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Molecular Mechanism and Current Therapies for Catecholaminergic Polymorphic Ventricular Tachycardia

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Abstract

The rhythmic contraction of the heart relies on tightly regulated calcium (Ca) release from the sarcoplasmic reticulum (SR) Ca release channel, Ryanodine receptor (RyR2). Genetic mutations in components of the calcium release unit such as RyR2, cardiac calsequestrin and other proteins have been shown to cause a genetic arrhythmic syndrome known as catecholaminergic polymorphic ventricular tachycardia (CPVT). This book chapter will focus on the following: (1) to describing CPVT as a stress-induced cardiac arrhythmia syndrome and its genetic causes. (2) Discussing the regulation of SR Ca release, and how dysregulation of Ca release contributes to arrhythmogenesis. (3) Discussing molecular mechanisms of CPVT with a focus on impaired Ca signaling refractoriness as a unifying mechanism underlying different genetic forms of CPVT. (4) Discussing pharmacological approaches as CPVT treatments as well as other potential future therapies. Since dysregulated SR Ca release has been implicated in multiple cardiac disorders including heart failure and metabolic heart diseases, knowledge obtained from CPVT studies will also shed light on the development of therapeutic approaches for these devastating cardiac dysfunctions as a whole.

Keywords: EC coupling, Ca-dependent arrhythmias, RyR2, Ca signaling, sudden cardiac death, DCR

1. Introduction

The rhythmic beating of the heart is controlled by an intricate and well-orchestrated flux of ions through a process called excitation–contraction coupling (ECC), where the electrical action potential leads to cellular contraction. Among all the ions involved in ECC, calcium (Ca) plays a critical role and serves as the signal for cardiac contraction. Briefly, upon cardiac excitation a small current of Ca enters the cytoplasm through the sarcolemmal L-type Ca channels (LTCC). This triggers a much larger Ca release from the sarcoplasmic reticulum (SR)—the intracellular Ca store—by opening the type 2 ryanodine receptor (RyR2) channel in a process called Ca-induced-Ca release (CICR) [1]. The resultant elevation of cytoplasmic Ca concentration activates the contractile apparatus, thus leading to myocyte contraction. For relaxation to occur, Ca must be extruded from the cytoplasm. Two main

Figure 1.
Cardiac excitation-contraction coupling.

mechanisms are involved in removing cytoplasmic Ca: one is by Ca re-sequestration into the SR to replenish the intracellular store, through the action of the SR Ca ATPase (SERCA). The other is by transporting calcium outside of the cell via the membrane-embedded protein, sodium calcium exchanger (NCX) (**Figure 1**). Other avenues for Ca removal do exist (e.g. sarcolemmal Ca-ATPase and mitochondria Ca uniporter), but only play a minor role in this process [1]. The rhythmic rise and fall of cytoplasmic Ca underlies the systolic and diastolic phases of the cardiac cycle.

2. Dysregulated SR Ca release is linked to cardiac pathologies

The RyR2 is a large protein with a molecular weight of ~560 kDa that forms homotetrameric channels in the SR membrane (**Figure 2**) [2]. Due to its crucial role in releasing Ca to trigger contraction, it is no surprise that there are a number of auxiliary proteins with likely overlapping/redundant functions acting from both the cytosolic and SR luminal sides to regulate the function of the channel complex. Moreover, the activity of the channel is also subject to regulation by post-translational modifications, including redox modifications, phosphorylation, and nitrosylation [3]. Unfortunately, both genetic and acquired defects due to mutation or posttranslational modification of the channel complex contribute to its dysfunction [3]. These defects typically make the channel hyperactive or leaky, giving rise to dysregulated Ca release (DCR). DCR is implicated in a spectrum of cardiac dysfunctions [3], and in particular, it directly causes a deadly cardiac arrhythmia syndrome, catecholaminergic polymorphic ventricular tachycardia (CPVT) [3, 4].

CPVT is a stress-induced arrhythmia that is triggered by elevated levels of catecholamines [5]. Patients do not exhibit the cardiac remodeling typical of structural heart diseases, which makes the diagnosis particularly challenging. Life-threatening cardiac arrhythmias occur following exercise or emotional stress, which elevates circulating catecholamine levels. CPVT mutations have been identified in the genes encoding the RyR2 channel and its several auxiliary proteins. The remainder of this chapter focuses on the regulation of SR Ca release, the molecular mechanisms of CPVT, and the current and future state of therapies targeted towards CPVT.

Figure 2.
 SR Ca release channel RyR2 is regulated by both cytosolic and SR luminal proteins.

Since dysregulated SR Ca release has been implicated in multiple cardiac disorders, knowledge obtained from CPVT studies will also shed light on the development of therapeutic approaches for these devastating cardiac dysfunctions as a whole.

3. Modulation of RyR2 Ca release

RyR2s form physically separated/isolated clusters to act as functionally independent Ca release units [6]. Within dyads, the structural element formed by the close apposition of T-tubules and junctional SR, Ca influx via LTCC in the T-tubule triggers more Ca release from RyR2 clusters in the junctional SR to initiate contraction (**Figure 1**). Ca release from individually activated RyR2 clusters are known as Ca sparks and can be experimentally observed during diastole [7]. The systolic Ca transient is the summation of tens of thousands of Ca sparks due to the synchronized Ca release of RyR2 clusters following sarcolemmal depolarization. The positive-feedback nature of CICR suggests that SR Ca release should terminate upon depletion of SR Ca store. However, only a fraction of the SR Ca store is released during EC coupling [8, 9]. This begs the question of how the Ca release process is terminated. Cytosolic Ca-dependent inactivation of RyR2 has been proposed as a mechanism for the termination of Ca release [10], but lacks widespread support. In contrast, evidence from different research groups collectively points to a mechanism that works to inhibit Ca release from the SR luminal side.

The first piece of evidence comes from an *in vitro* study conducted with single RyR2 channels reconstituted into lipid bilayers. This study demonstrated that the opening of RyR2 is significantly reduced at lowered luminal Ca [11]. The channel's luminal accessory proteins, calsequestrin 2 (CASQ2), junctin, and triadin, are required for this luminal Ca-dependent inhibition of the channel [12]. More

convincing evidence comes from a subsequent cellular study that manipulated the SR Ca buffering capacity by introducing exogenous Ca chelators into the SR to result in a slower Ca depletion [13]. Enhanced SR Ca buffering drastically increased the amplitude of Ca release (both Ca sparks and global Ca transients) and slowed its termination, hence supporting the role of SR luminal Ca in controlling RyR2 Ca release. Mathematical modelling studies provided further support that luminal Ca-dependent deactivation of RyR2 is involved in termination of Ca release [14].

While the role of luminal Ca-dependent deactivation of RyR2 has been established, it is unclear what the specific molecular mechanism is. There is evidence supporting either direct activation of RyR2 or through its luminal accessory proteins, i.e. CASQ2, junctin, and triadin, which form the SR Ca release unit with RyR2. Studies performed in human embryonic kidney cells (HEK293) overexpressing recombinant RyR2 support a direct activation of RyR2 by luminal Ca. Despite a lack of several Ca handling proteins, HEK293 cells with exogenously expressed RyR2 mutants of CPVT exhibit dysregulated Ca release that is sensitive to SR Ca load in a process called store-overload-induced Ca release (SOICR) [15]. The authors found that CPVT mutations of RyR2 reduce the threshold for SOICR, which is expected to increase the propensity of dysregulated Ca release, hence contributing to cellular arrhythmogenesis. Further, a more recent study from the same group proposed the amino acid E4872 as the luminal Ca sensor for the direct activation of RyR2 [16]. A point mutation of E4872A completely abolishes luminal Ca activation of RyR2 in single channel studies, and markedly reduces dysregulated Ca release in HEK293 and HL-1 cardiac cells. Moreover, mice harboring a heterozygous mutation of E4872Q are resistant to SOICR and protected from ventricular arrhythmias *in vivo*. E4872 is localized in the S6 helix bundle-crossing region, the putative cation binding pocket of RyR2. Despite recent breakthroughs in resolving the structure of RyR2, the composition of this putative cation binding pocket remains undetermined [17], and it is thought that other key amino acids in this region such as E4878 may also play a critical role in luminal Ca activation of RyR2.

On the other hand, independent labs have provided evidence supporting the participation of luminal proteins in regulating RyR2 Ca release. Lipid bilayer single-channel studies found that CASQ2 serves as a sensor to inhibit opening of RyR2 at low luminal [Ca], which notably required the presence of junctin and triadin, thus suggesting these proteins form a regulatory complex to control luminal Ca-dependent deactivation of RyR2 [12]. Additionally, increasing or decreasing the expression of CASQ2 in rat cardiac myocyte not only changes the SR Ca storage capacity, consistent with CASQ2's Ca buffer function, but also affects SR Ca release [18]. In particular, decreased expression of CASQ2 leads to dysregulated arrhythmogenic Ca release, supporting CASQ2 function as an inhibitor of RyR2 [18]. Interestingly, the expression of a competitive peptide in myocytes to disrupt the interaction between CASQ2 and triadin impairs the ability of CASQ2 to stabilize the Ca release channel [19], thus echoing conclusions from earlier bilayer studies that CASQ2 interacts with other luminal proteins to regulate RyR2 activity. The development of a genetic model of CASQ2 KO mouse provided additional evidence supporting CASQ2's regulation of SR Ca release [20]. CASQ2 KO mice phenocopied human CPVT by exhibiting catecholamine-induced tachyarrhythmias *in vivo*. Myocytes isolated from these mice are characterized with β agonist-induced dysregulated Ca release, a hallmark of cellular arrhythmogenesis. However, besides resulting in CPVT, the ablation of major SR Ca buffer protein CASQ2 does not seem to result in more severe cardiac dysfunctions, suggesting the existence of other Ca buffer proteins with similar function at the SR luminal side. Surprisingly, deletion of CASQ2 and another Ca binding protein histidine-rich calcium binding protein (HRC) in a double knockout (DKO) mouse model alleviates arrhythmias as

compared with the CASQ2 KO mouse [21]. HRC binds to RyR2 through the same CASQ2-binding domain on triadin, and results from this DKO mouse study suggest that rather than having redundant roles, CASQ2 and HRC play opposing roles to regulate RyR2 Ca release. Taken together, these studies not only support the notion that luminal accessory proteins of RyR2 participate in controlling SR Ca release, but also highlight the intricate nature of such regulation.

4. Molecular mechanisms of CPVT

CPVT mutations have been identified in 6 genes encoding 4 different proteins of the Ca release channel complex: *RYR2*, *CASQ2*, *TRDN*, *CALM1*, *CALM2*, and *CALM3* [22]. Among them, the 3 genes of calmodulin (*CALM1*, *CALM2*, and *CALM3*) encode the same protein. Of note, these mutations account for up to 60–75% of CPVT cases, with the genetic cause of the remaining clinical cases unknown [23, 24]. It is likely that more disease mutations will be discovered in other proteins of the Ca release channel complex. In this section, we will summarize the proposed molecular mechanisms for different genetic forms of CPVT.

4.1 CPVT linked to RyR2 mutations

Among the genetically confirmed cases of CPVT, over 90% are due to mutations of RyR2 [23]. CPVT linked to RyR2 mutations is autosomal dominant. Up to date, more than 200 gain-of-function mutations in RyR2 have been discovered. RyR2 loss-of-function mutations have also been detected but are less frequent and are associated with arrhythmias distinct from CPVT [25]. Additionally, the loss-of-function mutation appears to cause arrhythmias through an early afterdepolarization (EAD)-based mechanism [26] which is much less studied compared to the classic DAD-based mechanism. Never the less, EADs have been observed in CPVT patient specific induced pluripotent stem cells (iPSC)-derived cardiomyocytes [27] in addition to myocytes isolated from a mouse model harboring a loss-of-function CPVT mutation of RyR2 [26]. The focus of the rest of the chapter will be on the arrhythmias evoked by DADs, rather than EADs.

Three main theories of how gain-of-function RyR2 mutations lead to CPVT have been proposed by different groups. The first comes from the observation that CPVT mutants of RyR2 expressed in HEK293 cells decreased the threshold to induce SOICR [15]. Based on these results, it is proposed that CPVT mutations make RyR2 more sensitive to luminal Ca, thus susceptible to dysregulated arrhythmogenic Ca release. The second theory proposes that CPVT mutations reduce the binding of RyR2 to FKBP12.6, a cytosolic protein thought to stabilize the channel, thus increasing RyR2 activity and giving rise to arrhythmogenic diastolic calcium release [28]. However, this theory has been challenged by several labs [29–31], a conserved binding between CPVT mutant RyR2 and FKBP has been reported [32]. The majority of RyR2 mutations are found at three “hot spots” which are located in the N-terminal domain (amino acids 1–600), central domain (amino acids ~2100–2500) and C terminal domain (amino acids ~3900–end) of the protein [33, 34]. Structural studies show that many of them are found at the domain-domain interfaces, thus giving rise to the third theory that mutations impair the inter-domain interactions of RyR2 to cause CPVT. Specifically, the interaction between N-terminal and central domains of RyR2 is responsible for the so-called domain “zipping” and is thought to stabilize the channel; the third theory posits that CPVT mutants impair this interaction (causing domain unzipping) and causes channel dysfunction. This model of domain zipping-unzipping has been supported by experimental evidence [35–37].

4.2 CPVT linked to CASQ2 mutations

The second most common cause of CPVT is mutation of CASQ2, an SR luminal Ca binding protein thought to regulate deactivation of RyR2. CPVT linked to CASQ2 was considered as an autosomal recessive disease until the recent discovery of autosomal dominant disease-causing mutations [38]. CASQ2 is a low-affinity, high-capacity Ca binding protein. It does not contain Ca binding structural domains such as an EF-hand motif, a helix-loop-helix structural domain, found in “typical” Ca binding proteins (troponin C, calmodulin) [39]. Instead, it has multiple (~60–70) negatively charged amino acids, which facilitates electrostatic interactions between the protein and ~ 40–50 Ca ions [40]. CASQ2 monomers change their structure upon Ca binding, and form protein polymers in a Ca-dependent process. Structural studies show that monomeric CASQ2 contains three highly similar tandem domains, resembling that of bacterial thioredoxin. However, much less is known about the structure of the polymers. Based on *in vitro* biophysical studies by Park et al. [41], the following model of CASQ2 polymerization is proposed: CASQ2s exist as monomers at low luminal [Ca]; as [Ca] increases, CASQ2s form dimers, tetramers, and polymers in a [Ca]-dependent process. Thus, CASQ2s polymerize to bind additional Ca at high luminal Ca, but depolymerize to release Ca at low luminal Ca. Considering the Ca and protein concentrations in SR, CASQ2s likely exist as a mixture of monomers, dimers, and polymers of varying sizes [42]. As described above, monomeric CASQ2 is thought to be anchored to RyR2 via junctin and triadin to deactivate Ca release at low luminal Ca. The intriguing question remains whether this Ca-dependent change in the polymerization states of CASQ2 happens on a beat-to-beat basis in response to SR Ca load to regulate RyR2 Ca release. It has been shown that the polymerization state of CASQ2 changes upon depletion of luminal Ca in fibroblast by fluorescent approaches [43]. Additional evidence comes from studies conducted with skeletal muscle fibers demonstrating luminal-Ca dependent changes in polymerization of CASQ1, the skeletal counterpart of CASQ2 [44]. However, whether Ca-dependent changes in CASQ2 polymerization happens in beating cardiomyocytes at a time scale comparable with the cardiac cycle awaits further investigation.

At least 2 molecular mechanisms are proposed to explain how autosomal recessive CPVT mutations of CASQ2 cause the disease based on its two primary functions, buffering Ca and modulating RyR2 opening [4]. These mutations (nonsense or missense) lead to loss or reduced expression of CASQ2. Subsequently, reduced Ca buffering allows the free Ca to rise faster near the Ca release sites, thereby triggering dysregulated Ca release. Besides reduced Ca buffering power, some missense mutations of CASQ2 appear to work through another mechanism: by impairing RyR2 regulation from the luminal side. It's been shown that the mutation of R33Q leads to abnormal interaction between CASQ2 and the Ca release channel complex [45], and another mutation of D307H reduces the binding between CASQ2 and triadin [46]. These results support the notion that a regulatory complex involving several proteins (CASQ2, triadin, junctin, and potentially others) senses luminal Ca to regulate Ca release, and disruption of interactions between them leads to dysregulation of the channel and disease. Compared with the autosomal recessive mutations, less is known about the autosomal dominant mutations that were more recently identified. Two mutations (K180R and S173I) have been found to interfere the polymerization of CASQ2 [47], likely causing CPVT by reducing the Ca buffering capacity.

4.3 CPVT linked to triadin mutations

CPVT mutations have also been identified in triadin, a trans-SR membrane protein that helps anchor CASQ2 to the RyR2 channel complex. Triadin has a short

N-terminal region located on the cytosolic side of SR, a single membrane spanning domain, and a highly charged C-terminal region that comprises most of the protein and resides in the SR luminal side. The C-terminal tail of the protein contains KEKE motifs formed by stretches of alternating residues with opposite charges. A single KEKE motif consisting of 15 residues (210–224) has been suggested as the CASQ2 binding region [48]. The binding between triadin and CASQ2 is Ca-dependent and they dissociate at high [Ca] (10 mM). In contrast, triadin's binding to RyR2 is Ca-independent [48]. Due to its role of anchoring CASQ2 to Ca release sites, triadin is thought to facilitate SR Ca release by allowing CASQ2 to buffer Ca near the Ca release channel.

It is also proposed that triadin may play a direct role in regulating RyR2 channel activity. Both overexpression and knockout mouse models of triadin have been created to decipher its function. The overexpression model displayed hypertrophy and altered Ca handling, accompanied by compensatory changes in the expression of several proteins of the RyR2 channel complex, thus masking the functional role of triadin [49]. Similarly, loss of triadin in the KO model also caused compensatory changes [50]. Drastic reduction in the interface of junctional SR and T-tubules occurred due to structural remodeling, thus impaired the coupling between RyR2 and LTCC. As a result, inactivation of LTCC is reduced, which increased Ca current, prolonged action potential, and subsequently increased cellular and SR Ca. Due to Ca overload, myocytes from triadin KO model displayed elevated levels of arrhythmogenic Ca release, especially when stimulated with catecholamines [50]. While both mouse models support the notion that triadin plays an important role in myocyte Ca handling, the massive compensatory changes in these chronic models makes an explanation of the data challenging. Nevertheless, acute overexpression of triadin in cultured myocytes increased RyR2 opening, dysregulated Ca release, and membrane depolarization, mimicking the cellular phenotype of CPVT [51]. Relatively few CPVT mutations of triadin have been reported as of yet. In a 2012 study, three autosomal recessive mutations of triadin were discovered, with two of them (one deletion, one nonsense) resulting in loss of the protein. The third one, a missense mutation of T59R, results in a protein that is more susceptible to degradation [52]. Thus, loss or decreased expression of triadin appear to cause CPVT. Another two autosomal recessive triadin mutations were identified in a 2015 study [53], although the underlying disease-causing mechanisms await further investigation.

4.4 CPVT linked to calmodulin mutations

Calmodulin (CaM) is an EF-hand Ca binding protein that binds RyR2 from the cytosolic side to regulate Ca release. CaM has a dumbbell-shaped structure, with its two globular domains connected by a flexible central helix. Each of the two domains contain two EF-hand Ca binding sites. The N-domain of the protein has a lower Ca binding affinity than the C-domain [54, 55]. Upon Ca binding, the hydrophobic pockets in both domains become exposed, thereby allowing CaM to bind its several intracellular targets, including RyR2, LTCC and Na channel (Nav 1.5) [56]. Mutations of CaM have been linked to different types of arrhythmias, such as CPVT, long QT syndrome, and idiopathic ventricular fibrillation, likely due to its impaired regulation of various target proteins [22]. Following systolic Ca release and the ensuing increasing in Ca on the cytosolic side of RyR2, CaM binds to the channel and inhibits its opening during the diastole phase of the cardiac cycle [57, 58]. CPVT mutations of CaM appear to have impaired ability to inhibit the channel and promoted the generation of DCR in the form of Ca waves and Ca sparks in a cellular study [59]. They also exhibited higher binding affinity to RyR2 than WT CaM, thereby contributing to the autosomal dominant mode of action [59].

5. Impaired Ca signaling refractoriness and generation of DCR

Despite the differences in the molecular details on how the various CPVT mutations of the RyR2 channel complex cause the disease, they all seem to make the channel more susceptible to arrhythmogenic diastolic Ca release. Following systolic Ca release, RyR2 becomes functionally suppressed and remains that way for a brief period, known as Ca signaling refractoriness [60]. Refractoriness of the Ca release channel can be measured by myocyte experiments employing a two-pulse protocol to record the process of Ca transient restitution. It's been demonstrated that full recovery of Ca transient takes ~1 s (anywhere from ~0.8 – 1.5 s, depending on species) [61–65]. If Ca signaling refractoriness is impaired, the RyR2 channel is expected to recover earlier from the functionally suppressed state, thereby promoting the generation of DCR, thus causing cellular arrhythmogenesis. Indeed, multiple CPVT mutations have been found to shorten refractoriness of RyR2, including mutations of CASQ2 [65], CaM [66], and RyR2 [67]. Moreover, shortened Ca signaling refractoriness can also occur due to oxidation/hyperphosphorylation of RyR2 as seen in models of acquired heart diseases [63]. Thus, both genetic and acquired defects of RyR2 channel complex seem to converge on shortening Ca signaling refractoriness to cause arrhythmogenic Ca release. Further evidence supporting shortened refractoriness as a unifying mechanism for the generation of DCR comes from a recent study using an engineered therapeutic CaM in an attempt to restore refractoriness and treat CPVT [66], as discussed in a later section on future therapies for CPVT. Taken together, these studies suggest that disease mutations may change the SR Ca dynamics, the modulation of RyR2 by cytosolic or luminal proteins, or conformational changes of the channel protein itself, each of which has been experimentally demonstrated to shorten Ca signaling refractoriness and give rise to arrhythmogenic DCR.

6. Cellular arrhythmogenesis: SR Ca load

At the single myocyte level, DCR is manifested as different forms: Ca sparks, Ca wavelets, and propagating Ca waves. When large enough, DCR activates electrogenic NCX, resulting in an inward current that causes delayed afterdepolarization (DAD) [68–70]. With a large enough amplitude, DADs may surpass the voltage threshold to open Na channels, thus leading to the generation of an ectopic action potential or triggered activity [71, 72]. Both the amplitude and the rate of DCRs are important in determining if it will trigger an ectopic action potential [73]. Localized DCR events in the form of Ca sparks and wavelets are less likely to trigger ectopic action potentials as compared with propagating Ca waves. Ca waves are more likely to occur when SR Ca load is high, such as following activation of β -adrenergic signaling pathways.

β -adrenergic stimulation results in phosphorylation of key EC-coupling proteins and subsequent generation of a larger and faster Ca transient, underlying its positive inotropy (ability to contract) and lusitropy (ability to relax) effect [1]. One of these proteins, phospholamban (PLN), acts as an inhibitor of SERCA. Its inhibition on SERCA is relieved upon β -adrenergic-dependent phosphorylation, thus contributing to a faster Ca transient decay and also higher SR Ca content. This higher SR Ca load facilitates Ca wave generation, and explains the stress-induced arrhythmias that occur in CPVT. Therefore, SERCA's function to refill the SR with Ca is critical to maintain a certain SR Ca load to stimulate the generation of Ca waves. On the other hand, SERCA directs Ca out of the cytosol while refilling the SR with Ca, which opposes the formation of or “breaks” Ca waves.

Based on these seemingly contradictory effects of SERCA activity on Ca wave generation, an interesting question arises: what will be the consequences of upregulating SERCA activity in the setting of CPVT? Both beneficial and deleterious effects have been reported from studies conducted by different groups. When overexpressing a skeletal isoform of SERCA1a in the CPVT model of CASQ2 KO, the resultant CPVT-SERCA^{ox} mice developed severe Ca-dependent cardiomyopathy [74]. These mice suffered from early mortality and contractile dysfunction. Myocytes isolated from the hypertrophied hearts of these animals also displayed enhanced levels of DCR. While these results clearly demonstrate a detrimental effect, the severe cardiomyopathy phenotype due to chronic SERCA overexpression masks the effect of the genetic manipulation on arrhythmias. A follow-up study from the same group conditionally overexpressed SERCA2a in the same CASQ2 KO mice and found that both atrial and ventricular arrhythmias were exacerbated due to acute upregulation of SERCA activity [75]. In contrast, in another study employing a different CPVT model of RyR2 knock in mouse (R4496C^{+/-}), upregulation of SERCA activity by knocking out its inhibitor PLN suppressed arrhythmias *in vivo*. In cellular experiments, Ca waves were also suppressed, due to propagating Ca waves being converted into non-arrhythmogenic mini waves and Ca sparks [76]. Interestingly, a different study showed that although enhancing SERCA activity by PLB ablation alleviated arrhythmias, it exacerbated myocardial infarction and cardiac damage in a RyR2 model featuring elevated DCR due to a mutation (S2814D) mimicking constitutive CaMKII-mediated phosphorylation of RyR2 [77].

7. Synchronization of DCR in myocardium

It is well established how DCR triggers ectopic action potentials at the cellular level. However, the heart contains billions of cardiomyocytes, and how arrhythmogenesis at the level of isolated myocytes translates into arrhythmias at the level of a multi-cellular tissue preparation or even the whole heart remains unknown. Within the myocardium, individual myocytes are electrically coupled to their neighboring cells, hence Ca-dependent depolarizing currents generated in any random, isolated cells should be easily absorbed by neighboring cells that act as a current sink (the source-sink mismatch theory) [78]. Therefore, cellular depolarization, if happening randomly in individual cells, cannot generate sufficient current to trigger tissue-level depolarization.

Computational simulation studies suggest a very large number of cells—nearly 7×10^5 —have to depolarize simultaneously to overcome source-sink mismatch and trigger depolarization to generate an ectopic beat in normal myocardium. This number is reduced by modeling disease conditions such as fibrosis or heart failure related electrical remodeling, but the number of requisite cells still remains quite large [78]. Therefore, it's been proposed that DCR happens in a synchronous way in multiple cells of the CPVT hearts to cause a tissue-wide ectopic beat. Experimental evidence has been provided in support of the synchronization of DCR in a CPVT model carrying the CASQ2 R33Q mutation [65]. This study quantified the degree of DCR synchronization by measuring the latency, or the time interval to the first DCR, following systolic Ca release. It was found that DCR occurs in a highly synchronized way in both myocytes and cardiac muscle tissue obtained from the R33Q CPVT model. Importantly, two factors are important for the synchronization of DCR: 1) shortened Ca signaling refractoriness that increases the propensity of release sites to fire synchronously by facilitating CICR, and 2) the presence of a preceding systolic action potential acting as a synchronizing event that temporally aligns the release sites and primes them for recovery from refractoriness.

8. The cellular origin of CPVT: Purkinje cells or ventricular myocytes?

Purkinje fibers are a specialized network of electrically excitable cells found in the conduction system of the heart. They radiate throughout the ventricular muscle to ensure a rapid propagation of electrical impulse and a coordinated ventricular contraction. Compared with the myocardium, the Purkinje system has a smaller source-sink mismatch [78]. Based on this and other structural features [79], Purkinje cells have been proposed as the cellular origin of many arrhythmias including CPVT. Experimental evidence obtained from the CPVT model of RyR2 R4496C^{+/-} mouse supports this hypothesis [80]. Optical mapping of R4496C^{+/-} hearts demonstrates that ventricular tachycardia (VT) may originate from the His-Purkinje system in both ventricles. Cellular studies also found that Purkinje cells had a significantly higher rate of DCR and triggered activity compared to ventricular myocytes [81, 82].

However, a recent study attempting to establish the causal link between Purkinje cells and CPVT did not provide such evidence [83]. In this study, CASQ2 was conditionally knocked out in the cardiac conduction system, but not the myocardium, using a conduction system-specific Cre recombinase. Ablation of CASQ2 in the Purkinje fibers failed to produce a CPVT phenotype. Considering CASQ2 ablation is an established molecular cause of CPVT as demonstrated by a global CASQ2 KO model [20], this result argues against Purkinje cells as the origin of CPVT, at least not on their own. On the other hand, in support of myocytes as the origin of CPVT, cells isolated from the myocardium of CPVT mouse models have been shown to exhibit DCR, DAD, and ectopic action potentials in multiple studies [20, 66, 67, 80]. Human iPSC-derived cardiomyocytes generated from biopsies of human CPVT patients also displayed DCR, DAD, and ectopic action potentials characteristic of the above-mentioned CPVT cells [84, 85]. Drug studies based on isolated myocytes also serve as good indicators of drug efficacy in both mouse models and humans [22, 86, 87]. More evidence regarding the cellular origin of CPVT are discussed elsewhere [22].

9. Therapies for CPVT

Symptoms of CPVT vary from palpitations, syncope, or even cardiac arrest. Although a rare disease, the mortality rate of CPVT can reach as high as ~50% in untreated individuals before the age of 40 [23]. In this section, we will first discuss traditional therapies that are currently available to CPVT patients. Next, we will focus on novel therapeutic approaches, based on recent advances in understanding the molecular mechanisms of this life-threatening arrhythmia syndrome.

9.1 Current therapies for CPVT

9.1.1 Beta-blockers

Beta-blockers are the first-line drug therapy to treat CPVT. As discussed above, in CPVT, the β -adrenergic-dependent increase in SR Ca load is important in triggering DCR and subsequent cellular arrhythmogenesis. Thus, blocking the β -adrenergic signaling pathway is expected to decrease DCR and suppress arrhythmias. The most effective beta-blocker at the time of writing is nadolol [88, 89], but it remains unknown why it is more effective than other beta-blockers. Unfortunately, beta-blockers only offer limited protection even with the maximal tolerated dose. It has been reported that more than 30% patients still suffer from arrhythmic events after receiving beta-blockers [90].

Carvedilol, a beta-blocker that is highly effective in preventing VT in heart failure, has been shown to suppress CPVT through a dual inhibitory action on both β -adrenergic signaling and RyR2 channel activity [91]. Experimentally, an analog of carvedilol with minimal beta-blocking activity still prevented VT in a CPVT mouse model and exhibited improved efficacy when combined with a selective beta-blocker [91]. Nevertheless, further studies are required to assess its effectiveness in CPVT patients. However, this provides a new potential pharmacological approach where a combination of RyR2 channel inhibition and beta-blockade could provide a more effective therapeutic approach than current options based solely on beta-blockers.

9.1.2 Na channel blockers and flecainide

Na channel blockers may serve as anti-arrhythmic drugs due to the critical role of the Nav 1.5 channel in the depolarization phase of action potential. Flecainide, an FDA approved drug to treat arrhythmias, was originally thought to work by blocking the Na channel. Recent studies have found that flecainide prevents CPVT both in mouse models and human patients through a dual inhibition mechanism: inhibiting Na channels as well as RyR2 [86]. Studies show that flecainide appears to be a promising therapy for CPVT patients not responding well to beta-blockers. However, the working mechanism of flecainide was controversial.

A study conducted on the CPVT model of RyR2 R4496C^{+/-} showed that while flecainide was effective in preventing arrhythmias, it did not reduce DCR in the cellular experiments. Instead, it increased the threshold for triggered activity, thus pointing to the other possibility: that the drug works by solely acting as a Na channel blocker [92]. Several follow-up studies attempted to reconcile this discrepancy. Evidence has been provided that flecainide is effective in reducing DCR in cells harboring the RyR2 R4496C^{+/-} mutation, but this effect could be masked by experimental conditions such as Ca overload [93]. On the other hand, more convincing evidence comes from a recent study that employed a synthesized analog of flecainide with reduced inhibition on RyR2 activity but unaltered inhibition on Na channel [94]. This analog failed to reduce DCR at cellular level and arrhythmia burden *in vivo*, indicating that flecainide acts through inhibition of RyR2 activity. In support of this, flecainide reduced DCR in permeabilized CPVT cells lacking membrane-residing Na channels, and intact cells pretreated with Na channel blocker. Similar to flecainide, another approved drug propafenone also seems to work through dual inhibition of Na channel and RyR2 [95]. Further studies are required to fully understand its working mechanism.

9.1.3 Other treatment options

Ca channel blockers (LTCC blockers) such as verapamil, have been tested in cellular and animal studies, as well as clinical studies, to examine their efficacy in treating CPVT. Consistently, these studies found Ca channel blockers only confer limited benefits in both cellular preparations and patients already on beta-blockers [96–98]. However, it has been shown to be beneficial for some patients when used in combination with other pharmacological approaches including beta-blockers [98].

Left cardiac sympathetic denervation serves as an alternative treatment. It works by preventing the release of catecholamines from the sympathetic nerve endings. The procedure appears to be effective in reducing major arrhythmic events in clinical studies [99, 100], and thus has been recommended for patients who don't respond to more conventional pharmacological treatments such as beta-blocker therapy. In one case employing an extrapleural approach, the lower part of the

stellate ganglion was ablated together with the second and third thoracic ganglia [99]; another case used the thoracoscopic, the transaxillary, and the supraclavicular approaches as the main surgical approaches [100].

Implantable cardiac defibrillators (ICD) have been utilized in patients who still experience symptoms despite drug therapy and/or sympathetic denervation. A recent study systematically analyzed the efficacy of ICDs using existing clinical data containing 505 CPVT patients implanted with ICDs [101]. It was found that although effective for ventricular fibrillation, ICDs were not protective for VT. Another study of 136 CPVT patients also suggests ICD implant did not confer survival benefits [102]. Considering the potential complications and psychological burden of implantation, especially for pediatric patients, ICDs are not an optimal treatment for CPVT patients.

9.2 Potential future therapies for CPVT

The molecular mechanisms underlying CPVT have been intensively studied in the past several decades. Several novel therapeutic strategies have been proposed and tested in animal models and even pre-clinical studies. In this section, we will discuss these novel approaches, with a focus on gene therapy.

9.2.1 Gene therapy

With advances in the adeno-associated virus (AAV) vector-based gene transfer technology in the past a few decades, using gene therapy to treat CPVT is starting to become technically feasible. Several proof-of-principle studies have been conducted to test the efficacy of different therapeutic strategies. Considering several CPVT mutations, especially the ones identified in CASQ2, cause loss or reduced expression of the associated protein, it would seem that the most straightforward therapeutic approach is to deliver a normal gene encoding the protein. Indeed, AAV9-mediated gene transfer of a WT CASQ2 to both CASQ2 KO and R33Q mouse models restored the normal expression of CASQ2, improved abnormal electrophysiological properties of cells, and reduced arrhythmia burden *in vivo* [103, 104]. However, this gene replacement approach is limited by the size of the AAV vector, thus hindering the delivery of a normal gene for the large RyR2 protein, which accounts for the majority of the CPVT mutations. To solve this problem, AAV9 was instead used to deliver siRNA to silence mutant mRNA of RyR2 in an allele-specific way [105]. This RNA silencing approach increased the ratio of WT-RNA vs. mutant RNA, proving to be effective at normalizing cardiac electrophysiology, alleviating abnormal ultrastructural remodeling, and inhibiting *in vivo* VT when tested in the RyR2 R4496C^{+/-} mice. Alternatively, another study attempted *in vivo* genome editing using the CRISPR/Cas9 system delivered by AAV and also obtained promising results in a different CPVT model of RyR2 (R176Q^{+/-}) [106].

While these gene therapy strategies seem effective, one of the prerequisites for applying this technology is knowing the genetic cause of CPVT. However, the genetic cause of ~30–40% of clinical cases of CPVT remains undetermined. Several groups have developed novel approaches to tackle this problem. It has been found that the Ca binding properties of CaM—in particular, the kinetics of Ca dissociation from CaM—affects RyR2 refractoriness [66]. Based on this, a therapeutic CaM (TCaM) that specifically targets RyR2 and prolongs its refractoriness by slowing Ca dissociation from CaM was engineered. TCaM reduced DCR in CPVT cells and alleviated arrhythmias *in vivo* when delivered to a CPVT model of CASQ2 R33Q mice [66]. Instead of targeting the specific disease-causing gene, TCaM targets the

impaired RyR2 refractoriness, and thus it could potentially serve as a therapeutic avenue for distinct forms of CPVT. Another study chose to target CaMKII, an adrenergically activated kinase that is implicated in arrhythmogenesis and pathological remodeling in multiple cardiac disorders, including CPVT. Pharmacological inhibitors of CaMKII are limited in their efficacy due to their lack of specificity. In contrast, a CaMKII inhibitory peptide was delivered in a cardiomyocyte-specific way by AAV9 and found to be effective in reducing arrhythmias burden *in vivo* in the CPVT model of RyR2 (R176Q^{+/-}) [107]. Collectively, these studies provide strong evidence supporting AAV-based gene therapy as a promising future therapy for CPVT patients.

9.3 Targeting sinus node dysfunction

CPVT patients also present sinus node dysfunction and bradycardia, which are recapitulated in the mouse models of CPVT. The pathophysiological role and underlying mechanism for sinus node dysfunction are discussed in details elsewhere [22]. Targeting the impaired sinus node dysfunction has been proposed as a therapeutic approach for CPVT. It has been shown that increasing heart rate by (1) pharmacological intervention (atropine), (2) atrial overdrive pacing, or (3) re-expressing CASQ2 in the CASQ2 KO mouse all appear to reduce arrhythmia burden [83, 108]. Further, atropine has been tested in a small group of 6 CPVT patients and was found to be effective in reducing exercise-induced arrhythmic events [109].

9.4 Other potential therapies

Tremendous effort has been expended on identifying and developing small molecules that specifically target DCR of RyR2, since DCR is implicated in a spectrum of cardiac disorders. Dantrolene, a drug used clinically to treat a skeletal muscle condition of malignant hyperthermia, exhibited partial protection for a subset of CPVT patients [110]. The recently discovered ent-(+)-verticilide, an unnatural verticilide enantiomer, appears to be a potent and selective RyR2 inhibitor [87]. It reduced DCR, triggered activity in cells, and arrhythmias *in vivo* when tested with the CPVT model of CASQ2 KO. It seems to exert a stronger antiarrhythmic effect when compared with dantrolene or flecainide. More details on the current state of therapeutic small molecule development are reviewed elsewhere [111].

10. Conclusion

Great progress has been made in the past few decades to help us better understand CPVT and develop therapeutics for this deadly arrhythmia syndrome. These efforts will continue in both basic science and clinical studies and will provide deeper mechanistic insight on the molecular, cellular, and tissue mechanisms of CPVT. Since DCR is implicated in a spectrum of human diseases, knowledge obtained from these studies will also benefit the development of therapies for other cardiac dysfunctions including heart failure and metabolic heart disease.

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Abbreviation list

AAV	adeno-associated virus
Ca	calcium
CALM	calmodulin (gene)
CaM	calmodulin (protein)
CASQ2	calsequestrin 2
CICR	calcium-induced calcium release
CPVT	catecholaminergic polymorphic ventricular tachycardia
DAD	delayed afterdepolarization
DCR	dysregulated calcium release
DKO	double knockout
EAD	early afterdepolarization
ECC	excitation-contraction coupling
HEK29	human embryonic kidney cells
HRC	histidine-rich calcium binding protein
ICD	implantable cardiac defibrillators
iPSC	induced pluripotent stem cells
LTCC	L-type calcium channel
PLN	phospholamban
RyR2	type 2 ryanodine receptor
NCX	sodium calcium exchanger
SERCA	sarcoplasmic reticulum calcium ATPase
SOICR	store-overload-induced calcium release
SR	sarcoplasmic reticulum
TCaM	therapeutic calmodulin
TRDN	triadin
VT	ventricular tachycardia

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