Inherited Dysfunction of Sarcoplasmic Reticulum Ca\(^{2+}\) Handling and Arrhythmogenesis

Silvia G. Priori, S.R. Wayne Chen

Abstract: Catecholaminergic polymorphic ventricular tachycardia (CPVT) is an inherited arrhythmogenic disease occurring in patients with a structurally normal heart: the disease is characterized by life-threatening arrhythmias elicited by stress and emotion. In 2001, the ryanodine receptor was identified as the gene that is linked to CPVT; shortly thereafter, cardiac calsequestrin was implicated in the recessive form of the same disease. It became clear that abnormalities in intracellular Ca\(^{2+}\) regulation could profoundly disrupt the electrophysiological properties of the heart. In this article, we discuss the molecular basis of the disease and the pathophysiological mechanisms that are impacting clinical diagnosis and management of affected individuals. As of today, the interaction between basic scientists and clinicians to understand CPVT and identify new therapeutic strategies is one of the most compelling examples of the importance of translational research in cardiology. (Circ Res. 2011;108:871-883.)

Key Words: arrhythmias ■ genetics ■ ryanodine receptor ■ triggered activity ■ calcium regulation

Catecholaminergic polymorphic ventricular tachycardia (CPVT) is an inherited arrhythmogenic disease associated with cardiac arrest in the pediatric population. The disease was first described as a novel clinical entity by Coumel et al\(^1\) in 1978 and then in a follow-up study in 1995.\(^2\) With the advancements of molecular genetics the discovery of the genetic substrate of the disease\(^3,4\) showed that CPVT results from inherited abnormalities of intracellular Ca\(^{2+}\) regulation caused by dominant mutations in the \(RYR2\) gene, encoding the cardiac Ca\(^{2+}\) release channel (ryanodine receptor isoform 2 [RyR2])\(^3\) and by recessive mutations in the \(CASQ2\) gene, encoding cardiac calsequestrin isoform 2.\(^5\)

The discovery of the molecular substrate of CPVT has fuelled basic science studies to characterize \(RYR2\) and
CASQ2 mutations in vitro and in vivo, leading to important advancements in the understanding of intracellular Ca\textsuperscript{2+} regulation and its relevance to arrhythmogenesis.

In this article, we provide an overview of the developments that have occurred in the 10 years following the discovery of the first RYR2 mutations and the involvement of mutant RyR2 in CPVT patients. We start with the physiology of Ca\textsuperscript{2+} storage and release in the sarcoplasmic reticulum (SR) and its relevance to rhythm maintenance. We then discuss how mutations in RyR2 disrupt the Ca\textsuperscript{2+} handling system, leading to cardiac arrhythmias. Finally, we address how the understanding of the pathophysiology of the disease is leading to novel therapeutic strategies.

**Fundamental Mechanisms of Intracellular Ca\textsuperscript{2+} Handling**

Understanding of the physiology of Ca\textsuperscript{2+} handling in cardiac cells is critical for understanding how CPVT mutations alter intracellular Ca\textsuperscript{2+} regulation in the heart. Excitation–contraction (EC) coupling in cardiac muscle is mediated by a mechanism known as Ca\textsuperscript{2+}-induced Ca\textsuperscript{2+} release (CICR).\textsuperscript{6} During an action potential, the voltage-dependent L-type Ca\textsuperscript{2+} channel in the transverse T-tubular membrane is activated, resulting in a small influx of external Ca\textsuperscript{2+} into the cytosol. This Ca\textsuperscript{2+} binds to the cytosolic Ca\textsuperscript{2+} sensor in RyR2 located in the sarcoplasmic reticulum (SR) and opens the channel, leading to a large release of Ca\textsuperscript{2+} from the SR, the major intracellular Ca\textsuperscript{2+} store containing 1 to 1.5 mmol/L free Ca\textsuperscript{2+}.\textsuperscript{7} The Ca\textsuperscript{2+} released then binds to troponin C, causing a cascade of conformational changes in the myofilaments and ultimately muscle contraction. During the relaxation phase, SR Ca\textsuperscript{2+} release is terminated, and the released Ca\textsuperscript{2+} is recycled back to the SR by the SR Ca\textsuperscript{2+}-ATPase (SERCA) or extruded from the cell by the Na\textsuperscript{+}/Ca\textsuperscript{2+} exchanger (NCX), effectively lowering the cytosolic Ca\textsuperscript{2+} concentration and allowing the dissociation of Ca\textsuperscript{2+} from the myofilaments and muscle relaxation (Figure 1).\textsuperscript{8}

It has long been known that SR Ca\textsuperscript{2+} release can also occur in the absence of cellular depolarization through a mechanism referred to as spontaneous Ca\textsuperscript{2+} release\textsuperscript{9} that is facilitated by the presence of SR Ca\textsuperscript{2+} overload.\textsuperscript{10–12} Spontaneous SR Ca\textsuperscript{2+} release can also occur as a result of spontaneous membrane depolarization.\textsuperscript{13–15} To distinguish spontaneous Ca\textsuperscript{2+} release in the form of Ca\textsuperscript{2+} waves from depolarization-initiated Ca\textsuperscript{2+} release and considering its dependence on the size of the SR Ca\textsuperscript{2+} store, we have proposed that it be designated as Store Overload Induced Ca\textsuperscript{2+} Release (SOICR).\textsuperscript{16,17}

A number of conditions, including β-adrenergic stimulation, digitalis toxicity, elevated extracellular Ca\textsuperscript{2+}, and fast pacing can lead to SR Ca\textsuperscript{2+} overload and subsequently SOICR in cardiac cells.\textsuperscript{10,18} For instance, the release of catecholamines leads to the activation of β-adrenergic receptors and adenylate cyclase and to an increase of cAMP. The cAMP-dependent protein kinase (PKA) is then activated, leading to the phosphorylation of a number of protein targets, including the L-type Ca\textsuperscript{2+} channel and phospholamban, an inhibitor of SERCA. Phosphorylation of the L-type Ca\textsuperscript{2+} channel by PKA increases Ca\textsuperscript{2+} influx, whereas phosphorylation of phospholamban by PKA relieves its inhibition on SERCA and consequently increases SR Ca\textsuperscript{2+} uptake. Excessive β-adrenergic stimulation would, therefore, lead to augmented Ca\textsuperscript{2+} influx and SR Ca\textsuperscript{2+} uptake, resulting in SR Ca\textsuperscript{2+} overload and subsequently SOICR. In the case of digitalis toxicity, cardioglycosides such as ouabain or digoxin, inhibit the activity of Na\textsuperscript{+}/K\textsuperscript{+} ATPase, resulting in the accumulation of intracellular Na\textsuperscript{+}, which, in turn, inhibits the activity of the NCX. The decrease in NCX activity reduces Ca\textsuperscript{2+} extrusion from the cell, leading to more Ca\textsuperscript{2+} being recycled back to the SR, SR Ca\textsuperscript{2+} overload and SOICR. Similarly, elevated external Ca\textsuperscript{2+} or fast pacing will increase Ca\textsuperscript{2+} influx and SR Ca\textsuperscript{2+} loading and thus the propensity for SOICR.\textsuperscript{9} Overall, there are at least 3 major components of the progression to SR Ca\textsuperscript{2+} overload: (1) increased Ca\textsuperscript{2+} influx; (2) increased SR Ca\textsuperscript{2+} uptake; and (3) reduced Ca\textsuperscript{2+} extrusion (Figure 1).

**Electrophysiological Consequences of SOICR: Delayed Afterdepolarizations, Early Afterdepolarizations, and Triggered Arrhythmias**

It has long been recognized that SOICR can alter membrane potential. The large increase in cytosolic Ca\textsuperscript{2+} as a result of SOICR in the form of Ca\textsuperscript{2+} waves can activate NCX. Because NCX is electrogenic, activation of NCX will generate a transient inward current. This inward current can depolarize the surface membrane after the action potential is ended, and thus produce delayed afterdepolarizations (DADs).\textsuperscript{10–12} If the amplitude of DADs reaches the threshold for Na\textsuperscript{+} channel activation, DADs can trigger an action potential, which can lead to triggered arrhythmias.\textsuperscript{19–21} Recently, the mechanisms initiating early afterdepolarizations (EADs) have been reassessed.\textsuperscript{22} It was suggested that NCX may also be implicated in the generation of EADs.\textsuperscript{23} SOICR

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**Non-standard Abbreviations and Acronyms**

<table>
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<tr>
<th>Abbreviation</th>
<th>Definition</th>
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<tr>
<td>ARVD2</td>
<td>arrhythmogenic right ventricular cardiomyopathy type 2</td>
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<td>CASQ2</td>
<td>cardiac calsequestrin isoform 2</td>
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<td>CICR</td>
<td>Ca\textsuperscript{2+}-induced Ca\textsuperscript{2+} release</td>
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<td>CPVT</td>
<td>catecholaminergic polymorphic ventricular tachycardia</td>
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<td>DAD</td>
<td>delayed afterdepolarization</td>
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<td>DCM</td>
<td>dilated cardiomyopathy</td>
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<tr>
<td>EAD</td>
<td>early afterdepolarization</td>
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<tr>
<td>EC</td>
<td>excitation–contraction</td>
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<tr>
<td>NCX</td>
<td>Na\textsuperscript{+}/Ca\textsuperscript{2+} exchanger</td>
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<tr>
<td>PKA</td>
<td>protein kinase</td>
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<tr>
<td>RyR2</td>
<td>ryanodine receptor isoform 2</td>
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<tr>
<td>SERCA</td>
<td>sarcoplasmic reticulum Ca\textsuperscript{2+}-ATPase</td>
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<td>SOICR</td>
<td>store overload–induced Ca\textsuperscript{2+} release</td>
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<td>SR</td>
<td>sarcoplasmic reticulum</td>
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in the form of Ca\(^{2+}\) waves can also trigger intracellular Ca\(^{2+}\) alternans and sudden changes in action potential duration.\(^{24}\) Interestingly, both the magnitude and the rate of spontaneous SR Ca\(^{2+}\) release events are critical for the determination of whether triggered activity could occur.\(^{25}\) It has been estimated that a total SR Ca\(^{2+}\) release of 50 to 60 \(\mu\)mol/L cytosol, or 50% to 70% of the SR Ca\(^{2+}\) load, is required to produce DADs with an amplitude that is sufficient to trigger an action potential.\(^{21}\) In addition to Ca\(^{2+}\) waves, spontaneous SR Ca\(^{2+}\) release can occur in the form of brief, localized Ca\(^{2+}\) transients (Ca\(^{2+}\) sparks)\(^{26}\) or invisible Ca\(^{2+}\) leak through “rogue” RyR\(_{2}\).\(^{27}\) These small visible or invisible SR Ca\(^{2+}\) leaks as a result of “leaky” RyR\(_{2}\) channels by themselves are unlikely to generate DADs with amplitudes that are high enough to produce an action potential or triggered activity. It is the Ca\(^{2+}\) overload-induced spontaneous SR Ca\(^{2+}\) release in the form of Ca\(^{2+}\) waves (SOICR) that is capable of producing DAD-mediated triggered arrhythmias.

**Mechanisms of Store Overload Induced Ca\(^{2+}\) Release**

Spontaneous SR Ca\(^{2+}\) release occurs when the SR Ca\(^{2+}\) content reaches a critical load,\(^{28}\) suggesting that luminal Ca\(^{2+}\) concentration is the most plausible trigger for spontaneous SR Ca\(^{2+}\) release during SR Ca\(^{2+}\) overload. However, the evidence that Ca\(^{2+}\) release increases in a steep and nonlinear fashion with increasing SR luminal Ca\(^{2+}\) concentration\(^{29}\) suggests that Ca\(^{2+}\) release is not a passive flow driven by Ca\(^{2+}\) gradient, rather it is an active process regulated by more complex and perhaps multiple mechanisms.\(^{30}\)

It has been proposed that luminal Ca\(^{2+}\) activates RyR\(_{2}\) by passing through the open channel and acting on the cytosolic Ca\(^{2+}\) activation site of the channel. This is known as the “feed-through” hypothesis.\(^{31,32}\) However, the finding that single RyR\(_{2}\) channels are still activated by luminal Ca\(^{2+}\) under conditions where luminal-to-cytosolic Ca\(^{2+}\) flux is absent does not support the feed-through hypothesis.\(^{33,34}\) As a consequence it was suggested that the luminal Ca\(^{2+}\) is sensed by a luminal Ca\(^{2+}\) activation site distinct from the cytosolic Ca\(^{2+}\) sensor.\(^{33}\) Recently, it has been proposed that luminal-to-cytosolic Ca\(^{2+}\) flux may be required for a full activation of the RyR\(_{2}\) by luminal Ca\(^{2+}\).\(^{35}\) At present, most evidence supports the view that activation of RyR\(_{2}\) by luminal Ca\(^{2+}\) is mediated by a luminal Ca\(^{2+}\) sensor that is distinct from the cytosolic Ca\(^{2+}\) sensor, yet the molecular identity of this proposed luminal Ca\(^{2+}\) sensor is still undefined.

**Cytosolic Ca\(^{2+}\) Versus Luminal Ca\(^{2+}\) in the Regulation of RyR\(_{2}\)**

Activation of RyR\(_{2}\) by cytosolic Ca\(^{2+}\) underlies the physiological release of Ca\(^{2+}\) from SR (CICR); activation of RyR\(_{2}\)
by luminal Ca\(^{2+}\) during SR Ca\(^{2+}\) overload leads to spontaneous Ca\(^{2+}\) release (store-overload induced-Ca\(^{2+}\) release or SOICR). Despite the fact that RyR2 mediates CICR, moderate modulation of RyR2 does not have a sustained impact on CICR. This phenomenon, often referred to as “SR auto-regulation,” is thought to be the result of RyR2 regulation by SR luminal Ca\(^{2+}\). \(^{36}\) Moderate changes in RyR2 activity are compensated for by the SR Ca\(^{2+}\) content. For instance, increasing RyR2 activity with low concentrations of caffeine would result in an increase in Ca\(^{2+}\) release and a decrease in SR Ca\(^{2+}\) content, which, in turn, would reduce the channel activity via the luminal Ca\(^{2+}\) regulatory mechanism. \(^{36}\) Although modulation of RyR2, to some extent, may not have a sustained impact on CICR, it can significantly influence the properties of SOICR. For example, increasing RyR2 activity with low concentrations of caffeine lowers the critical SR Ca\(^{2+}\) concentration at which SOICR occurs and increases its frequency. Conversely, inhibiting RyR2 activity by tetracaine increases the threshold for SOICR and lowers its frequency. \(^{37}\)

As a consequence, even small alterations of RyR2 will have a significant impact on SOICR and thus on the occurrence of DADs and triggered arrhythmias (Figure 1).

Role of Calsequestrin in SOICR

Gyorke and colleagues\(^{38,39}\) have provided data supporting the view that CASQ2 is the luminal Ca\(^{2+}\) sensor responsible for regulation of RyR2 by luminal Ca\(^{2+}\), and for the initiation and termination of SOICR. The authors proposed that CASQ2 monomers inhibit RyR2 at low SR Ca\(^{2+}\) concentrations by binding to the complex formed by triadin, junctin, and RyR2. \(^{39}\) At high SR Ca\(^{2+}\) concentrations, however, CASQ2 monomers assemble into polymers and dissociate from the RyR2 channel complex, thus relieving the inhibition of RyR2, leading to channel activation and spontaneous Ca\(^{2+}\) release. \(^{38}\)

Knollmann et al.\(^{40}\) have provided data that is inconsistent with the theory by Gyorke showing instead that cardiac myocytes from CASQ2-null mice still display a steep and nonlinear relationship between SR Ca\(^{2+}\) release and SR Ca\(^{2+}\) load, indicating that the RyR2 channel can sense luminal Ca\(^{2+}\) in the absence of CASQ2. Furthermore, CASQ2-null cardiac myocytes exhibit normal intracellular Ca\(^{2+}\) handling at low SR Ca\(^{2+}\) concentrations, indicating that the removal of CASQ2 does not lead to a marked activation of RyR2 at low SR Ca\(^{2+}\) concentrations. Finally, CASQ2-null cardiac myocytes show spontaneous Ca\(^{2+}\) waves at high SR Ca\(^{2+}\) load, indicating that in the absence of CASQ2, spontaneous SR Ca\(^{2+}\) release can be still initiated and terminated. \(^{40}\) Taken together, these observations demonstrate that CASQ2 may modulate SR Ca\(^{2+}\) release, but is not essential for luminal Ca\(^{2+}\) regulation of RyR2 or for the initiation and termination of spontaneous SR Ca\(^{2+}\) release in cardiac myocytes.

Because purified native and recombinant RyRs remain sensitive to luminal Ca\(^{2+}\), it is likely that the luminal Ca\(^{2+}\) sensor lies within the primary structure of RyR2, and is potentially regulated by a number of factors and proteins associated with the Ca\(^{2+}\) release complex, including CASQ2, triadin, junctin, FKBP12.6, calmodulin, kinases, or other RyR2-associated proteins.

Linking Intracellular Ca\(^{2+}\) Handling With CPVT

The diagnosis of CPVT is most commonly established in apparently healthy pediatric patients suddenly and unexpectedly manifesting stress- or emotion-induced syncopal episodes. Occasionally, sudden cardiac death is the first clinical event in an otherwise healthy young individual.\(^{44–46}\) Unless diagnosed and treated the disease is lethal: in our series of patients, up to 30% of misdiagnosed and affected individuals died suddenly before the fourth decade of life.

The diagnosis of CPVT is challenging considering that the absence of a family history indicative of a genetic arrhythmogenic condition, CPVT can be suspected only during exercise stress testing when patients develop exercise-related ventricular tachycardia. During exercise stress test, CPVT patients show isolated premature beats at the beginning of exercise with a progressive worsening of the complexity of ventricular arrhythmias in response to an increased workload. Typically, when the heart rate reaches 90 to 110 bpm, runs of nonsustained or sustained VT appear and they may degenerate into sustained ventricular tachycardia (VT) and ventricular fibrillation (VF) unless exercise is promptly terminated. The morphology of VT is often the hallmark of the disease: the so-called bidirectional VT,\(^{1,3}\) which is characterized by a 180° beat-to-beat rotation of the axis of the QRS complexes on the frontal plane (Figure 2). Interestingly, bidirectional VT is also the typical morphology of ventricular tachycardia in the setting of digitalis intoxication thus suggesting that the 2 arrhythmogenic conditions may share similar electrophysiological bases.

Bidirectional VT is considered the diagnostic marker of CPVT, however, not all patients with the disease manifest this form of arrhythmias. Based on our patient population, we were able to identify different phenotypic manifestations of
CPVT among patients with RyR2 mutations: (1) patients presenting reproducible bidirectional VT and polymorphic VT at exercise stress testing; (2) patients presenting only with polymorphic VT; and (3) survivors of cardiac arrest and lacking induction of arrhythmias during exercise stress test.3 The latter group is intriguing and it is arguable whether they should be considered part of the CPVT phenotype. A subset of these patients, referred for genetic testing with the diagnosis of idiopathic VF, carry RyR2 mutations. It will be of interest to determine whether the impact of these idiopathic VF RyR2 mutations differ from that of the typical CPVT RyR2 mutations.

Molecular screening of the RYR2 and CASQ2 genes importantly contributes to diagnosis in patients with less typical phenotypic manifestations. Functional characterization in vitro as well as knock-in mouse models carrying clinical mutations also provide important information that helps us to understand how abnormalities in RyR2 and CASQ2 disrupt the physiology of intracellular Ca\(^{2+}\) regulation, leading to arrhythmic storms.

**RyR2 Mutations Linked to CPVT**

The RyR2 channel is a homotetramer with each subunit containing a large cytosolic domain formed by the first \(\approx 4300\) N-terminal residues and a smaller transmembrane (TM) domain formed by the last \(\approx 500\) C-terminal residues. The TM domain of RyR2 encompasses the channel pore, whereas the cytosolic domain contains binding sites for a number of channel regulators. In such a large and complex protein, it is challenging to investigate the topology of mutations in an attempt to derive structure-function information.

Mutations in genes encoding the voltage dependent ion channels that cause several clinical diseases, are often distributed across the entire coding region of the genes of interest. RyR2 mutations appear to be preferentially located in four regions (Figure 3). The term “domains” indicates those regions, according to this classification, domain I includes amino acids 77 to 466, domain II amino acids 2246 to 2534, domain III amino acids 3778 to 4201 and domain IV amino acids 4497 to 4959 (Figure 4). These regions are highly conserved in RyR across species and are superimposable (except for region III) to the localization of RyR1 mutations associated with central core disease and malignant hyperthermia.47

Mutations in RyR2 are not uniformly distributed across the four domains: mutations in domains III and IV collectively account for 46% of all mutations reported, followed by mutations located in domain II (21%) and by mutations in the N terminus (18%). It has been suggested that only a small number of mutations are positioned in less conserved regions outside the above mentioned domains: Medeiros Domingo et al46 reported that only 10% of mutations are outside domains I-IV. At variance with this estimation, analysis of our CPVT cohort by direct ORF sequencing indicates that 24% of RyR2 mutations identified in CPVT patients are located outside the four canonical domains.

Most mutations identified in RyR2 are single nucleotide replacements (also called “point mutations”) leading to an amino acid substitution: this is at variance with the case for other channelopathies where mutations such as truncations, deletions and insertions are more common. Premature stop codons, frame shifts, and out-of-frame insertions or deletions have not been identified in CPVT patients screened for mutation on the RYR2 gene. The lack of identification of loss of function mutations in RyR2 may be the consequence of the fact that the screening of RyR2 is usually targeted to patients with the CPVT phenotype and structurally intact heart. Therefore, it is possible that loss of function mutations result in different diseases possibly including structural abnormalities of the heart consistent with a cardiomyopathic phenotype. Some authors49–52 have suggested that RyR2 mutations may cause arrhythmogenic right ventricular cardiomyopathy type 2 (ARVD2).
Small in-frame duplications or in-frame insertions are present in patients with the CPVT phenotype, suggesting that their functional consequences are similar to that of point mutations, rendering the channel more prone to spontaneous SR Ca\(^{2+}\) release during adrenergic stimulation. We have identified 82 \(R\!Y\!R\!2\) mutations in our CPVT probands: 79 were point mutations and 3 (3.6%) were small deletions/insertions (one in frame insertion and 2 in frame deletions). Carriers of insertion/deletions had clinical manifestations consistent with CPVT and no structural abnormalities. Bhuiyan et al.\(^{54}\) using multiplex ligation-dependent probe amplification (MLPA) analysis, identified a large deletion encompassing part of intron 2, exon 3, and part of intron 3 associated with a complex clinical phenotype that included progressive AV block and SAN dysfunction, AF, atrial standstill, and depressed LV function to dilated cardiomyopathy (DCM). However, other reports of large deletions in the same region failed to confirm the association with DCM: large exon 3 deletions were reported by Marjamaa et al.\(^{55}\) in 2 apparently unrelated patients. One of these patients showed no major structural abnormalities, whereas the other patient showed increased trabeculation of the left ventricle, suggestive of noncompaction cardiomyopathy. Medeiros-Domingo et al.\(^{48}\) reported a large 3.6 kb exon 3 deletion, but the phenotype of the patient was not reported. Large deletions encompassing exon 3 seem to be relatively frequent, possibly because the region presents \(Alu\) repeats that may predispose genomic rearrangements.\(^{56}\) Whether these cause distinguishing phenotypes in addition to CPVT is still undefined.

**Functional Consequences of RyR2 Mutations**

Delayed afterdepolarization and triggered activity has been shown in knock-in models of CPVT (Figure 5).\(^{57}\) A key and fundamental question is what aspects of RyR2 function are impaired by these disease-causing RyR2 mutations. Considering the important role of luminal Ca\(^{2+}\) in triggering SOICR and DADs, Jiang and colleagues\(^{16,17}\) directly determined the impact of a number of CPVT RyR2 mutations on the sensitivity of single RyR2 channels to luminal Ca\(^{2+}\) activation in the near absence of cytosolic Ca\(^{2+}\) using single channel recordings in planar lipid bilayers. In these studies, a spectrum of RyR2 mutations were used, extending from the N- to the C-terminus, with small phenotypic differences among carriers: L433P and R176Q/T2504M located in the N-terminal region of the channel; mutations S2246L and R2474S located in the central region; and Q4201R, N4104K, R4496C, I4867M, N4895D, and V4653F\(^{58}\) located in the C-terminal region. All of these mutations showed a consistent behavior characterized by an enhanced response of the channel to luminal Ca\(^{2+}\) activation. Interestingly, \([3H]\)ryanodine binding or single channel studies revealed that most of the CPVT RyR2 mutations tested did not markedly affect the response of the RyR2 channel to cytosolic Ca\(^{2+}\). Exceptions were N4104K and V4653F, which also significantly increased the activation of \([3H]\)ryanodine binding by low concentrations of cytosolic Ca\(^{2+}\). The R4496C mutation\(^{1}\) also increases the cytosolic Ca\(^{2+}\) activation of \([3H]\)ryanodine binding.\(^{59}\) These observations indicate that CPVT RyR2 mutations preferentially sensitize the channel to luminal Ca\(^{2+}\) activation, whereas only a few CPVT RyR2 mutations sensitize the channel to both cytosolic and luminal Ca\(^{2+}\) activation.

In agreement with the role of luminal Ca\(^{2+}\) activation of RyR2 in SOICR, CPVT RyR2 mutations that increase the response of the channel to luminal Ca\(^{2+}\) also enhance the propensity for SOICR in HEK293 cells by reducing the threshold luminal Ca\(^{2+}\) level at which SOICR is triggered. Enhanced SOICR has also been observed in cardiomyocytes isolated from various knock-in mice harboring CPVT RyR2 mutations, R176Q,\(^{60}\) R4496C,\(^{59,61}\) or R2474S.\(^{62}\) The same manifestation of CPVT RyR2 mutations in both cardiomyo-
cytes and in HEK293 cells, which lack a number of cardiac specific proteins, including CASQ2, suggests that SOICR is not unique to cardiac cells, and is primarily determined by the intrinsic properties of the RyR2 channel. Together, these observations support the view that the luminal Ca$^{2+}$ sensor of RyR2 is located within its primary sequence and is responsible for the initiation and termination of SOICR.

Although CPVT RyR2 mutations alter the sensitivity of the channel to Ca$^{2+}$ activation, they apparently have little or no impact on excitation-contraction (EC) coupling, as patients with CPVT RyR2 mutations do not show arrhythmias in unstimulated conditions.63 This is likely attributable to the unique auto-regulatory property of SR Ca$^{2+}$ release. The SR Ca$^{2+}$ content may compensate for the defective luminal or cytosolic Ca$^{2+}$ activation of RyR2. This is because an enhanced sensitivity to luminal or cytosolic Ca$^{2+}$ activation would lead to increased SR Ca$^{2+}$ release and a reduction in SR Ca$^{2+}$ content, which, in turn, would decrease luminal Ca$^{2+}$ activation of RyR2. Hence, because of the autoregulation of SR Ca$^{2+}$ release, altered Ca$^{2+}$ activation of RyR2 would not have a sustained impact on EC coupling under normal conditions.64 However, under conditions of SR Ca$^{2+}$ overload, SR auto-regulation becomes ineffective, resulting in SOICR and, consequently, in DADs and triggered arrhythmias (Figure 6).

How RyR2 Mutations Alter the Sensitivity of the Channel to Luminal or Cytosolic Ca$^{2+}$ Activation?

In the previous section, we have presented robust evidence supporting the view that CPVT RyR2 mutations alter the sensitivity of the channel to luminal and/or cytosolic Ca$^{2+}$ activation, leading to enhanced spontaneous Ca$^{2+}$ release during SR Ca$^{2+}$ overload (SOICR). What remains unclear today is how CPVT RyR2 mutations exert these effects. Two mechanisms have been proposed and will be presented below: Domain unzipping and FKBP12.6 unbinding.

**Domain Unzipping**

It has been proposed that the N-terminal domain in RyR2 interacts with the central domain, and that CPVT RyR2 mutations in the N-terminal and central domains weaken this interaction (domain unzipping).65,66 These domain interactions are believed to be involved in the stabilization of the closed state of the channel. Hence, domain unzipping as a result of mutations would destabilize the closed state of the channel, rendering the channel more sensitive to stimuli, such as luminal or cytosolic Ca$^{2+}$.

Evidence for unzipping of the interaction between the N-terminal and central domains raises the question of whether “domain unzipping” is present in other regions of RyR2 in which CPVT mutations are located. In support of
this view are data showing that multiple, large conformational changes in the RyR2 structure occur during channel gating. Therefore, it is reasonable to hypothesize that mutations located throughout the molecule could alter physiologically important conformational changes, resulting in channel dysfunction. Recently investigators reported the 3D structures of fragments of the N-terminal region of RyR1 and RyR2 that contain a number of disease-linked RyR1 or RyR2 mutations and showed that most of them are located on the surface of domains and within domain interfaces where they could disrupt domain–domain interactions.\textsuperscript{67–69}

\textbf{FKBP12.6 Unbinding}

Another mechanism by which mutations may alter the sensitivity of the channel to Ca\textsuperscript{2+} activation is the disruption of critical protein–protein interactions. In this regard, it has been proposed that RyR2 mutations may specifically impair the interaction between RyR2 and the 12.6 kDa FK506 binding protein (FKBP12.6).\textsuperscript{70} FKBP12.6 is thought to play an important role in stabilizing the RyR2 channel and dissociation of FKBP12.6 from RyR2 as a result of phosphorylation of RyR2 by PKA during \(\beta\)-adrenergic stimulation has been shown to increase the sensitivity of the channel to cytosolic Ca\textsuperscript{2+} activation.\textsuperscript{71} In the hypothesis proposed by Wehrens et al.,\textsuperscript{70} RyR2 mutations may impair FKBP12.6 binding to RyR2 making the channel leaky. A number of studies have been carried out to determine the impact of CPVT RyR2 mutations on RyR2-FKBP12.6 interaction. Wehrens et al.\textsuperscript{70} showed that CPVT RyR2 mutations, S2246L and P2328S located in the central region, and Q4201R, R4496C, and V4653F located in the C-terminal region, reduced the affinity of FKBP12.6 binding to RyR2. Based on these observations they proposed that reduced FKBP12.6 binding affinity is a common defect of CPVT RyR2 mutations. Subsequently, Wehrens et al.\textsuperscript{70} and Lehnart et al.\textsuperscript{72} showed that the R2474S mutation also decreases the affinity of FKBP12.6 binding. These findings concerning causative alterations in the binding of FKBP12.6 to RyR2 have not been confirmed in more recent studies. For example, Tiso et al.\textsuperscript{73} showed that the same R2474S mutation was found to increase the affinity of FKBP12.6 binding. Moreover, Liu et al.\textsuperscript{57} studying the R4496C knock-in mouse model, showed that a compound

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\includegraphics[width=\textwidth]{figure6.png}
\caption{A unifying theory for CPVT? The SOICR thresholds and free luminal Ca\textsuperscript{2+} levels in normal SR (B) and abnormal SR associated with CASQ2 mutations (A) or RyR2 mutations (C) in the resting state (top) and under the conditions of SR Ca\textsuperscript{2+} overload (bottom) are shown. The normal SOICR threshold is depicted as a dashed red bar, whereas the mutation-lowered SOICR threshold is depicted as a solid red bar. The blue area represents the free SR luminal Ca\textsuperscript{2+} concentration, whereas the yellow area represents the increased free SR luminal Ca\textsuperscript{2+} level during a sudden increase in SR Ca\textsuperscript{2+} loading. The RyR2 channel complex is depicted as pink diamond structures. Mutations in RyR2 lower the SOICR threshold (C), whereas mutations in CASQ2 reduce the level of CASQ2 protein and/or Ca\textsuperscript{2+} buffering capability (A). The R33Q CASQ2 mutation or a reduction in the CASQ2 protein level has also been shown to lower the SOICR threshold (A). During SR Ca\textsuperscript{2+} overload, the free luminal Ca\textsuperscript{2+} level is more likely to exceed the RyR2 mutation-lowered SOICR threshold (C, bottom) or the normal or reduced SOICR threshold in the absence or lack of SR Ca\textsuperscript{2+} buffering as a result of CASQ2 mutations (A, bottom), leading to SOICR that can produce DADs and triggered arrhythmias. (Adapted from MacLennan DH, Chen SR. Store overload-induced Ca\textsuperscript{2+} release as a triggering mechanism for CPVT and MH episodes caused by mutations in RYR and CASQ genes. J Physiol. 2009;587[Pt 13]:3113–3115.) (Illustration Credit: Cosmocyte/Ben Smith).}
\end{figure}
that promotes the binding of FKBP12.6 to RyR2, K201, did not alter arrhythmogenesis in this mouse model. These findings suggest that either FKBP12.6 binding is not critical for arrhythmogenesis in CPVT or that it is critical only for selected mutations. Studies by George et al. and Jiang and colleagues. then demonstrated that CPVT RyR2 mutations had no effect on FKBP12.6 binding. Recent data from Bers and coworkers demonstrated that PKA is not involved in the dissociation of FKBP12.6 from RyR2, thus questioning any link between RyR2 phosphorylation and FKBP12.6 dissociation. Therefore, whether CPVT RyR2 mutations alter the link between RyR2 phosphorylation and FKBP12.6 dissociation, and whether CPVT RyR2 mutations affect the dissociation of FKBP12.6 will be critical to the understanding of CPVT RyR2 channel complex and affect the response of the channel to luminal Ca2+ concentrations. However, it is important to point out that CASQ2 null cardiomyocytes exhibit no abnormal SR Ca2+ release at low SR Ca2+ concentrations, and that CASQ2 null mice show no ventricular arrhythmias at rest. Therefore, the significance of CASQ2’s inhibitory effect on RyR2 at low SR Ca2+ concentrations is unclear.

The level of CASQ2 has also been suggested to be important in modulating the activity of RyR2. Chopra et al. showed that heterozygous CASQ2-null cardiac cells displayed more SR Ca2+ leak than the wild-type control cells at the same free SR Ca2+ concentration. This observation led to the conclusion that a modest reduction in CASQ2 protein level (≈25%) can reduce the threshold for spontaneous SR Ca2+ leak. However, Kubalova et al. have demonstrated that increasing (≈3.5 fold) or decreasing (to 30% of control) the level of CASQ2 protein has no effect on the threshold SR Ca2+ level at which spontaneous Ca2+ waves occur. Changing the level of CASQ2 protein, however, markedly alter the dynamics of SR Ca2+ recovery after SOICR. These observations indicate that CASQ2 via its Ca2+ buffering function mainly affects the dynamics of SR Ca2+ recovery or the frequency of SOICR, but not the threshold for SOICR. In this regard, it will be important to determine whether a reduction (25%) of CASQ2 in heterozygous CASQ2-null cardiac cells alters the dynamics and amplitude of free SR Ca2+ concentrations during spontaneous Ca2+ waves.

The clinical phenotype of patients affected by the recessive variant of CPVT is virtually identical to the autosomal dominant form except that carriers of homozygous CASQ2 mutation have more severe manifestations than carriers of heterozygous RyR2 mutations. In analogy with humans, mice with homozygous CPVT CASQ2 mutations display phenotypes virtually identical to, but more severe than, those observed in mice with heterozygous CPVT RyR2 mutations, suggesting that CASQ2-linked CPVT and RyR2-associated CPVT share a common causal mechanism (Figure 6). In other words, DADs induced by spontaneous SR Ca2+ release during Ca2+ overload (SOICR) is likely to be the cause for CASQ2-linked CPVT. An unresolved question is how CPVT CASQ2 mutations lead to abnormal activation of normal RyR2 by elevating luminal Ca2+, thus leading to enhanced SOICR and DADs. Some important clues to this question are emerging. An increasing body of evidence indicates that a reduction in the level of CASQ2 protein and

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**Functional and Structural Consequences of CASQ2 Mutations**

Mutations in the gene encoding the cardiac calsequestrin isoform 2 (CASQ2), mapping to chromosome 1p13.3-p11, causes an autosomal recessive form of CPVT (CPVT2). The first homozygous CASQ2 mutation (D307H) was identified in a large consanguineous Bedouin family and reported by Lahat et al. in 2001. As expected for a recessive disease, CPVT2 is much less common than the autosomal dominant form of the disease. As a consequence, eight years after the original description of the first CASQ2 mutations, only a few additional variants have been identified. As of today 12 CPVT-associated mutations and 3 non synonymous polymorphisms (cSNP) are known (http://www.fsm.it/cardmoc/). Among the 12 CPVT CASQ2 mutations, 4 are nonsense mutations that will lead to the expression of a truncated protein. Among the remaining 8 missense mutations, R33Q and D307H have been shown to reduce the level of CASQ2 protein to 5% and 45%, respectively. Hence, both the nonsense and some missense mutations lead to reduced CASQ2 protein levels and consequently reduced SR Ca2+ buffering capacity. The R33Q, K206N, L167H, and D307H mutations have also been shown to alter the Ca2+ binding capacity and/or the Ca2+-dependent polymerization of CASQ2. Because the CASQ2 polymer is responsible for high capacity Ca2+ binding, such alterations will also lead to a reduction in SR Ca2+ buffering capacity. The molecular defects of other missense mutations (Y55C, P308L, E177Q, and F189L) have not been characterized.

The Y55C and P308L are compound heterozygous CASQ2 mutations associated with CPVT. Nevertheless, these observations clearly demonstrate that reduced SR Ca2+ buffering capacity is a common consequence of CPVT CASQ2 mutations. Reduced SR Ca2+ buffering will result in a fast recovery of SR free Ca2+ after each Ca2+ release and a potentially higher level of SR free Ca2+ during a sudden increase in SR Ca2+ loading, both of which will increase the propensity for SOICR and thus DADs and triggered activity (Figure 6).

Some CASQ2 mutations may alter the interactions between CASQ2 and the RyR2 channel complex, thus affecting the response of RyR2 to elevating luminal Ca2+. For example, unlike the CASQ2 wt, the R33Q CASQ2 mutant has been shown to be unable to effectively inhibit the RyR2 channel at low SR Ca2+ concentrations. Cardiomyocytes overexpressing the R33Q mutant showed a reduced threshold SR Ca2+ level at which spontaneous Ca2+ release occurs. These observations have led to the proposal that the R33Q mutation may alter the interaction of CASQ2 with the RyR2 channel complex and affect the response of the channel to luminal Ca2+. However, it is important to point out that CASQ2 null cardiomyocytes exhibit no abnormal SR Ca2+ release at low SR Ca2+ concentrations, and that CASQ2 null mice show no ventricular arrhythmias at rest. Therefore, the significance of CASQ2’s inhibitory effect on RyR2 at low SR Ca2+ concentrations is unclear.

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Ca\(^{2+}\) buffering capacity is a common defect of CPVT CASQ2 mutations.\(^{93}\)

**Therapeutic Approaches to CPVT**

The understanding of the basic mechanisms of arrhythmogenesis in CPVT should guide the identification of novel therapeutic strategies. It seems that promising approaches for suppressing CPVT are (1) the prevention of SR Ca\(^{2+}\) overload and (2) the resolution of the defect in SOICR by RyR2 modulation.

Agents like \(\beta\)-blockers and Ca\(^{2+}\) channel inhibitors have been used in experimental as well as clinical settings, primarily act by reducing calcium overload via the reduction of heart rate and L-type Ca\(^{2+}\) channel current, and through the inhibition of phosphorylation of phospholamban and thus the activation of SERCA.

Inhibition of RyR2 may also be an important determinant of the effectiveness of antiarrhythmic drugs in CPVT. As suggested by recent data discussed below, flecainide may cause an open state block of the RyR2 channel, which may prevent the arrhythmogenic Ca\(^{2+}\) waves without affecting SR leak-load balance.

As of today the clinical management of CPVT aims at attenuating the arrhythmogenic effect of adrenergic stimulation through the use of \(\beta\)-blockers.

However, already in 2002, we reported\(^4\) that \(\beta\)-blockers can only confer partial protection from life-threatening arrhythmias among patients compliant to therapy with the maximal tolerated dose of \(\beta\)-blockers. When patients present recurrence of arrhythmic events on \(\beta\)-blockers, they are often implanted with an implantable cardioverter defibrillator. However, given the young age of patients, the use of the defibrillator is often challenging and associated with complications such as lead-fracture and infection of the pocket that contains the generator.

Clearly there are now expectations that recent basic science advancements in the understanding of the disease may guide the development of novel therapies. Prompted by the evidence that CPVT is caused by abnormalities in Ca\(^{2+}\) handling, some authors decided to test the efficacy of verapamil to RyR2 modulation.

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Clearly there are now expectations that recent basic science advancements in the understanding of the disease may guide the development of novel therapies. Prompted by the evidence that CPVT is caused by abnormalities in Ca\(^{2+}\) handling, some authors decided to test the efficacy of verapamil to RyR2 modulation. Whether this mechanism is present at clinically relevant concentrations of the drug, has not been established.

Recently, in vitro data showed that, in analogy with Ca\(^{2+}\) channel blockers, flecainide, a Na\(^{+}\) channel blocker, inhibits single RyR2 channel activity and suppresses spontaneous SR Ca\(^{2+}\) release in cardiac cells.\(^{97,98}\) Interestingly, the drug was also found to suppress ventricular arrhythmias in the CASQ2- knock-out mouse model of CPVT. Whether the antiarrhythmic activity of flecainide is dependent on its RyR2 blockade\(^9\) or on the sodium channel blocking activity that reduces the probability for DADs to reach the threshold for action potential generation,\(^9\) is still uncertain. The antiarrhythmic effect of flecainide, however, seems to be confirmed in patients, thus providing an important therapeutic breakthrough.

Animal studies have proposed additional therapeutic strategies that have not been tested in humans. K201(JTV519), a 1,4-benzothiazepine derivative that shares a high degree of structural similarity with the L-type channel blocker diltiazem, was originally discovered on the basis of its ability to protect cardiomyocytes from cell injury and death resulting from Ca\(^{2+}\) overload induced by epinephrine, caffeine, and high external Ca\(^{2+}\).\(^{100}\) K201 is thought to inhibit RyR2 and prevent SR Ca\(^{2+}\) leak by stabilizing FKBP12.6 binding to RyR2. However, recent studies revealed that K201 binds to the central region of RyR2 (2114 to 2149 aa)\(^101\) and suppresses SR Ca\(^{2+}\) leak and spontaneous Ca\(^{2+}\) waves irrespective of FKBP12.6 association.\(^101,102\) Despite its inhibitory action on RyR2 and spontaneous Ca\(^{2+}\) release, K201 was found to be unable to prevent ventricular arrhythmias in the R4496C\(^+/−\) knock-in mouse model of CPVT,\(^57\) although it is able to prevent ventricular arrhythmias in FKBP12.6\(^+/−\) mice.\(^103,104\) Because human studies with K201 or its derivative have not yet been completed, the clinical role of these agents remains to be defined.

An increasing body of evidence indicates that the Ca\(^{2+}\) and calmodulin-dependent protein kinase (CaMKII) plays an important role in the generation of spontaneous SR Ca\(^{2+}\) waves on \(\beta\)-adrenergic stimulation.\(^105\) Suppression of the CaMKII pathway may, therefore, represent another effective therapeutic approach for the suppression of Ca\(^{2+}\)-mediated arrhythmias. In support of this idea, preliminary evidence shows that KN93, an inhibitor of CaMKII, is able to prevent ventricular arrhythmias in the R4496C\(^+/−\) mouse model of CPVT.\(^106\)

**Summary**

Proteins responsible for regulation of intracellular Ca\(^{2+}\) handling have been implicated in genetic diseases. In particular, mutations in the \(RYR2\) gene encoding the cardiac ryanodine receptor isofrom 2 and the \(CASQ2\) gene encoding the cardiac calsequestrin isofrom 2 cause a clinical condition called CPVT that is highly lethal and often manifests in the pediatric population. RyR2 mutations result in an abnormal protein that is prone to spontaneous Ca\(^{2+}\) release from the SR. Mutations in the cardiac calsequestrin reduce the amount of protein and Ca\(^{2+}\) buffering in the SR. In vitro studies and development of knock-in mice have provided important information that have advanced the field and suggest that SOICR is likely to be the common mechanism for a variety of mutations in these 2 genes. The field has been particularly productive in bringing advancements to the clinics where new therapies have already been introduced. Advances in our understanding of the regulation of intracellular Ca\(^{2+}\) in health and disease will facilitate the development of novel risk
stratification and management scheme to improve survival and quality of life of CPVT patients.

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Inherited Dysfunction of Sarcoplasmic Reticulum Ca$^{2+}$ Handling and Arrhythmogenesis
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