Review Article

Arrhythmogenic Mechanism of Catecholaminergic Polymorphic Ventricular Tachycardia

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Catecholaminergic polymorphic ventricular tachycardia (CPVT) is a highly lethal form of inherited arrhythmogenic disease characterized by adrenergically mediated polymorphic VT. The identification of the genetic substrate of the disease has allowed to achieve important milestones in the understanding of the arrhythmogenic mechanisms of the disease. Abnormal calcium leak from the mutant cardiac ryanodine receptor has been associated with the induction of delayed afterdepolarization suggesting that arrhythmogenesis in CPVT is likely to be induced by triggered activity. Here we review the current knowledge and some controversial issues about the molecular mechanism of arrhythmias initiation in CPVT and we discuss their implications for the development of novel therapeutic strategies in CPVT.
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Key words: Catecholaminergic polymorphic ventricular tachycardia, Mechanism, Ryanodine receptor

Catecholaminergic polymorphic ventricular tachycardia (CPVT) is an inherited disease characterized by adrenergically mediated polymorphic ventricular tachycardia leading to syncope and sudden cardiac death.[1,2] Since 2001, molecular genetic studies have unveiled that CPVT results from inherited defects of intracellular calcium handling in cardiac myocytes, two genetic variants of CPVT have been identified, one transmitted as an autosomal dominant trait caused by mutations in the gene encoding the cardiac ryanodine receptor (RyR2)[3] and one recessive form caused by mutations in the cardiac-specific isoform of the calsequestrin gene (CASQ2).[4] In the last six years, rapid basic advances have been made in CPVT, a simplified models clarifying intracellular calcium regulation in heart, which open an exciting scenario for the potential treatment of cardiac diseases associated with dysfunctional intracellular calcium handling. Since the CASQ2-associated recessive variant of CPVT is a relatively uncommon,[5] in this review, we will primarily focus on the molecular mechanism of arrhythmogenesis in RyR2-associated CPVT and some promising therapeutic approaches, based on recent advances in the understanding of the cellular mechanisms underlying arrhythmias in CPVT.

Intracellular Calcium Cycling and Cardiac Ryanodine Receptor

Under normal conditions during the plateau phase of the cardiac action potential a small amount of calcium enters the cardiac myocytes through the voltage-dependent L-type calcium channels, causing

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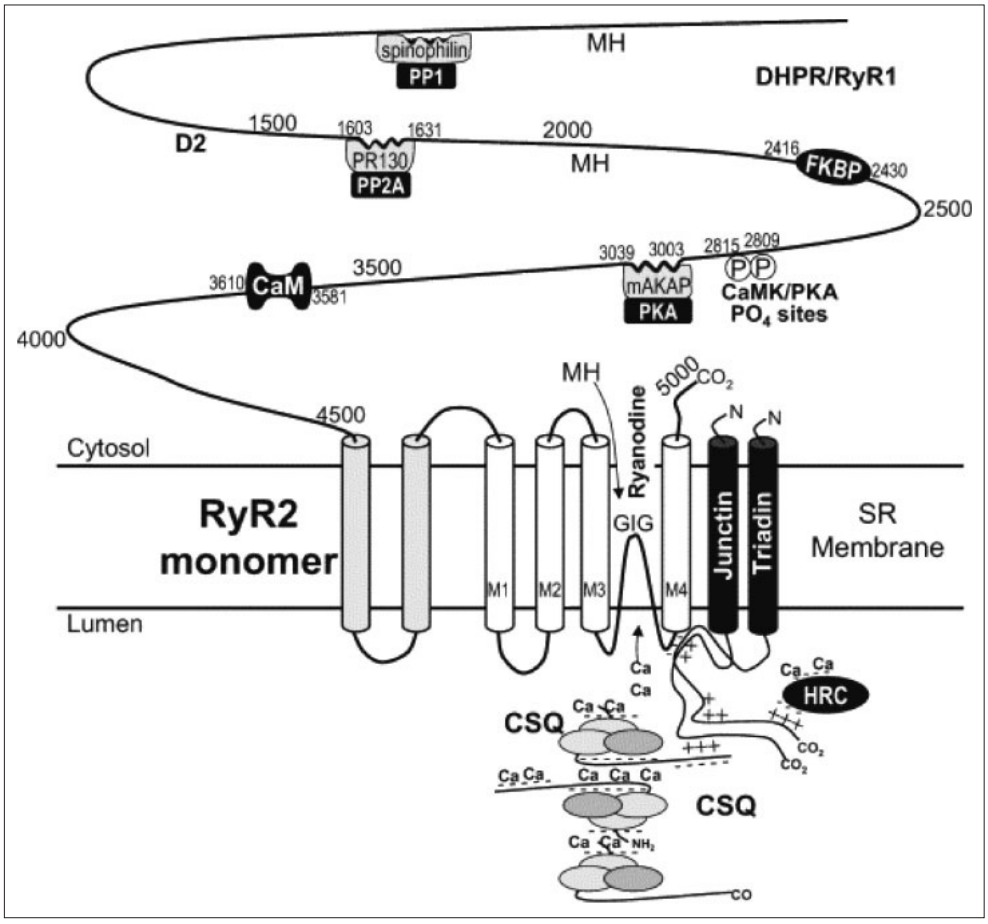

Figure 1 Schematic showing the predicted structure of the cardiac ryanodine receptor, RyR2, including the sites of interaction with ancillary proteins and the phosphorylation sites. Calsequestrin, junctin, and triadin, proteins interacting with ryanodine receptor in the SR, are also depicted. PP, protein phosphatase; P, phosphorylation sites; CaM, calmodulin; CaMK, calmodulin-dependent protein kinase. (Reprinted from Bers DM: Macromolecular complexes regulating cardiac ryanodine receptor function. J Mol Cell Cardiol 2004; 37: 417–429,[8] with permission from Elsevier)

calcium release into the cytosol through the RyR2 which is located in the membrane of the sarcoplasmic reticulum (SR). This process, called calcium-induced calcium release (CICR),[6] is the base of cardiac excitation–contraction (E–C) coupling: it activates the contractile apparatus. Calcium release terminates when SR luminal calcium falls below a threshold level, causing a decline in RyR2 activity via a mechanism called *luminal calcium dependent deactivation*.[7] To preserve the proper function of the contractile apparatus therefore cytosolic calcium levels are lowered by two systems: the first is represented by the calcium-pump ATPase (SERCA2) that reuptakes calcium back into the SR; the second mechanism is represented by the sodium-calcium exchanger (NCX) that extrudes the remaining smaller portion of cytosolic calcium from the sarcolemma and allows initiation of cardiomyocytes' relaxation.

It is known that the cardiac ryanodine receptor (RyR2) plays a central role in cardiac E–C coupling. RyR2 is a large tetrameric channels (2.2 MDa) located in the membrane of sarcoplasmic reticulum (SR). The transmembrane segments and the carboxy-terminal only comprise approximately 10% of the human RyR2 polypeptide (4967 amino acids), the remaining 90% of the molecule corresponds to the large cytoplasmic domain which binds several proteins, such as calmodulin (CaM), CaMkinase II, FKBP12.6 and sorcin (see **Figure 1**). that form a macromolecular complex.[8] Therefore, RyR2 activity is highly regulated by cytoplasmic signalling pathways that play an important role in the arrhythmogenesis of CPVT.

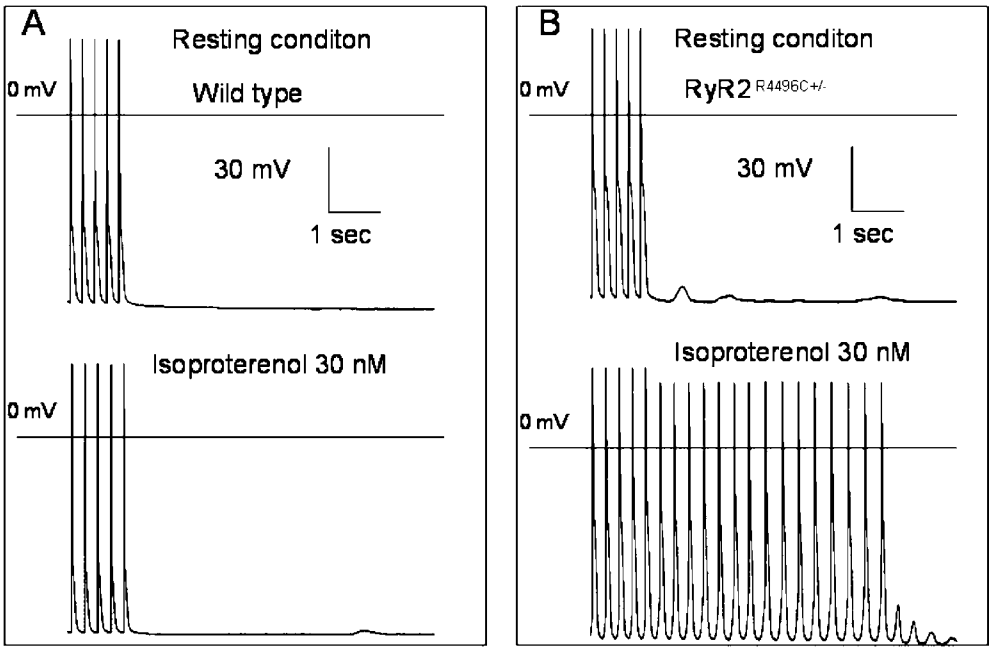

Figure 2 Action potential recording from a WT myocyte (panel A) and a $RyR2^{R4496C+/-}$ myocyte (panel B) in the absence (top panel) and in the presence (bottom panel) of isoproterenol (30 nM). Arrows indicate the last five paced action potentials. (Reprinted from Liu N et al.: Arrhythmogenesis in catecholaminergic polymorphic ventricular tachycardia: insights from a RyR2 R4496C knock-in mouse model. Circ Res 2006; 99: 292–298,[15] with permission from LWW)

RyR2 Mutations in CPVT Produce Gain of Function

To date, more than 60 RyR2 mutations have been reported (more information is available online at: http://www.fsm.it/cardmoc/). All RyR2 mutations identified so far in CPVT are mostly single-base-pair substitutions leading to the replacement of highly conserved amino acids. Most RyR2 mutations are localized in the C-terminal region or in the central region, a minority of RyR2 mutations are located in the other regions of the gene. In 2002, Jiang et al.[9] first characterized the properties of mutation R4496C of mouse RyR2, which is equivalent to a disease-causing human RyR2 mutation R4497C, by heterologous expression of the mutant in HEK293 cells and reported that R4496C mutation resulted in increased basal channel activity and enhanced the sensitivity to activation by Ca^{2+} and by caffeine. Subsequently other investigators confirmed that RyR2 mutations produce a "gain of function" and cause diastolic Ca^{2+} "leakage" from the SR in condition of sympathetic activation in lipid bilayers and HL1-cardiomyocytes experiments,[10,11] however, they did not demonstrate that RyR2 mutations increase resting channel activity. In 2004, Thomas et al. reported that RyR2 mutation L433P exhibits desensitised caffeine-induced activation[12] while shortly after Jiang et al. found that the L433P mutation increases, rather than decreasing, the sensitivity of the RyR2 channel to caffeine activation.[9] One potential explanation for this discrepancy may be related to the DNA constructs used. In the study of Thomas et al, the human RyR2 wild type and mutants were tagged at the N terminus with enhanced green fluorescence protein (GFP). This insertion of GFP into the N terminus may interfere with the action of the L433P mutation. Only few CASQ2 mutations have been identified and functionally characterized in vitro. In collaboration with the group of Gyorke, we demonstrated that all CASQ2 mutations produce a "gain of function" (increased response to RyR2 agonists that promote calcium release from the SR).[7,13] Taken together the basic science investigations suggest that all RyR2 and CASQ2 mutations that cause CPVT share some common features: they increase sensitivity to calcium overload in SR and enhance the propensity for spontaneous calcium release from the SR in the condition of sympathetic activation that is consistent with the clinical phenotype of CPVT.

Triggered Activity is the Electrophysiological Mechanism for CPVT

The hypothesis that arrhythmias in CPVT are initiated by delayed afterdepolarizations (DADs) and triggered activity had been advanced based on the observation that the bidirectional VT observed in CPVT patients closely resembles digitalis-induced arrhythmias.[2] Digitalis-induced intracellular Ca^{2+} overload leads to the activation of sodium-calcium exchanger that, in turn, generates a net inward current (the so-called "transient inward" Iti current). Iti underlies diastolic membrane depolarizazions, DADs, that may reach threshold for sodium current activation and trigger an abnormal beats. This mechanism for arrhythmia initiation is defined as "triggered activity".

Recently, the role of DADs in CPVT arrhythmogenesis was confirmed in our knock-in mouse model carrier of the RyR2 R4496C mutation in which develops the typical bidirectional VT upon exposure to caffeine and epinephrine.[14] In vitro investigation demonstrated that DADs are already spontaneously present in $RyR2^{R4496C+/-}$ myocytes in the absence of adrenergic stimulation but not in WT myocytes. Upon exposure to beta adrenergic stimulation we observed further enhancement of DADs and the development of multiple triggered action potentials arising from DADs (see **Figure 2**).[15] These data have been shortly after confirmed in $R176Q^{+/-}$ knock in mice model and $Casq2^{-/-}$ knock out mice model.[16,17] Since it is well known that Purkinje fibers are more sensitive to calcium overload than ventricular myocytes,[18] it is likely that bidirectional VT is triggered by DADs alternatively originating from right and left branches of the Purkinje fibers: preliminary evidence that this might be the case has been recently obtained by mapping the heart of our knock in mice during an episode of bidirectional VT.[19]

Molecular Mechanism of Abnormal Calcium Leaky from RyR2 in CPVT

In vitro studies (performed in lipid bilayers, HEK293 cells, HL1-cardiomyocytes) suggested that the RyR2 mutations produce a "gain of function" and cause diastolic Ca^{2+} "leakage" from the SR in condition of sympathetic activation leading to intracellular calcium overload that is responsible for the development of DADs and triggered activity.[9–11] Despite intensive investigations, the molecular mechanisms by which RyR2 mutations alter the physiological properties of RyR2 in CPVT to initiate such arrhythmogenic cascade remain highly controversial. In the following sections we will briefly review some of the leading mechanisms that have been proposed to explain the detrimental effect of RyR2 mutations identified in CPVT patients.

Reduced binding affinity of FKBP12.6 to CPVT-associated RyR2 mutations

Each RyR2 monomer binds a single FKBP12.6 molecule that stabilizes the RyR2 channel in the closed state and reduces its activity.[20] Marks and collaborators[11,21] have performed a very elegant series experiments and demonstrated that RyR2 mutants (S2246L, R2474S, R4497C, P2328S, Q4201R, and V4653F) cause a reduced affinity of the RyR2 channel for binding of the regulatory protein FKBP12.6 and that this defective interaction is further aggravated when the phosphorylation of RyR2 by PKA during adrenergic stimulation dissociates FKBP12.6 from the RyR2 channel complex, causing Ca^{2+} to leak out of the SR. Furthermore, on account of this hypothesis, the same group demonstrate that K201, a derivative of 1,4-benzothiazepine formerly called JTV 519, is able to enhance the binding of FKBP12.6 to RyR2 in FKBP12.6 $+/-$ mice thus preventing adrenergically induced arrhythmias and sudden death.[22] These observations provide compelling evidence that FKBP12.6 plays an important role in determining the arrhythmogenic response of RyR2 mutation in CPVT to PKA phosphorylation.

However, other investigators challenged this hypothesis. Two independent groups demonstrated that phosphorylation of RyR2 by PKA does not dissociate FKBP12.6 from CPVT-associated RyR2 mutant channels (S2246L, N4104K, R4497C, Q4201R, I4867M, S2246L, R2474S, R176Q(T2504M), and L433P).[10,23] Recently, we assessed the RyR2-FKBP12.6 association in WT and $RyR2^{R4496C+/-}$ mice, western blot analysis indicated that the relative amounts of FKBP12.6 to RyR2 found in heavy SR of the stimulated hearts was similar to that found in unstimulated controls, for both WT and $RyR2^{R4496C+/-}$ mice. These observations indicate normal RyR2–FKBP12.6 interaction in the heart from both WT and $RyR2^{R4496C+/-}$ animals, which is not altered following treatment with caffeine and epinephrine.[15] The reasons for the discrepancies in the FKBP12.6-binding affinity for CPVT-associated RyR2 mutant channels observed by different groups are not clear, it is possible that they depend on differences in experimental conditions.[24]

Enhanced store overload–induced calcium release in CPVT-associated RyR2 mutations

It is well known that when SR Ca^{2+} content reaches a critical level, spontaneous SR Ca^{2+} release occurs in the absence of membrane depolarization. Jiang et al. firstly demonstrated the link between defective luminal Ca^{2+} activation of RyR2 and CPVT, and referred to this process with the term enhanced store overload–induced calcium release (SOICR).[25] These authors used HEK293 cell lines stably expressing the CPVT RyR2 mutants, N4104K, R4496C, and N4895D and demonstrated that they exhibited an enhanced propensity for SOICR. They also showed that these RyR2 mutations increase the sensitivity of single RyR2 channels to luminal calcium dependent activation. Subsequently, this group confirmed and extended this hypothesis to other 6 RyR2 mutations located in different regions of the channel (Q4201R, I4867M, S2246L, R2474, R176Q(T2504M) and L433P) and demonstrated that HEK cell line and HL-1 cardiac cells expressing these CPVT RyR2 mutants all exhibited increased SOICR activity. Single channel analyses revealed that disease-linked RyR2 mutations primarily increase the channel sensitivity to luminal calcium, but not to cytosolic calcium.[23] The hypothesis of SOICR is attractive and it seem to provide another exclusive molecular mechanism for CPVT. However, Thomas et al.[26] demonstrated that L433P, N2386I, and R176Q(T2504M) exhibited significantly altered Ca^{2+} sensitivity characterised by a marked loss of cytosolic Ca^{2+}-dependent channel inhibition. Since some studies exhibited that luminal Ca^{2+} effects can be manifest as changes in cytosolic Ca^{2+} sensitivity,[27] so it is difficult to give a final conclusion in present time. RyR2 is a macromolecular complex that can be regulated by many accessory protein and intracellular signal pathway. Accordingly, although all CPVT associated RyR2 mutations exhibit a similar pathological phenotype, it is unlikely that a single unifying mechanism of RyR2 dysfunction underlie CPVT. Since mutations linked to the CPVT phenotype are located in physically and functionally distinct regions of RyR2, it is likely that the position of each mutation is the mechanistic determinant of RyR2 dysfunction.

Defective intermolecular domain interactions in CPVT-associated RyR2 mutations

Intramolecular interaction between discrete RyR2 domains is necessary for the normal function of the RyR2 channel.[28] It has been proposed that RYR2 mutations may cause dysfunction of these regions (unzipping) leading to RyR2 hyperactivation or hypersensitization that may result in Ca^{2+} leak from the SR. Recently, George et al,[29] using high-resolution confocal microscopy and fluorescence resonance energy transfer analysis, provided the first cell-based evidence to support the hypothesis that RyR2 mutations occurring in the central domain (S2246L) and the C-terminal domain (N4104K and R4497C) directly cause RyR2 channel instability via defective interdomain interaction, resulting in Ca^{2+} release dysfunction. More interestingly the same authors, using noise analysis, a powerful tool to elucidate mechanistically relevant information in the amplitude patterning of experimental traces, demonstrated that there are differences in the precise mode of Ca^{2+} release dysfunction and conformational instability arising from central or C-terminal domain mutations, C-terminal domain mutations exhibited postactivation channel instability that did not occur with central domain mutation. These findings support the view that abnormal interdomain interaction is a fundamental event in RyR2-mediated arrhythmogenesis and the mutational locus may be an important underlying mechanistic determinant of channel dysfunction.

Cardiac Ryanodine Receptor as a Novel Antiarrhythmic Target in CPVT

Beta adrenergic blockers is the first choice for CPVT patients. In the initial clinical observations, it appeared to be able to prevent the occurrence of cardiac events in CPVT patients,[2] however, incomplete protection from sudden cardiac death has been subsequently reported.[30–32] It is common practice to consider these individuals candidates for an ICD, given the young age of the patients, it would be certainly important to have other pharmacological options available. Since the defect of RyR2 mutation is the central issue of pathophysiology in CPVT, targeting cardiac ryanodine receptor emerges as therapeutic strategy in CPVT.

According to the hypothesis proposed by Marks, abnormal FKBP12.6–RyR2 interaction may be central in the pathogenesis of CPVT. Marks' group proposed that stabilizing cardiac ryanodine receptor by that increasing FKBP12.6 binding to mutant RyR2 channels might provide a very specific therapeutic strategy for preventing triggered arrhythmias in CPVT. In their planar lipid bilayers experiment, JTV 519, a derivative of 1,4-benzothiazepine, can increase the affinity of FKBP12.6 with CPVT-associated RyR2 mutations (P2328S, G4201A and V4653P), which normalized single channel gating.[11] Further, this group demonstrated that JTV 519 can

prevent adrenergically induced arrhythmias and sudden death in FKBP12.6 deficiency mouse model.[22] These findings suggest that JTV519 and its derivatives may represent a novel class of drugs for the treatment of the patients CPVT. Although we were unable to confirm the validity of this approach in our knock in animal model, the possibility that CPVT associated with other mutations may respond positively to the drug remains open.

As discussed above, albeit there exist debates in the underlying molecular mechanism of CPVT, all CPVT associated RyR2 mutations produce "gain of function" and lead to the final common pathway of promoting diastolic calcium leak from RyR2. It appears logical to hypothesize that reducing RyR2 open probability may be a straightforward strategy to seal the aberrant calcium leaky from mutant RyR2. Recently, Venetucci et al.[33] provided experimental evidence for clarifying this issue by using the analytic techniques to examine calcium fluxes in rat ventricular myocytes. After myocytes inducing the diastolic calcium release in presence of isoproterenol, application of tetracaine(50 μmol/L), a common drug for reducing RyR open possibility, abolish the diastolic calcium release. Surprisingly, this was accompanied by an increase in the amplitude and duration of the systolic calcium transient. These data suggest that reducing ryanodine receptor open probability without altering systolic function may provide another successful antiarrhythmic strategy in CPVT.

Conclusions

In the last six year, impressive advancements have been achieved in elucidating the arrhythmogenic mechanism of RyR2-associated CPVT. Although CPVT is uncommon genetic disorders, they have been considered as simplified human and experimental models that may help to clarify intracellular calcium regulation in heart what the Long QT Syndrome has represented for the understanding of the role of voltage-dependent channels in the study of cardiac excitability. Albert there exist debates, an innovative curative strategy thought the modulation of RyR2 function for calcium-associated arrhythmia disease emerges, it may apply not only to patients with CPVT, but also to individuals with acquired heart disease associated with dysfunctional intracellular calcium handling, such as heart failure.

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