L433P}$ mutant fragment resulted in the appearance of the tetramer; however, its abundance was rather low relative to WT (*Figure 2B* and *E*). Cumulative data ($n \geq 6$) following densitometry analysis demonstrated that the presence of the L433P mutation resulted in an ~60% decrease in the tetramer-to-monomer ratio (*Figure 2G*, 60 min). This effect was also seen under reducing conditions, where dithiothreitol (DTT, 10 mM) was used prior to chemical cross-linking to reduce the endogenous disulfide bonds that covalently link BT4L tetramers. Following DTT pre-treatment, the tetramer-to-monomer ratio for $BT4L^{L433P}$ was decreased by ~60% compared with the WT fragment (*Figure 2H*, 60 min).

Dantrolene is a drug used for the treatment of the RyR1-mediated neuromuscular disorder MH,[29] and despite having no effect on WT RyR2,[30] dantrolene was reported to restore normal cardiac function in RyR2-related cardiac disease.[14,31–34] Since its putative binding site is contained within the RyR2 N-terminus (residues 601–620, identical to RyR1 residues 590–609),[35,36] we investigated the effects of dantrolene on RyR2 N-terminus oligomerization. $BT4L^{WT}$ and $BT4L^{L433P}$, expressed in mammalian HEK293 cells, were treated with 1 μM dantrolene prior to chemical cross-linking and analysed by western blotting. Dantrolene treatment did not produce any appreciable effect on $BT4L^{WT}$ tetramer formation (see Supplementary material online, *Figure S1*). Remarkably, dantrolene induced a substantial increase in $BT4L^{L433P}$ tetramer formation under both ambient and reducing conditions (*Figure 2C* and *F*). Cumulative data ($n \geq 6$) following densitometry analysis revealed that the drug increased the tetramer-to-monomer ratio by approximately two-fold (*Figure 2G* and *H*, 60 min). These findings suggest that dantrolene is able to partially reverse the L433P mutation-induced defect in N-terminus self-association.

3.3 The L433P mutation results in unstable RyR2 tetrameric channels

The Y2H, co-IP, and chemical cross-linking experiments described above indicate that the L433P mutation significantly impairs the ability of the RyR2 N-terminus to form tetramers, but this remained to be determined for the full-length protein. To test this, RyR2 oligomerization was assessed by sucrose density gradient centrifugation of CHAPS-solubilized HEK293 microsomes expressing the WT or mutant protein. This technique separates proteins according to their size under non-denaturing conditions, therefore allowing for non-covalent protein–protein interactions to be retained. Western blot analysis of density gradient fractions indicated that solubilized $RyR2^{WT}$ was almost exclusively present in 'heavy' sucrose fractions (*Figure 3A*), a sedimentation

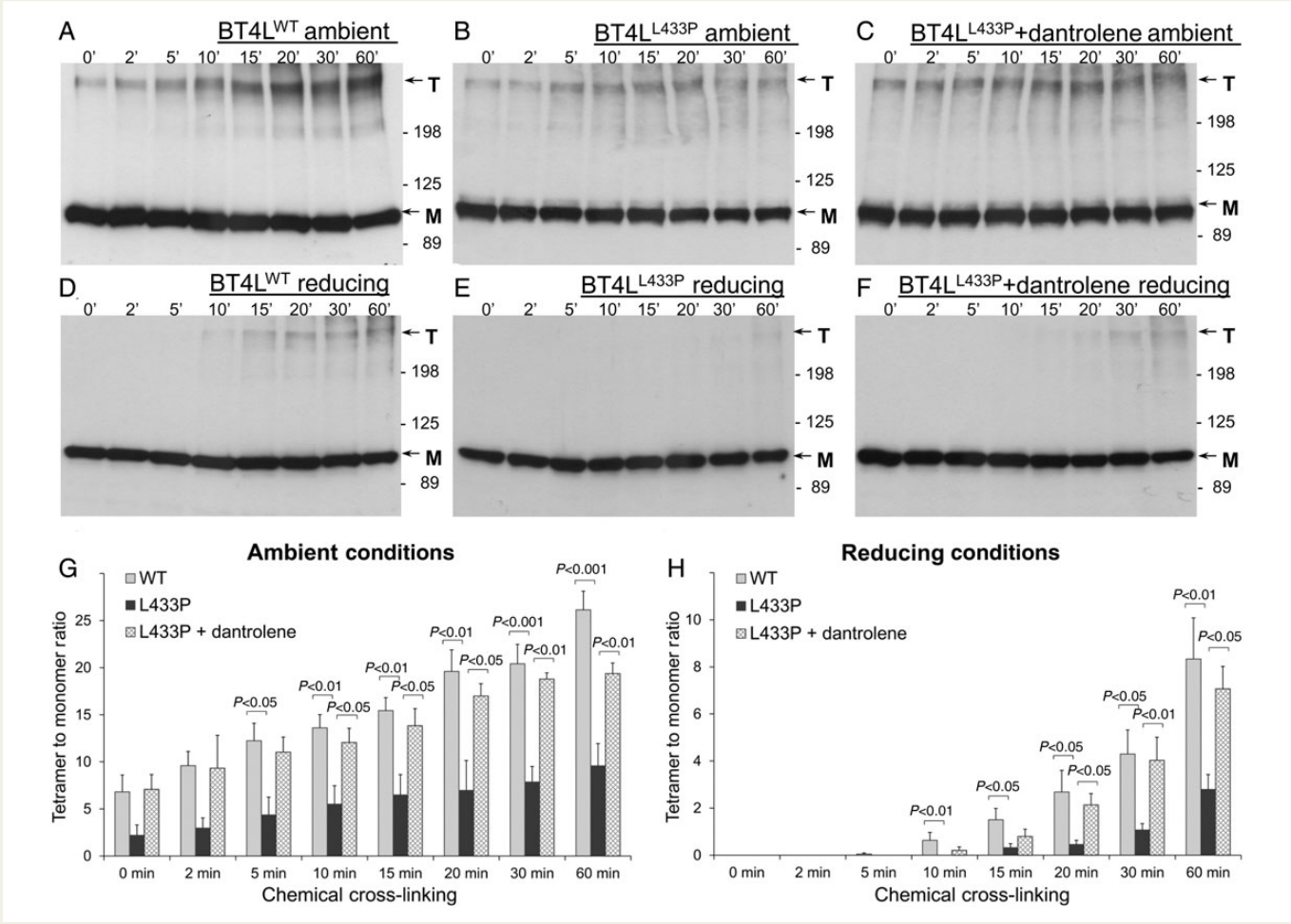

Figure 2 Dantrolene enhances tetramerization of L433P mutant RyR2 N-terminus. Chemical cross-linking assays of HEK293 cell homogenates expressing BT4L^{WT} (*A* and *D*) or BT4L^{L433P} pre-treated without (*B* and *E*) or with 1 μM dantrolene (*C* and *F*). Cell homogenates were incubated with glutaraldehyde for the indicated time points at ambient (*A*–*C*) or reducing (10 mM DTT) conditions (*D*–*F*), and analysed by western blotting using Ab^{cMyc}; monomer (M) and tetramer (T) are indicated with the arrows. Densitometry analysis ($n \geq 6$) was carried out on the bands corresponding to tetramer and monomer moieties, and used to calculate tetramer formation at (*G*) ambient or (*H*) reducing conditions. Data are given as mean value ± SEM; statistical analysis was carried out using one-way ANOVA with the Bonferroni *post hoc* test (*G*) or non-parametric (Kruskal–Wallis) test with Dunn's *post hoc* test (*H*).

profile consistent with the known tetrameric assembly of the channel, whereas minimal monomer was detected in 'light' fractions. Notably, the distribution of RyR2^{L433P} was considerably different from RyR2^{WT}, with the mutant protein widely distributed over almost the entire sucrose gradient (*Figure 3B*). Some RyR2^{L433P} protein was indeed detected in 'heavy' fractions (25–28%) corresponding to the tetramer, but the mutant was enriched in 'light' sucrose fractions (15–17%) corresponding to dissociated subunits. Quantification using densitometry analysis ($n \geq 3$) demonstrates that RyR2^{L433P} forms substantially less tetramers compared with RyR2^{WT} ($P < 0.05$; *Figure 3D* and see Supplementary material online, *Figure S2*). RyR2^{L433P} channel tetramer formation was also tested by [^{3}H]ryanodine-binding assays under conditions promoting maximum channel activation (100 μM Ca^{2+} and 10 mM caffeine) and reporting on the total number of functional tetrameric channels. The amount of bound [^{3}H]ryanodine for RyR2^{L433P} was very low relative to RyR2^{WT} (16.3 ± 2.0 vs. 39.7 ± 2.4 fmol/mg, respectively, $P < 0.01$), despite having equivalent protein levels of RyR2^{L433P} and RyR2^{WT} expressed in HEK293 cells (see Supplementary material online, *Figure S3*). Given that high-affinity ryanodine binding requires an intact tetrameric RyR2 channel,[37] the most plausible explanation is that the RyR2^{L433P} tetramer assembly is partially impaired, which is consistent with the density gradient centrifugation profile.

We subsequently tested whether dantrolene treatment affects tetramer stability of the full-length RyR2^{L433P}. Using sucrose density gradient centrifugation, we found that dantrolene results in substantial redistribution of the RyR2^{L433P} sedimentation profile, with most of the protein enriched in 'heavy' sucrose fractions resembling the RyR2^{WT} pattern (*Figure 3C*). Cumulative data ($n \geq 3$) indicated that dantrolene shifted the abundance of the RyR2^{L433P} protein from 'light' sucrose fractions to predominantly 'heavy' fractions, similar to RyR2^{WT} (*Figure 3D* and see Supplementary material online, *Figure S2*). These findings suggest that dantrolene promotes tetramer stability of full-length RyR2^{L433P}. Dantrolene (1 μM) also resulted in a substantial (~50%) increase in [^{3}H]ryanodine binding to RyR2^{L433P} at 100 μM Ca^{2+} (13.8 ± 1.7 vs. 20.7 ± 2.4 fmol/mg for dantrolene, $P < 0.05$), providing further evidence that the drug enhances the functional tetrameric assembly of the mutant channel.

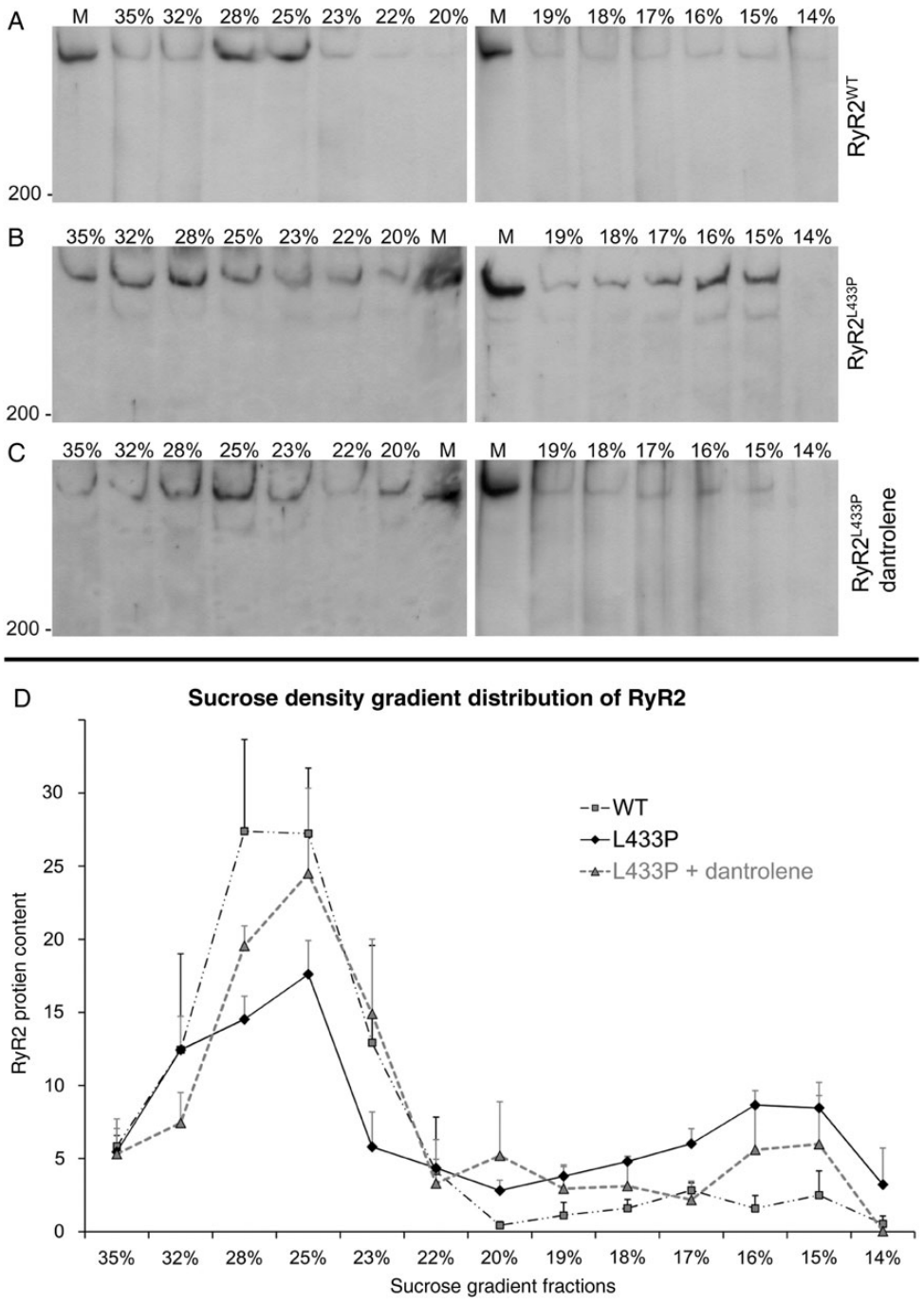

Figure 3 Dantrolene promotes oligomerization of the full-length RyR2^{L433P}. Distribution of RyR2^{WT} (*A*) or RyR2^{L433P} pre-treated without (*B*) or with 1 μM dantrolene (*C*) in fractions collected following sucrose density gradient centrifugation. RyR2^{WT} and RyR2^{L433P} were expressed in HEK293 cells, and CHAPS-solubilized microsomal membranes were subjected to sucrose density gradient centrifugation. The protein distribution profile was analysed by western blotting using RyR2 Ab^{1093}. Microsomes (25 μg, M) were also included to assess protein expression; sucrose concentration as indicated. Cumulative data ($n \geq 3$) following densitometry analysis are presented in (*D*); data are given as mean value ± SEM.

3.4 Defective RyR2^{L433P} N-terminus self-association underlies altered Ca^{2+} sensitivity

To obtain some insights into the functional effects of the L433P mutation, we tested the Ca^{2+} dependence of the mutant channel using [^{3}H]ryanodine-binding assays. Despite RyR2^{L433P} tetramer instability resulting in reduced ryanodine-binding capacity relative to RyR2^{WT}, the mutant channel was responsive to Ca^{2+} activation (*Figure 4A*). A typical sigmoid Ca^{2+}-dependence curve was obtained for RyR2^{L433P}; however, it was much flatter compared with WT. This might suggest that the L433P mutation results in depressed channel activity at high Ca^{2+} concentrations; however, this interpretation would be misleading

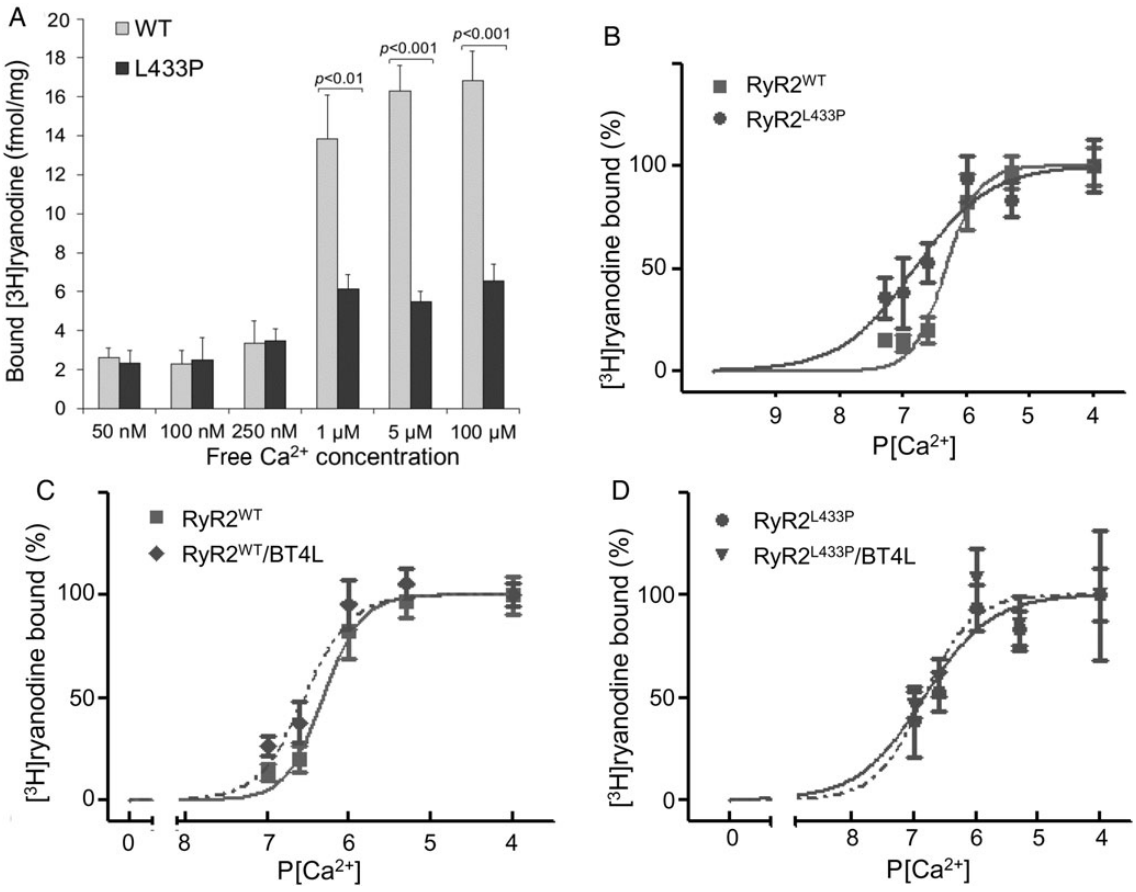

Figure 4 RyR2L433P displays altered Ca^{2+} sensitivity due to defective N-terminus self-association. (*A*) [^{3}H]ryanodine-binding assays of $RyR2^{WT}$ or $RyR2^{L433P}$ expressed in HEK293 cell homogenates, over a range of free Ca^{2+} concentrations. Summary of three separate experiments each performed at least in duplicate. Data are expressed as mean value ± SEM; statistical analysis was carried out using unpaired, two-tailed Student's *t*-test. (*B*) Ca^{2+} dose–response curve for $RyR2^{WT}$ and $RyR2^{L433P}$ where the data obtained in (*A*) are normalized against maximum binding (100 μM Ca^{2+}). The EC_{50} values for Ca^{2+} activation, calculated from nonlinear regression analysis using the GraphPad Prism, were 465.2 nM for $RyR2^{WT}$ vs. 147.8 nM for $RyR2^{L433P}$, $P < 0.001$. (*C*) A dose–response curve for Ca^{2+} dependence of [^{3}H]ryanodine binding to $RyR2^{WT}$ alone and $RyR2^{WT}$ + BT4L and normalized against maximum binding (100 μM Ca^{2+}). The EC_{50} values for Ca^{2+} activation were 470.9 nM for $RyR2^{WT}$ alone vs. 280.6 nM for $RyR2^{WT}$ + BT4L ($P < 0.05$). (*D*) Dose–response curve for Ca^{2+} dependence of [^{3}H]ryanodine binding to $RyR2^{L433P}$ alone and $RyR2^{L433P}$ + BT4L and normalized against maximum binding (100 μM Ca^{2+}). The EC_{50} values for Ca^{2+} activation were 182.6 nM for $RyR2^{L433P}$ alone vs. 129.2 nM for $RyR2^{L433P}$ + BT4L ($P = 0.511$).

because depressed [^{3}H]ryanodine binding is more likely due to tetrameric assembly impairment. Normalization of L433P-binding data relative to its own maximum-binding value (obtained at 100 μM Ca^{2+}) produced a dose–response curve for $RyR2^{L433P}$ that is evidently left-shifted relative to $RyR2^{WT}$ (*Figure 4B*), consistent with an increased $RyR2^{L433P}$ sensitivity to Ca^{2+} activation with a significantly lower EC_{50} ($EC_{50} = 147.8$ nM Ca^{2+} for $RyR2^{L433P}$ vs. $EC_{50} = 465.2$ nM Ca^{2+} for $RyR2^{WT}$, $P < 0.001$).

The observed increased $RyR2^{L433P}$ sensitivity to Ca^{2+} activation could be due to mutation-induced defective N-terminus self-association. We have shown previously that the presence of exogenous RyR2 N-terminus domain (BT4L) increases RyR2 channel activity at low diastolic Ca^{2+} concentrations, most likely by disrupting the endogenous N-terminal intersubunit interactions.[28] Thus, BT4L can be used as a probe to assess the state of N-terminal intersubunit interactions within $RyR2^{L433P}$. Western blot analysis of subcellular HEK293 fractions showed that BT4L is expressed at similar levels when co-expressed with the full-length $RyR2^{L433P}$ or $RyR2^{WT}$ (see Supplementary material online, *Figure S4*). However, the amount of BT4L detected in the microsomal fraction was lower upon co-expression with $RyR2^{L433P}$ than with $RyR2^{WT}$, despite comparable RyR2 protein levels, indicating reduced BT4L interaction with the mutant channel relative to WT. This is entirely compatible with our Y2H and co-IP assays on the isolated N-terminal fragments, described above. To ensure the presence of equivalent BT4L protein levels, [^{3}H]ryanodine-binding assays were carried out on cell homogenates rather than microsomes. As previously observed,[28] BT4L co-expression left-shifted the Ca^{2+} dose–response relationship of $RyR2^{WT}$ ($EC_{50} = 470.9$ nM Ca^{2+} for $RyR2^{WT}$ alone vs. $EC_{50} = 280.6$ nM for $RyR2^{WT}$ + BT4L, $P < 0.05$; *Figure 4C*), indicating that BT4L enhances the Ca^{2+} sensitivity of the channel. Notably, we found no significant change in the dose–response curve of $RyR2^{L433P}$ upon BT4L co-expression ($EC_{50} = 182.6$ nM Ca^{2+} for $RyR2^{L433P}$ alone vs. $EC_{50} = 129.2$ nM for $RyR2^{L433P}$ + BT4L, $P = 0.511$; *Figure 4D*). Thus, contrary to WT, the mutant L433P channel is not affected by BT4L, an observation that is most probably due to the N-terminal self-association within $RyR2^{L433P}$ already being disrupted.

3.5 Dantrolene corrects abnormal RyR2^{L433P} intracellular Ca^{2+} transients

To assess the effect of the L433P mutation on RyR2-mediated intracellular Ca^{2+} handling, we employed a cellular assay developed by Jiang *et al.*[19] referred to as 'store overload-induced Ca^{2+} release'. They found that, at elevated extracellular Ca^{2+} levels resulting in Ca^{2+} store overload, HEK293 cells expressing RyR2 display spontaneous Ca^{2+} release events in a manner indistinguishable from cardiac myocytes. Untransfected cells that do not have endogenous RyR2 did not display Ca^{2+} transients and did not respond to caffeine.[19] To monitor cytosolic Ca^{2+} transients, HEK293 cells transfected with RyR2^{WT} or RyR2^{L433P} were loaded with the fluorescent Ca^{2+} indicator Fluo-3 and were maintained in 1.3 mM extracellular Ca^{2+}. Cells expressing either the WT or the mutant RyR2 displayed spontaneous Ca^{2+} transients but with two clear differences (*Figure 5*). First, RyR2^{L433P}-expressing cells displayed fewer Ca^{2+} transients compared with RyR2^{WT} (*Figure 5*, 'number of events' panel), most likely due to the reduced number of functional channels and compatible with the density gradient centrifugation and [^{3}H]ryanodine-binding data described above. Secondly, Ca^{2+} transients had substantially increased duration and the Ca^{2+} store content, measured with application of 10 mM caffeine, was reduced in RyR2^{L433P}-expressing cells compared with RyR2^{WT}. These observations are consistent with defective RyR2^{L433P} N-terminus self-association resulting in compromised channel closure.

To test the functional effects of dantrolene, cells were treated with 1 μM of the drug and their Ca^{2+} handling properties were analysed by Ca^{2+} imaging. Dantrolene treatment had insignificant effects on RyR2^{WT}-expressing cells, but induced pronounced changes in RyR2^{L433P}-mediated Ca^{2+} mobilization (*Figure 5*). In particular, 1 μM dantrolene increased the Ca^{2+} store content and the number of RyR2^{L433P} Ca^{2+} transients, and decreased transient duration to levels similar to RyR2^{WT}. These findings suggest that dantrolene restores WT channel function to RyR2^{L433P} and normalizes Ca^{2+} handling in cells.

4. Discussion

In the present study, we investigated the molecular mechanism underlying RyR2 channel dysfunction in arrhythmogenic cardiac disease. Our major findings are: (i) the arrhythmogenic L433P mutation compromises RyR2 N-terminus self-association resulting in defective channel closure and (ii) dantrolene promotes N-terminus self-association thereby restoring RyR2^{L433P} channel function.

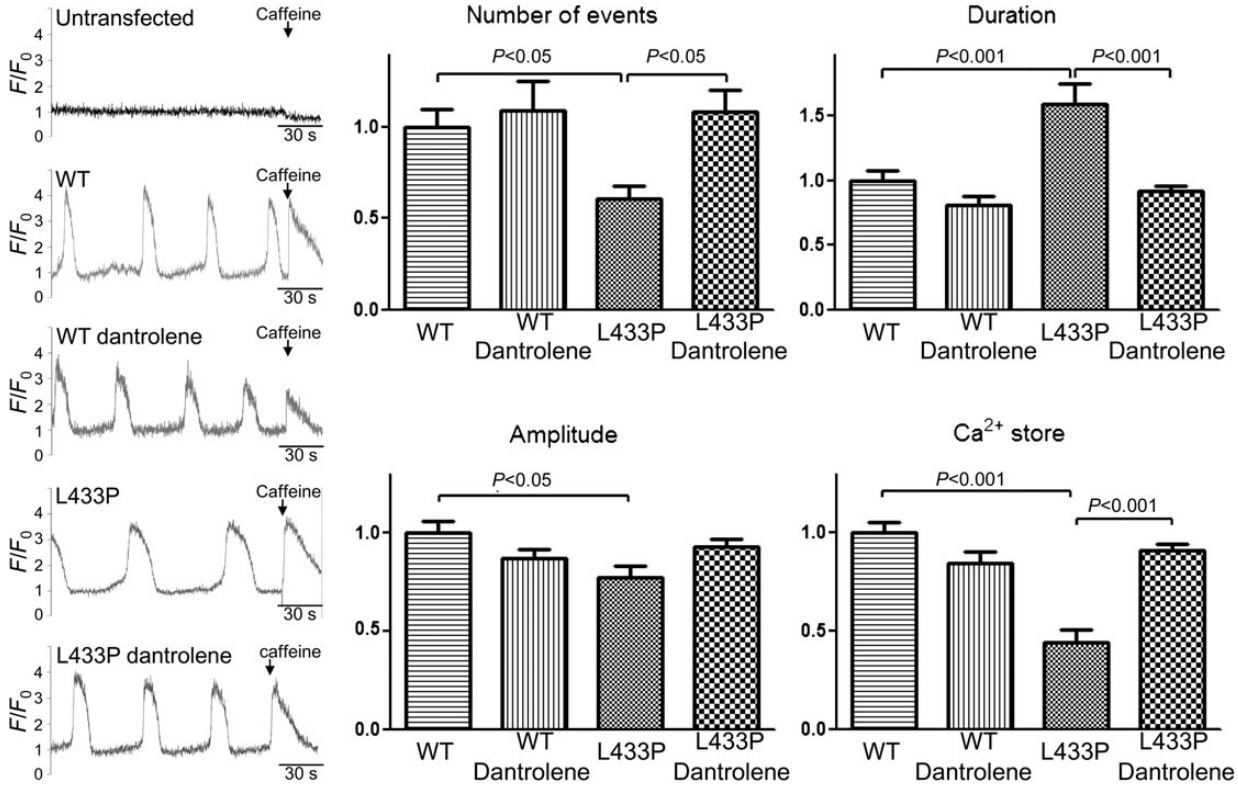

Figure 5 Dantrolene normalizes abnormal RyR2^{L433P}-mediated Ca^{2+} handling in cells. Single-cell Ca^{2+} imaging using confocal laser scanning microscopy to monitor intracellular Ca^{2+} mobilization in RyR2-expressing cells. HEK293 cells transfected with RyR2^{WT} or RyR2^{L433P}, and treated with or without 1 μM dantrolene, were imaged at 1.3 mM extracellular Ca^{2+} to induce spontaneous Ca^{2+}- release transient events. The Ca^{2+} store content was estimated from the amplitude of the (10 mM) caffeine-induced Ca^{2+} release at the end of each experiment. Ca^{2+} transient characteristics were analysed from 39 cells for RyR2^{WT}, 41 for dantrolene-treated RyR2^{WT}, 29 for RyR2^{L433P}, and 53 for dantrolene-treated RyR2^{L433P}. Data are normalized for RyR2^{WT} and expressed as mean value ± SEM; statistical analysis was carried out using one-way ANOVA with the Bonferroni *post hoc* test (amplitude, duration, and store load) or non-parametric (Kruskal–Wallis) test with Dunn's *post hoc* test (frequency).

4.1 The N-terminus domain is an important structural determinant for RyR2 tetramerization

Using independent but complementary experimental techniques, we found that the L433P mutation results in substantially reduced self-association of the human RyR2 N-terminus domain (*Figures 1* and *2*). This mutation cannot affect intersubunit interactions directly, because it is buried within the three-dimensional structure of the RyR2 N-terminus.[38] In particular, L433 lies within an α-helix, and its substitution for proline, a rigid body amino acid, is likely to disrupt the helix and alter the structure locally. This may in turn induce allosteric rearrangements within the RyR2 N-terminus, indirectly affecting the intersubunit interface, as suggested for some RyR1 mutations.[39]

Moreover, density gradient centrifugation indicated that the full-length $RyR2^{L433P}$ has reduced tetramer stability (*Figure 3*). This is further supported by measurements of maximal [^{3}H]ryanodine binding to $RyR2^{L433P}$, which was substantially decreased compared with WT (despite equivalent protein amounts). It is generally believed that the C-terminal region is necessary and sufficient for oligomerization of the native RyR. This is based on observations that large RyR1/2 C-terminal fragments containing the transmembrane domains are able to form a Ca^{2+}-conducting pore and bind ryanodine,[21,22,24,25] whereas the extreme cytoplasmic C-terminal tail is capable of tetramerization.[40] In addition, RyR1 lacking the last 15 amino acids does not display high-affinity ryanodine binding, an effect attributed to impaired tetrameric assembly.[23] Recently, we have reported that the N-terminus of all three mammalian RyR isoforms is also capable of tetramerization.[27,28] The present study demonstrates that the arrhythmia-linked L433P mutation disturbs the tetramerization of not only the RyR N-terminus, but also the full-length protein. Although we cannot exclude the possibility of long-range alterations affecting C-terminal domain organization, the most likely explanation is that the N-terminus self-association is a significant RyR oligomerization determinant contributing to the tetrameric stability of the protein complex.

4.2 Defective N-terminus self-association underpins $RyR2^{L433P}$ channel deregulation

Our functional studies suggest that defective N-terminus self-association within the L433P mutant channel results in increased Ca^{2+} sensitivity (*Figure 4*). Exogenous N-terminus domain (BT4L) was previously shown to enhance native RyR2 [^{3}H]ryanodine binding at low Ca^{2+} concentrations most likely due to disruption of endogenous RyR2 N-terminal intersubunit interactions.[28] This was also found in the current study with $BT4L^{WT}$ lowering the EC_{50} for Ca^{2+} activation of $RyR2^{WT}$ (*Figure 4C*). In contrast, exogenous $BT4L^{WT}$ had a minimal effect on $RyR2^{L433P}$ [^{3}H]ryanodine binding (*Figure 4D*), and the EC_{50} for Ca^{2+} activation of the mutant channel was comparable to $RyR2^{WT}$ + $BT4L^{WT}$, suggesting that the N-terminal intersubunit interactions within $RyR2^{L433P}$ are already disrupted. It was recently reported that $RyR2^{L433P}$ knock-in cardiomyocytes display increased Ca^{2+} spark frequency and SR Ca^{2+} leak.[41] The present characterization of recombinant human $RyR2^{L433P}$ expressed in HEK293 cells, where prolonged Ca^{2+} transients and reduced Ca^{2+} store content were observed (*Figure 5*), concurs with the transgenic mouse model. Based on these findings, we propose a model where the arrhythmia-linked L433P mutation impairs N-terminal intersubunit interactions resulting in defective channel closure (*Figure 6*).

Our findings indicate that the functional defect produced by the L433P mutation is two-fold: (i) reduced number of functional channels and (ii) increased Ca^{2+} sensitivity. The interplay between these two empirical observations, which could have seemingly opposite effects, namely depressed or enhanced Ca^{2+} mobilization, may explain the distinctive Ca^{2+} release profile previously reported for $RyR2^{L433P}$ and also observed in the present study. Thomas *et al.*[13] reported that, unlike other CPVT1/ARVD2 mutations, $RyR2^{L433P}$ has reduced caffeine sensitivity, which could be due to the reduced number of functional channels (*Figure 3*). On the other hand, they also found that, similar to other mutations, $RyR2^{L433P}$ channels result in increased Ca^{2+} release rate and prolonged Ca^{2+} transient,[13] which could be due to enhanced Ca^{2+} sensitivity (*Figure 4*). We note that prolonged Ca^{2+} transients were also observed in the present study (*Figure 5*), where Ca^{2+} mobilization was induced by Ca^{2+} store overload instead of caffeine application. The L433P mutation has also been functionally characterized by an independent group, which reported increased propensity for spontaneous Ca^{2+} oscillations due to enhanced luminal Ca^{2+} activation, similar to other RyR2 mutations.[10] Jiang *et al.*[10] also reported no change in cytoplasmic Ca^{2+} activation for $RyR2^{L433P}$, in disagreement with our results. The reason for these discrepancies is unclear since we used the same expression system (HEK293 cells) and functional assays

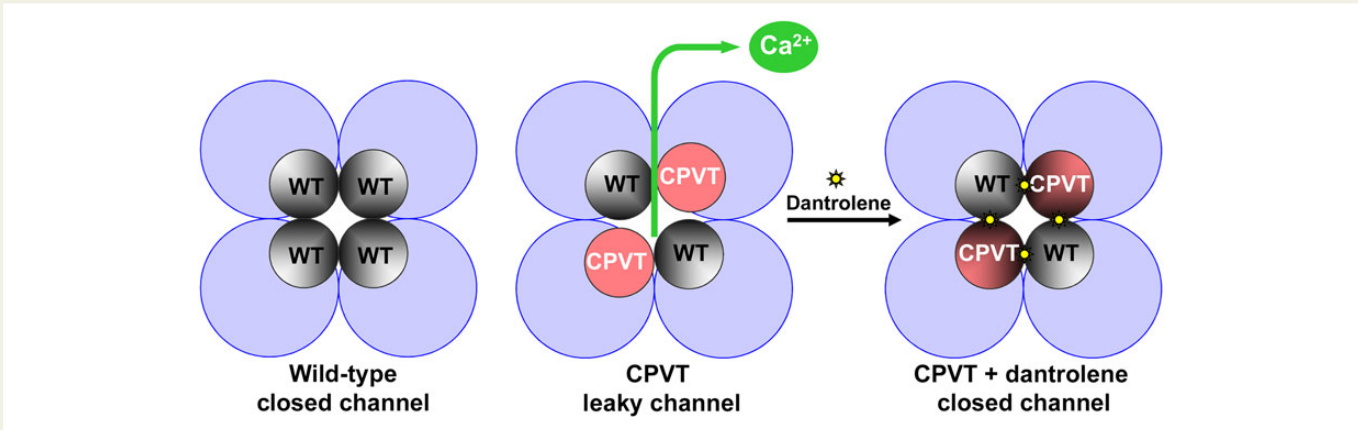

Figure 6 Working model for dantrolene rescue of defective RyR2 in CPVT1/ARVD2. Schematic representation of the putative role of N-terminus tetramerization in stabilizing the closed conformation of the RyR2 channel. In CPVT1/ARVD2, N-terminal intersubunit interactions are defective, resulting in compromised channel closure and prolonged Ca^{2+} mobilization. Dantrolene restores RyR2 N-terminus self-association and prevents excessive diastolic SR Ca^{2+} release.

([^{3}H]ryanodine-binding and store overload-induced Ca^{2+} release), but it could be because of the different species of RyR2 characterized, namely human vs. mouse. We note that although the human sequence shows 97% overall identity (98% similarity) with the mouse RyR2 used by Jiang and colleagues, there are several non-conserved substitutions in the vicinity of L433 (residues 440–443, see Supplementary material online, *Figure S5*), which form a flexible loop connecting two α-helices,[38] and this disparity could therefore result in local structural differences.

Importantly, our present work identifies the putative mechanism underlying RyR2^{L433P} channel dysfunction, namely defective N-terminal intersubunit interactions. This molecular defect is functionally manifested as perturbed cellular Ca^{2+} handling with unique characteristics (*Figure 5*). In particular, a reduced number of spontaneous Ca^{2+} transients would suggest a loss-of-function phenotype, whereas enhanced Ca^{2+} transient duration would classify this mutation as gain of function. Despite their lower frequency, prolonged Ca^{2+} transients resulted in reduced Ca^{2+} store content implying that the L433P mutation is primarily gain of function. It should be noted that we monitored spontaneous RyR2^{L433P} Ca^{2+} transients in non-cardiac cells, whereas in the cardiomyocyte context, Ca^{2+} mobilization is triggered by the action potential and normal heart beating rate is fixed by the sinus node. Thus, the reduced frequency of spontaneous events is likely to be less pertinent in the pathogenesis of cardiac arrhythmias, compared with the increased duration of the Ca^{2+} transient and the consequent decreased Ca^{2+} store content.

4.3 Dantrolene reverses the L433P mutation-induced impairment and restores channel function

Remarkably, we found that dantrolene was able to enhance the tetramer formation of the L433P mutant RyR2 N-terminus domain (*Figure 2*) and improve the tetrameric stability of the full-length mutant protein (*Figure 3*). Moreover, dantrolene was found to normalize the abnormal Ca^{2+} mobilization pattern in cells expressing RyR2^{L433P} (*Figure 5*). Dantrolene was synthesized in 1967 as a new class of skeletal muscle relaxant and since 1977 has been widely used to treat MH, a pharmacogenetic disorder associated predominantly with RyR1 mutations.[29] Although dantrolene inhibits RyR1 channel activity, it has no effect on RyR2 either in native cardiomyocytes or when heterologously expressed in HEK293 cells.[30] The drug-binding site has been mapped to residues 590–609 within RyR1, which are identical to RyR2 residues 601–620; yet the WT RyR2 surprisingly does not bind dantrolene.[35,36] However, a number of recent studies reported that dantrolene prevents the SR Ca^{2+} leak mediated from dysfunctional RyR2,[14,31–34] in agreement with our present findings (*Figure 5*). Collectively, these studies suggest that the dantrolene-binding site only becomes accessible in the altered RyR2 conformation presented in genetic or acquired cardiac disease.

It was previously postulated that the drug's mechanism of action involves stabilization of inter-domain interactions between the N-terminal and central regions of RyR.[14,32,33,42] However, a recent study reported that the location of the dantrolene-binding site is distal to the RyR2 central region, and it was suggested that dantrolene may allosterically modulate the interaction between the N-terminal and central domains.[43] In light of our present observation of direct effects on RyR2 N-terminus self-association, we propose that dantrolene's therapeutic effect is mediated by the restoration of the compromised N-terminal intersubunit interactions within arrhythmogenic mutant RyR2 (*Figure 6*). We should note that dantrolene was used in our studies at 1 μM, a clinically relevant concentration since this drug reaches a plasma concentration of 4.2 mg/L (~12 μM) in MH patients.[29] Dantrolene has not been associated with any deleterious effects on cardiac contractility, and while its use is only temporary in MH, it is chronically administered for the treatment of skeletal muscle spasticity. The fact that dantrolene does not alter normal RyR2 and cardiac function, but affects only the diseased heart, is of particular importance for its potential clinical application to attenuate arrhythmias.

4.4 Implications for CPVT/ARVD and heart failure

Abnormal RyR2 channel regulation results in arrhythmias and has been implicated in inherited (CPVT1/ARVD2) and acquired cardiac diseases (heart failure).[1–4] A common defect is believed to be diastolic Ca^{2+} overload due to SR Ca^{2+} leak through dysregulated RyR2. This in turn could result in Na^+ influx via the plasma membrane Na^+/Ca^{2+} exchanger reaching a threshold for delayed after-depolarizations and triggered arrhythmias.[4,44] Here, we demonstrate that RyR2^{L433P} results in substantially prolonged Ca^{2+} transients providing a potential explanation for the generation of fatal arrhythmias. The RyR2 L433P mutation was originally identified in members of one Italian family, where electrical instability and cases of sudden cardiac death were reported.[6] This mutation has been classified as causative of ARVD2 because affected patients displayed symptoms of right ventricle degeneration and fibrosis. ARVD2 and CPVT1 have very similar clinical manifestation characterized by catecholamine-induced ventricular arrhythmias and both are linked with RyR2 mutations that are also co-clustered in three hot spots on the peptide sequence, suggesting that they may share common mechanism(s) for channel dysfunction. Our present findings on an ARVD2-linked mutation may therefore be applicable to CPVT1. We note that prolonged Ca^{2+} transients and reduced Ca^{2+} store content, observed for RyR2^{L433P}, are hallmark features of heart failure.[3,45,46] Moreover, we show that dantrolene is able to normalize cellular Ca^{2+} handling by restoring RyR2^{L433P} N-terminus self-association, a drug previously shown to stabilize RyR2 function and prevent SR Ca^{2+} leak in failing cardiomyocytes.[32,34] Thus, we propose that defective N-terminal intersubunit interactions may be integral to the molecular mechanism underlying RyR2 channel deregulation in both genetic and acquired arrhythmogenic cardiac disease.

At present, it is unclear how a RyR2 mutation can lead to right ventricle degeneration. Arrhythmogenic right ventricular cardiomyopathy or dysplasia has been described as a disease of the desmosome, because it is associated predominantly with mutations in desmosomal proteins.[47–49] ARVD has incomplete penetrance and highly variable clinical manifestation, indicating that there is not a straightforward genotype–phenotype relationship. Arrhythmias can also appear early in the disease before significant structural remodelling of the myocardium and contractile dysfunction develop. Several mechanisms have been proposed to explain the development of right ventricle dysfunction and fibrofatty scar formation, including disruption of cell–cell adhesion, gene transcription involved in adipogenesis, and cardiomyocyte apoptosis. Perturbed Ca^{2+} handling due to dysregulated RyR2 leads to apoptosis and cell loss,[50] providing a potential explanation for the development of ARVD by the RyR2 L433P mutation. It should be noted that a RyR2^{L433P} mouse knock-in model was recently generated, and while it was found to display the CPVT phenotype (as well as atrial fibrillation), the heart did not present any structural abnormalities.[41] It is

also known that the same RyR2 mutation (e.g. A77V and R176Q) can lead to either CPVT or ARVD,[6–8] suggesting the involvement of additional factors associated with fibrofatty replacement of the right ventricle. Indeed, it has been suggested that ARVD progression and disease expression are due to the complex effect of multiple genetic and/or epigenetic modifiers interacting with environmental factors such as exercise or inflammation.[49]

In summary, we report on a novel molecular mechanism for the pathogenesis of ARVD2, which is likely to stimulate further investigations on RyR2 dysfunction in inherited and acquired cardiac pathologies. Our findings suggest that targeting defective RyR2 N-terminal intersubunit interactions represents a potentially effective therapy for the treatment of RyR2-related arrhythmogenic cardiac disease.

Supplementary material

Supplementary material is available at *Cardiovascular Research* online

Conflict of interest: none declared.

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