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Molecular and tissue mechanisms of catecholaminergic polymorphic ventricular tachycardia

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Abstract

Catecholaminergic polymorphic ventricular tachycardia (CPVT) is a stress-induced cardiac channelopathy that has a high mortality in untreated patients. Our understanding has grown tremendously since CPVT was first described as a clinical syndrome in 1995. It is now established that the deadly arrhythmias are caused by unregulated 'pathological' calcium release from the sarcoplasmic reticulum (SR), the major calcium storage organelle in striated muscle. Important questions remain regarding the molecular mechanisms that are responsible for the pathological calcium release, regarding the tissue origin of the arrhythmic beats that initiate ventricular tachycardia, and regarding optimal therapeutic approaches. At present, mutations in six genes involved in SR calcium release have been identified as the genetic cause of CPVT: *RYR2* (encoding ryanodine receptor calcium release channel), *CASQ2* (encoding cardiac calsequestrin), *TRDN* (encoding triadin), *CALM1*, *CALM2* and *CALM3* (encoding identical calmodulin protein). Here, we review each CPVT subtype and how CPVT mutations alter protein function, RyR2 calcium release channel regulation, and cellular calcium handling. We then discuss research and hypotheses surrounding the tissue mechanisms underlying CPVT, such as the pathophysiological role of sinus node dysfunction in CPVT, and whether the arrhythmogenic beats originate from the conduction system or the ventricular working myocardium. Finally, we review the treatments that are available for patients with CPVT, their efficacy, and how therapy could be improved in the future.

Graphical Abstract

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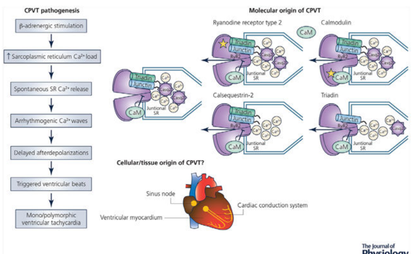

Catecholaminergic polymorphic ventricular tachycardia (CPVT) is a cardiac arrhythmia characterized by the presence of ventricular tachycardia in response to β-adrenergic receptor stimulation. Here, we describe the mechanistic progression from β-adrenergic stimulation to the formation of mono/polymorphic ventricular tachycardia. The goal of this review is to highlight the current molecular mechanisms that lead to CPVT followed by a discussion of the current hypotheses and research around the cellular/tissue origin of the arrhythmias.

Keywords

arrhythmia; calcium; heart excitation

Introduction

Catecholaminergic polymorphic ventricular tachycardia (CPVT) is a lethal, stress-induced cardiac channelopathy. First described in 1995, CPVT is characterized by polymorphic ventricular arrhythmias that are triggered by catecholamines released during exercise, stress or sudden emotion in individuals with structurally normal hearts (Leenhardt *et al.* 1995). Symptoms range from palpitations to cardiac arrest, with mortality rates between 30 and 50% in untreated individuals by age 40 (Pérez-Riera *et al.* 2018). Patients are normally diagnosed during early childhood but initial symptoms can occur in patients as old as 40 years of age (Pérez-Riera *et al.* 2018). CPVT is rare, with an estimated prevalence of 1:5000 to 1:10,000 depending on the population studied (Modell *et al.* 2012; Pérez-Riera *et al.* 2018). The true prevalence of CPVT is likely higher, since CPVT cases are frequently missed as most patients present with a normal resting electrocardiogram and structurally normal heart on cardiac workup (Imberti *et al.* 2016).

Human genetic studies have established that CPVT is caused by mutations in genes that encode proteins of the sarcoplasmic reticulum (SR) calcium release complex depicted in Fig. 1 (Swan *et al.* 1999; Lahat *et al.* 2001a,b). Supported mostly by experimental studies in mouse CPVT models (Cerrone *et al.* 2005; Knollmann *et al.* 2006; Rizzi *et al.* 2008; Uchinoumi *et al.* 2010), the current understanding of cellular CPVT pathophysiology is that catecholamines released during stress or exercise activate β-adrenergic receptor signalling, leading to a cellular chain reaction that culminates in pathological calcium release during diastole and calcium-triggered action potentials as described in Fig. 2.

Open questions remain as to how exactly mutations in CPVT genes cause functional alterations in the SR calcium release machinery that leads to pathological calcium release during diastole (molecular mechanisms). Even less well understood is how the altered

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cellular function causes CPVT at the whole-heart and *in vivo* level. It remains to be determined if and to what extent the dysfunction of the sinus node, the cardiac conduction system and the ventricular working myocardium contribute mechanistically to CPVT (tissue mechanisms). Here, we review the current understanding of the molecular and tissue mechanisms of CPVT to help consolidate the information and shed light on the areas where more work is needed. Our goal is to help advance our understanding of CPVT pathophysiology and its treatment.

Molecular mechanisms of CPVT

As of 2019, six different CPVT disease genes have been identified, which account for 60–75% of CPVT cases. The genetic cause of the remaining clinical CPVT cases is not yet known (Pérez-Riera *et al.* 2018; Roston *et al.* 2018b). All six CPVT genes (*RYR2, CASQ2, TRDN, CALM1, CALM2, CALM3*) encode proteins that are directly involved in regulating SR calcium release during excitation-contraction (EC) coupling (Fig. 1). Intheheart, electrical activation couples to mechanical force via the secondary messenger calcium (Bers, 2002). Membrane depolarization during the cardiac action potential opens L-type calcium channels (Fig. 1), which bring calcium into the cell. Calcium binds to and opens RyR2 located in the terminal cisternae of the SR, the junctional SR (Fig. 1), a process known as calcium-induced calcium release (CICR) (Fabiato, 1985). During systole, cytosolic calcium initiates myofilament contraction before being taken back up into the SR or pumped out into extracellular space during diastole. Proteins regulating SR calcium release can be categorized based on function and location. Proteins that are located within the SR lumen (e.g. calsequestrin, histidine-rich calcium-binding protein) affect the levels of free calcium present in the SR during the EC cycle. Proteins located within the junctional membrane of the SR (e.g. triadin, junctin) facilitate the interaction between calcium-handling proteins (e.g. calsequestrin and RyR2). Finally, there are a group of proteins that bind to the cytoplasmic surface of RyR2 (e.g. calmodulin, FK506 binding proteins) and regulate RyR2 sensitivity to cytoplasmic and SR luminal calcium levels. The following section reviews the proteins of interest in CPVT, their physiological role, and how mutations lead to CPVT.

Ryanodine receptor type 2

Gain-of-function mutations in the *RYR2* gene are found in about 95% of patients with a genetically confirmed diagnosis of CPVT (Pérez-Riera *et al.* 2018) and are designated as CPVT type 1 (CPVT1). CPVT1 is autosomal-dominant and was first described in 1999 (Swan *et al.* 1999) before being mapped to *RYR2* in 2001 (Priori *et al.* 2001). Since then, more than 200 gain-of-function variants in *RYR2* have been discovered. Loss-of-function *RYR2* variants also exist but are less common and associated with ventricular arrhythmia syndromes distinct from CPVT (Roston *et al.* 2017). *RYR2* encodes the cardiac ryanodine receptor (RyR2), a 565 kD protein that forms a homotetrameric, high-conductance, cation-selective channel that releases calcium from the SR (Fig. 1) (Seidel *et al.* 2015). RyR2 interacts with many other proteins including calsequestrin 2 (Costello *et al.* 1986; Franzini-Armstrong *et al.* 1987), triadin (Guo *et al.* 1996), junctin (Zhang *et al.* 1997), calmodulin (Yamaguchi *et al.* 2003, 2007), junctophilin, and the immunophilins FKBP 12 and FKBP 12.6 (Jayaraman *et al.* 1992; Yano *et al.* 2009). FKBPs are thought to stabilize the closed

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conformation of RyR2 and prevent diastolic release of calcium from the SR (Wehrens *et al.* 2003). Currently, there are several hypotheses as to why mutations in *RYR2* lead to CPVT, see Fig. 3 (Ikemoto & Yamamoto, 2002; Wehrens *et al.* 2003; Jiang *et al.* 2004; Liu *et al.* 2009). One theory is that mutations in *RYR2* affect the ability of FKBP 12.6 to interact with RyR2, leading to dissociation of FKBP 12.6 and the opening of RyR2 during diastole (Wehrens *et al.* 2003). However, others have challenged this hypothesis (Xiao *et al.* 2007), and at the present time it seems unlikely that loss of FKBP 12.6 is responsible for CPVT. Rather, FKBPs still bind to mutant RyR2 but fail to inhibit them (Zhang *et al.* 2016). The most widely held hypothesis states that CPVT mutations sensitize RyR2 channels to SR luminal calcium, causing them to open at a lower intra-SR calcium concentration, termed store overload-induced calcium release (SOICR) (Jiang *et al.* 2004). According to the SOICR hypothesis, mutations in *RYR2* decrease the threshold of SR calcium that is required to activate RyR2, leading to an increased probability of calcium leak and diastolic SR calcium release. A third hypothesis focuses on the interactions within the structure of RyR2. Normally, intramolecular interactions occur between the N-terminal and central domain of RyR2 monomers, termed ‘zipping’, which are critical to stabilizing the protein. When RyR2 is activated during the EC coupling cycle, the intramolecular interactions are weakened, ‘unzipping’ the domains, opening the channel, and causing the release of calcium. Mutations in *RYR2* that occur within the interaction domains have been shown to cause a similar unzipping, which results in calcium leak (Ikemoto & Yamamoto, 2002; George *et al.* 2006; Sumitomo, 2016). While it is still debated which hypothesis is correct, common to all of them is that CPVT-linked *RYR2* mutations increase the likelihood of spontaneous RyR2 openings and pathological calcium release during diastole.

Calsequestrin 2

The second most common cause of CPVT is mutations in the *CASQ2* gene, termed CPVT2 (Lahat *et al.* 2001a,b). Calsequestrin was first associated with CPVT when missense mutations were discovered in a family with autosomal recessive CPVT in 2001 and nonsense mutations in a similar family in 2002 (Postma *et al.* 2002). Calsequestrin-linked CPVT was thought to be an autosomal recessive disease only (Postma *et al.* 2002; de la Fuente *et al.* 2008; Kirchhefer *et al.* 2010), but in 2016 a report of a novel *CASQ2* mutation (K180R) in a family with autosomal-dominant inheritance of CPVT was published (Gray *et al.* 2016). Calsequestrin is a calcium-binding protein located in the terminal cisternae that form the junctional SR (Fig. 1). Calsequestrin is a high-capacity, low-affinity calcium-binding protein that binds 40–50 calcium ions through its 60–70 negatively charged amino acid residues (Yano & Zarain-Herzberg, 1994). While it is known that calsequestrin forms homo-polymers in a calcium-dependent manner (Park *et al.* 2003), not much is known about the structure of the polymers. A recent report utilized very low pH (3.5) conditions to crystalize a novel structure for the calsequestrin polymer filament (Fig. 4) (Titus *et al.* 2019). The filaments are an assembly of calsequestrin dimers that interact with one another to form three different thioredoxin helices that come together to form the filament (Fig. 4a). Two of the three domains form a double helix at the core of the filament, while the final domain creates an outer ‘collar’ and winds around the inner double helix (Fig. 4b). The new structure was then used to identify novel potential calcium-binding sites. The analysis found calcium-binding sites that bridged both intra- and inter-dimer interfaces of calsequestrin,

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suggesting that calcium is involved in the ability for calsequestrin to form dimers and those dimers to form subsequent filaments (Titus *et al.* 2019). Functionally, calsequestrin regulates the amount of calcium released from the SR during EC coupling by buffering intra-SR calcium (Zhang *et al.* 1997; Bers, 2002).

To regulate calcium release, calsequestrin is anchored to RyR2 by two proteins, triadin and junctin. It has been suggested that the interaction between calsequestrin and RyR2 may contribute to the refractory period of calcium release that occurs after each physiological CICR, but the mechanism is still not well understood (Györke *et al.* 2009; Katz *et al.* 2009; Liu *et al.* 2009). Studies using skeletal calsequestrin (Casq1, not expressed in the heart) showed changes in diffusional mobility of Casq1 after depletion of SR calcium (Manno *et al.* 2017). If cardiac calsequestrin (Casq2) acts in a similar manner in the heart, the findings from Casq1 would provide a plausible mechanism for how calsequestrin is regulating calcium release and termination. Hence, the simplest explanation of why mutations in calsequestrin cause CPVT is that they impair calsequestrin's ability to buffer calcium in the SR, resulting in a much faster rise of intra-SR free calcium concentration close to RyR2 release channels. Although intra-SR calcium kinetics have not yet been measured experimentally in calsequestrin CPVT models, there is extensive evidence that absence of calsequestrin in mice leads to hyperactive RyR2 channels, impaired calcium-release termination, a shortened calcium release refractory period, and enhanced spontaneous release of calcium (Knollmann *et al.* 2006; Chopra *et al.* 2007; Kryshtal *et al.* 2015). While impaired calcium buffering is the generally accepted mechanism for calsequestrin nonsense mutations that result in loss of calsequestrin, missense mutations (e.g. R33Q) may alter calsequestrin interaction with RyR2 in addition to reducing calcium buffering (Terentyev *et al.* 2006). For autosomal-dominant CPVT2, the current hypothesis is that calsequestrin mutations affect the ability for polymerization to occur. Two autosomal-dominant mutations (K180R and S173I) were unable to form polymers in a turbidity assay. Interestingly, the defect for the K180R mutation only occurred in the presence of magnesium. While the exact mechanism is unknown, magnesium may affect the ability of calsequestrin to form filaments and could be involved in the pathogenesis of the K180R mutation (Titus *et al.* 2019). More work is needed to understand the physiological role of calsequestrin in the EC coupling cycle, to determine the prevalence of autosomal-dominant calsequestrin mutations, and to understand how they cause CPVT.

Calmodulin

Three different genes - *CALM1, CALM2, CALM3* - encode identical calmodulin proteins and are located on chromosomes 14q32, 2p21 and 19q13, respectively. Mutations in each of the three calmodulin genes have been linked to three distinct genetic arrhythmia disorders: CPVT (Nyegaard *et al.* 2012; Yin *et al.* 2014; Gomez-Hurtado *et al.* 2016), idiopathic ventricular fibrillation (Marsman *et al.* 2014), and long-QT syndrome (Jiménez-Jáimez *et al.* 2016). Structurally, calmodulin has an alpha-helical structure and contains four classical calcium-binding EF hand motifs, two on each N- and C-terminal lobe. The motifs can bind to one calcium ion each with the N-terminal lobe having a lower affinity for calcium (Linse *et al.* 1991; VanScyoc *et al.* 2002). When calcium binds to calmodulin, the protein will undergo a conformational change that exposes the hydrophobic patches in each lobe. The

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patches contain a large amount of methionine residue that utilizes a central linker between the two lobes to interact with a large number of protein targets (Yamniuk & Vogel, 2004). Within the EC coupling machinery, calmodulin binds to RyR2 and the L-type calcium channel (LTCC) (Fig. 1). The binding of calmodulin to RyR2 inhibits calcium release from the SR during diastole (Yamaguchi *et al.* 2003, 2007). Calcium binding to calmodulin inactivates LTCC channels (Peterson *et al.* 1999; Zühlke *et al.* 1999), a process termed calcium-dependent inactivation. In addition to RyR2 and LTCC, calmodulin regulates other membrane ion channels important for the cardiac action potential (e.g. Nav 1.5, KCNQ1). Mutations in the three *CALM* genes have a wide range of effects, which may explain the diverse arrhythmia phenotypes associated with calmodulin mutations. For example, calmodulin mutants linked to CPVT either fail to inhibit or even activate RyR2 (Hwang *et al.* 2014; Gomez-Hurtado *et al.* 2016). CPVT calmodulin mutants tend to bind to RyR2 with higher affinity than wild-type calmodulin (Hwang *et al.* 2014; Gomez-Hurtado *et al.* 2016), which can explain their autosomal-dominant mode of action. On the other hand, calmodulin mutants associated with LQTS do not affect RyR2, but rather impair LTCC inactivation, leading to a profound action potential lengthening and QT prolongation (Limpitikul *et al.* 2014). Due to the many calmodulin targets within the cell, calmodulin mutations may also cause ventricular arrhythmias by other mechanisms, but so far none have been definitively confirmed.

Triadin

Triadin is a trans SR membrane protein that forms a complex with RyR2, calsequestrin and junctin to create the SR calcium release unit (Jones *et al.* 1995; Kobayashi & Jones, 1999). Triadin localizes to the junctional SR membrane in both cardiac and skeletal muscle (Fig. 1). Triadin has a single membrane-spanning domain, a short N-terminal segment located in the cytoplasm, a long C-terminal tail that projects intraluminally and is highly basic and charged, and a long run of charged amino acid residues called 'KEKE' association motifs that promote protein-protein interactions (Knudson *et al.* 1993; Jones *et al.* 1995; Guo *et al.* 1996; Zhang *et al.* 1997). Triadin is thought to act mainly as a scaffolding protein to help anchor calsequestrin to RyR2 near the junctional SR membrane. The anchoring properties of triadin are important for maintaining the ultrastructure of the terminal cisterna. When triadin is knocked out in mice (Chopra *et al.* 2009), protein levels of RyR2, calsequestrin and junctin were all reduced despite normal RNA expression. Consistent with the role of triadin as the primary anchoring protein that retains calsequestrin in the terminal cisterna, calsequestrin can be detected in the free SR of triadin knockout myocytes (Chopra *et al.* 2009). Loss of triadin also resulted in profound structural remodelling of the terminal cisterna, with an approximately 50% reduction of the junctional SR-t-tubule interface. As a result of the structural remodelling, coupling efficiency between LTCC and RyR2 was impaired and LTCC calcium-dependent inactivation reduced. Due to the impaired LTCC inactivation, triadin loss results in a gain-of-function effect on LTCC, increased calcium current and prolonged cardiac action potential. The ensuing cellular and SR calcium overload causes an increase in spontaneous calcium release events especially during catecholaminergic stimulation (Chopra *et al.* 2009). Other roles of triadin have been proposed, such as the ability of overexpressed triadin to enhance SR calcium release by directly affecting the RyR2 channel, but the work was conducted in cultured myocytes and

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needs to be confirmed at the single channel level (Terentyev *et al.* 2005). The first triadin mutation linked to CPVT was discovered in 2012 and another was found in 2015 (Roux-Buisson *et al.* 2012; Rooryck *et al.* 2015). Triadin mutations are thought to result in decreased levels of the protein. For example, the triadin-T59R mutation renders the triadin protein unstable and leads to enhanced degradation (Roux-Buisson *et al.* 2012). More recently, triadin mutations were also found in patients suffering from long-QT. The mutations caused a frameshift in the *TRDN* gene leading to a syndrome termed 'triadin knockout syndrome' (Altmann *et al.* 2015). Patients suffered from multiple episodes of exertion-induced syncope and cardiac arrest in early childhood. Other symptoms include mild to moderate muscle weakness. Further electrocardiographic workup demonstrated extensive T-wave inversion and QT prolongation. Normally, patients with similar findings would be classified as having long-QT syndrome. It has been proposed that patients with triadin mutations that decrease the levels of triadin should be diagnosed with 'triadin knockout syndrome' (Altmann *et al.* 2015), which is essentially an overlap syndrome with features of both CPVT and long-QT.

Other candidate genes for CPVT

Although not yet identified, mutations in other genes that are integral to SR calcium handling could potentially cause CPVT and should be screened for in patients carrying a clinical diagnosis of CPVT. Junctin, encoded by the gene aspartyl-beta-hydroxylase (*ASPH*), is a junctional SR protein (Fig. 1) that interacts with triadin and RyR2 (Jones *et al.* 1995; Kobayashi & Jones, 1999). Like triadin, junctin functions as a scaffold for calsequestrin and is involved in the polymerization of calsequestrin as calcium increases in the SR (Lee *et al.* 2012). Other CPVT candidates are proteins that regulate RyR2 calcium sensitivity such as FKBP 12 and FKBP 12.6 (Fig. 1). Both proteins are expressed in cardiac myocytes and are thought to promote the closed state of the channel. FKBP 12 and 12.6 bind to the same region of RyR2 which suggests that the competition between them is important for the functional outcome of RyR2 (Gonano & Jones, 2017). Another candidate is histidine-rich calcium (HRC) binding protein, a SR luminal calcium-binding buffering that is upregulated when calsequestrin is knocked out (Murphy *et al.* 2011). Increased HRC may activate RyR2 calcium release channels and contribute to CPVT (Liu *et al.* 2015).

Tissue mechanisms underlying CPVT

It is generally agreed upon that CPVT mutations render the RyR2 calcium release channels hyperactive, generating pathological calcium release during diastole. What remain unclear are the tissue mechanisms of CPVT. For example, a key question is the mechanistic contribution of low sinus heart rates, since sinus node dysfunction is a hallmark of CPVT in patients and animal models (Faggioni *et al.* 2014b; Miyata *et al.* 2018). Another key question is whether the arrhythmic trigger that causes the ventricular arrhythmias originates from ventricular cardiomyocytes in the working myocardium or from specialized cells of the cardiac conduction system, the Purkinje cells. Theoretical considerations and modelling studies favour the ventricular Purkinje network as the primary cellular source of CPVT (Xie *et al.* 2010) but conclusive experimental evidence is lacking.

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Pathophysiological role of sinus node dysfunction in CPVT

Sinus node dysfunction and bradycardia are well-documented phenotypes of CPVT in humans and in mouse models of CPVT (Faggioni *et al.* 2014b; Miyata *et al.* 2018). The mechanisms of how CPVT mutations cause sinus node dysfunction are thought to be multifactorial. For example, one theory is that the loss of calsequestrin causes a regional microfibrosis in the sinus node, which would alter the generation and propagation of an electrical impulse through the node (Glukhov *et al.* 2015). It is hypothesized that the microfibrosis could form from apoptosis and fibrogenesis due to an overload of diastolic calcium (Swaminathan *et al.* 2011). The increase in calcium levels could also trigger the activation of CaMKII, which has been shown to be involved in the upregulation of genes that could promote structural remodelling (Huke & Knollmann, 2011). Understanding how sinus node dysfunction originates is important as sinus node chronotropic incompetence has been shown to be a risk predictor for ventricular arrhythmia in children and young adults with CPVT (Franciosi *et al.* 2019). A likely explanation is that low sinus rates prolong the diastolic interval, allowing the spontaneous SR calcium release to occur before CICR during the next action potential can empty the SR and reset the SR calcium clock. Our group tested this hypothesis experimentally at the cellular level and *in vivo* using the Casq2 KO mouse CPVT model (Faggioni *et al.* 2013). We found that increasing the pacing rate reduced the likelihood of spontaneous calcium release and triggered beats in isolated cardiomyocytes. *In vivo*, artificially raising resting heart rates with atropine or by overdrive pacing prevented CPVT (Faggioni *et al.* 2013). To study the role of the cardiac conduction system in CPVT in more detail, our group recently developed two novel mouse models where the *CASQ2* gene can be either inactivated (i.e. conditional KO) or activated (i.e. conditional rescue) by Cre-mediated recombination (Flores *et al.* 2018). Thus, we were able to modulate *CASQ2* gene expression in a tissue and temporal manner by crossing our new mouse models with mice that carry Cre controlled by tissue selective and/or inducible promotors. For example, using the HCN4KiT-Cre system (Hoesl *et al.* 2008), we were able to re-express Casq2 in the SA node of adult Casq2 KO mice, which accelerated sinus heart rates and prevented CPVT (Flores *et al.* 2018), as illustrated in Fig. 5. Hence, dysfunction of the sinoatrial node and the ensuing slow sinus heart rates may independently contribute to arrhythmia risk in CPVT.

Purkinje cells as the cellular source of CPVT

Theoretical considerations and modelling studies favour the ventricular Purkinje network as the primary cellular source of CPVT because of a favourable source-sink relationship (Xie *et al.* 2010). There are also key differences between Purkinje cells and ventricular myocytes that could explain why Purkinje cells are more likely to generate arrhythmias. Purkinje cells have a much lower number of T-tubules compared with ventricular cardiomyocytes and hence more corbular SR (Sommer & Johnson, 1968). The differences in organelle structure result in a calcium activation process that is unique to Purkinje cells, termed 'reverse mode-EC coupling'. The calcium release units within the centre of Purkinje fibres do not respond to voltage but instead are activated by calcium waves (Stuyvers *et al.* 2005). Experimental evidence to support the proposition that CPVT originates from the Purkinje system stems from a mouse model with a heterozygous mutation in *RYR2*(*RYR2*/*RYR2*^{R4496C}) (Cerrone *et al.* 2005). Optical mapping of whole mouse hearts demonstrated that breakthrough patterns from ventricular tachycardias may originate from the His-Purkinje network in both

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ventricles (Cerrone *et al.* 2007). To confirm that the arrhythmias were generated from the Purkinje fibres, chemical ablation (Lugol's solution) of the right ventricular endocardial cavity was performed. Mice treated with Lugol's had conversion of bi-directional VT into monomorphic VT, suggesting that endocardial Purkinje fibre ablation prevented the development of arrhythmias from the right ventricle (Cerrone *et al.* 2007). Follow-up studies in single cells found that *RYR2*/*RYR2*^{R4496C} Purkinje cells developed spontaneous calcium release events and triggered beats at a significantly higher rate than ventricular myocytes (Herron *et al.* 2010; Kang *et al.* 2010).

The experimental studies in the *RYR2*/*RYR2*^{R4496C} mice had suggested that alternating beats of bi-directional VT originated from the right and left bundle branches, but the mechanism by which the triggered activity generated an alternating pattern was not addressed (Cerrone *et al.* 2007). Using a simulated two-dimensional anatomic model of rabbit ventricles with a simplified His-Purkinje system, a computer modelling study attempted to gain a better understanding of the mechanism underlying bi-directional ventricular tachycardia, which is a hallmark of CPVT (Leenhardt *et al.* 1995). Based on the results of the modelling, the authors proposed that a 'ping-pong' mechanism, called reciprocating bigeminy, could account for the bi-directional VT pattern that was seen in CPVT (Baher *et al.* 2011). The ping-pong mechanism is based on the findings that above a certain threshold heart rate, a delayed afterdepolarization (DAD) triggers an action potential that initiates ventricular bigeminy, and that the threshold heart rate for bigeminy varies at different locations in the heart (Baher *et al.* 2011). The findings from the model suggested that DAD-triggered arrhythmias could cause ventricular bigeminy when a single site in the His-Purkinje system or the ventricular myocardium developed a DAD-triggered beat following a sinus beat. Bi-directional VT occurred when a second site developed ventricular bigeminy and reciprocally activated the first site by the ping-pong mechanism. Bi-directional VT could degenerate into polymorphic VT when the increase in heart rate recruited additional sites in the His-Purkinje to develop bigeminy (Baher *et al.* 2011). The data from the *RYR2*^{R4496C} model suggests that the His-Purkinje system could serve as both the source of initiation and the propagation of ventricular arrhythmias.

While the above evidence suggests Purkinje cells in the conduction system as the source for CPVT, direct experimental evidence was lacking until recently, when Flores *et al.* (2018) tried to establish causation by knocking out calsequestrin only in the conduction system using the HCN4KiT-Cre recombinase system. Cre expression is driven by the promoter of the *HCN4* gene, which is expressed in the sinus node and the cardiac conduction system but not in the ventricular working myocardium. Surprisingly, the deletion of calsequestrin in the Purkinje network did not produce a CPVT phenotype (Fig. 5) (Flores *et al.* 2018). Since deletion of calsequestrin is an established molecular mechanism of CPVT, the results of the study suggest that Purkinje cells in the conduction system may not be capable of generating CPVT on their own. Rather, ventricular myocytes may be the cellular culprit responsible for CPVT, which is discussed next.

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Ventricular cardiomyocytes as the cellular source of CPVT

Based on experimental studies of cells isolated from the ventricular myocardium of CPVT mouse models, ventricular cardiomyocytes are clearly capable of generating spontaneous calcium release, DADs, and spontaneous action potentials in response to catecholaminergic stimulation, making them candidates to be the origin of ventricular arrhythmias in CPVT (Knollmann *et al.* 2006; Liu *et al.* 2006; Cerrone *et al.* 2007). Other studies support this. For example, human cardiomyocyte models of CPVT generated from patient-specific induced pluripotent stem cells (iPSCs) also exhibit DADs and action potentials triggered by spontaneous calcium release (Novak *et al.* 2012, 2015). Drug efficacy in isolated ventricular cardiomyocytes can predict anti-arrhythmic efficacy in mouse models and in humans with CPVT (Knollmann, 2011; Batiste *et al.* 2019). Furthermore, CPVT patients frequently present with atrial tachycardia or atrial fibrillation that can occur prior to or during their ventricular tachycardia (Leenhardt *et al.* 1995). Up to 74% of patients with CPVT experience supraventricular tachycardias at slower heart rates than the ventricular arrhythmias (Sumitomo *et al.* 2007; Cerrone *et al.* 2009; Sy *et al.* 2011). Based on optical mapping data from a calsequestrin null mouse model, the atrial tachyarrhythmias are driven by spontaneous calcium release events in atrial myocardium that cause DADs and atrial triggered beats (Faggioni *et al.* 2014a), supporting the suggestion that that calcium-triggered tachyarrhythmias can originate outside the specialized conduction system. Finally, in clinical studies of CPVT patients, 60–70% of ventricular ectopy originated from the right and left ventricular outflow tracts (Sumitomo *et al.* 2003; Sy *et al.* 2011). Although there is no universal agreement on where to find Purkinje fibres in the human ventricle, researchers have suggested that the outflow tract has little or no Purkinje fibres present (Shimizu, 2009). However, a recent anatomical study of the human heart identified what appear to be specialized conducting cells in the right ventricular outflow tract. The specialized cells are thought to be the ramifications of the right bundle branch that do not extend past the pulmonary valves, but more studies are needed to understand the nature of these cells. Of note, some of the arrhythmia foci identified within the right ventricular outflow tract were at anatomical locations distinct from those of the specialized conduction cells (De Almeida *et al.* 2020). Together, the human data could indicate that ventricular cardiomyocytes are capable of triggering CPVT, at least in portions of the left or right outflow tract. More research with tissue-targeted genetic models such as those in Fig. 5 is needed to determine the tissue origin of CPVT, and to help understand how ventricular cardiomyocytes can overcome the sink–source mismatch to trigger CPVT.

Treatment of CPVT patients

For CPVT patients, there are several therapeutic options recommended by consensus guidelines (Priori *et al.* 2013). First-line therapy is beta-adrenergic receptor blockers (Class I recommendation). If patients on maximally tolerated beta-blocker therapy continue to have syncope or recurrent sustained VT, treatment should be intensified with combination medical therapy (e.g. adding flecainide, Class IIA) or left cardiac sympathetic denervation (Class IIB). There is no role for an implantable cardiac defibrillator (ICD) as standalone therapy, nor programmed electrical stimulation in CPVT (Class III).

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Beta-adrenergic receptor inhibitors (beta-blockers)

Beta-blockers are the first-line drug therapy to treat CPVT since CPVT is triggered by beta-adrenergic stimulation. Based on clinical studies (Hayashi *et al.* 2009; Leren *et al.* 2016), the most effective beta-blocker is nadolol, although it is not known why nadolol is superior to other beta-blockers. Although beta-blockers are recommended as a first-line therapy, they are not completely protective: over 30% of patients experienced events during an eight-year follow-up period (van der Werf *et al.* 2012).

Flecainide and propafenone

The Class 1C anti-arrhythmic drugs flecainide and propafenone both prevent exercise-induced VT in mice and humans with CPVT (Watanabe *et al.* 2009; Hwang *et al.* 2011). At the cellular level, their efficacy can be attributed to their dual inhibition of sodium and RyR2 calcium release channels, resulting in a profound suppression of spontaneous calcium release and triggered beats. Since the initial report in 2009, five clinical studies including a randomized placebo-controlled trial (Kannankeril *et al.* 2017) have investigated flecainide therapy in CPVT patients that were experiencing exercise-induced ventricular arrhythmia despite therapy with beta-blockers. After the initiation of flecainide, approximately 80% of patients had either a partial or a complete suppression of exercise-induced VT (van der Werf *et al.* 2011; Khoury *et al.* 2013; Watanabe *et al.* 2013; Kannankeril *et al.* 2017; Wangüemert Pérez *et al.* 2018). Propafenone, another Class 1C anti-arrhythmic drug, has also been used to treat CPVT (Marx *et al.* 2019). Similar to flecainide, propafenone inhibits RyR2 single channels (Hwang *et al.* 2011) and inhibits arrhythmogenic calcium waves in CPVT cardiomyocytes (Savio-Galimberti & Knollmann, 2015). While both anti-arrhythmic drugs are effective clinically, additional studies are still needed to gain a better understanding of the role of sodium channel *versus* RyR2 inhibition, and the potential as a first-line therapy (Behere & Weindling, 2016).

Calcium channel blockers

Although effective in cell and animal models of CPVT (Katz *et al.* 2010; Alcalai *et al.* 2011), clinically LTCC blockers by and large provide only transient or partial benefit in CPVT patients already treated with beta-blockers (Swan *et al.* 2005; Rosso *et al.* 2007; Katz *et al.* 2010). Both beta-blockers and flecainide are superior at preventing VT. Calcium channel blockers could be considered for patients that are unable to take flecainide or in refractory cases of CPVT.

Cardiac sympathetic denervation

Left or bilateral cardiac sympathetic denervation reduces catecholamine signalling in the heart by preventing the release of norepinephrine from sympathetic nerve terminals. It was first reported to be successful for CPVT in 2008 when three patients that were refractory to their medications became symptom free after the procedure (Wilde *et al.* 2008). A larger follow-up study found a reduction of major cardiac events from 86% to 21% (De Ferrari *et al.* 2015). The most recent guidelines recommend sympathetic denervation as an option for patients that are still experiencing symptoms on maximum beta-blocker therapy (Class IIB).

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Implantable cardiac defibrillator

The 2013 guidelines recommended (Class I) that a patient with CPVT has an ICD implanted if the patient has survived a cardiac arrest or if a patient has syncope/documented sustained VT despite optimal medical management and/or left cardiac sympathetic denervation(Al-Khatib *et al.* 2018). A recent meta-analysis reviewed 53 studies containing 1429 CPVT patients, 35% of whom had an ICD implanted (Roston *et al.* 2018a). During follow-up, 40% of patients received at least one appropriate shock, 21% of patients received at least one inappropriate shock, and electrical storm (three or more sustained episodes of ventricular tachycardia, ventricular fibrillation, or appropriate shocks from an ICD within 24 hours) occurred in 20% of patients. Seven patients died despite ICD placement, with four deaths associated with electrical storm. The effectiveness of the shocks was also assessed. Some 99% of shocks for ventricular tachycardia failed despite being appropriate whereas 94% of shocks for ventricular fibrillation were successful (Roston *et al.* 2018a). Thus, the efficacy of ICD shocks in CPVT appears dependent on the arrhythmia mechanism - effective for ventricular fibrillation but ineffective for VT. (Miyake *et al.* 2013) A recent multicenter study of 136 CPVT patients who presented with cardiac arrest showed no survival benefit associated with ICD implant (van der Werf *et al.* 2019). These data indicate that ICD therapy can be both ineffective and proarrhythmic, and can cause serious medical complications and psychological burden, especially in paediatric populations. Hence, ICD implantation is controversial and of questionable utility for CPVT patients.

Possible future CPVT therapies

One promising approach is to target the sinus node dysfunction that is characteristic of CPVT. As proof of concept, artificially raising heart rates with atropine, by atrial overdrive pacing or by re-expressing Casq2 in the sinoatrial node prevented catecholamine-induced ventricular arrhythmia in the Casq2 KO mouse CPVT model (Faggioni *et al.* 2013; Flores *et al.* 2018). Observational patient data also suggests the efficacy of raising sinus heart rates as a novel therapeutic approach that can prevent exercise-induced VT (Faggioni *et al.* 2013), which we recently confirmed in a small open-label clinical trial, where raising sinus heart rates with atropine prevented or reduced exercise-induced ventricular ectopy in six CPVT patients (Kannankeril *et al.* 2019).

Another approach that has been tested successfully in CPVT mouse models is gene therapy via adeno-associated viral (AAV) vectors. Multiple studies have seen beneficial effects from delivering various calcium handling proteins such as calsequestrin (Denegri *et al.* 2014; Kurtzwald-Josefson *et al.* 2017; Cacheux *et al.* 2019) and an engineered calmodulin that inhibits RyR2 calcium release (Liu *et al.* 2018). AAV was also successfully used for *in vivo* CRISPR/Cas9-mediated gene editing of an *RYR2* CPVT mutation in mice (Pan *et al.* 2018). As gene therapy continues to improve, the above studies demonstrate the power that a targeted approach could have on arrhythmic diseases, and potentially prevent patients from needing any pharmacological or surgical interventions.

More selective small molecule inhibitors of RyR2-mediated calcium release could provide a better tolerated and effective CPVT therapy. Dantrolene, a drug used clinically to treat hyperactive skeletal muscle calcium release, decreased exercise-induced ventricular ectopy

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in some but not all CPVT patients (Penttinen *et al.* 2015). Our group recently reported the discovery of ent-verticilide, a cyclic depsipeptide that has nanomolar potency, is selective for RyR2 over RyR1, and exhibits anti-arrhythmic efficacy *in vivo* (Batiste *et al.* 2019). Another promising drug target is the calmodulin-dependent serine-threonine protein kinase II (CaMKII) (Fig. 1). RyR2 phosphorylation by CaMKII increases RyR2 calcium leak. CaMKII inhibition with KN-93 (Liu *et al.* 2011) or with AAV-mediated delivery of a CaMKII peptide inhibitor (Bezzerides *et al.* 2019) was effective in suppressing arrhythmias in a murine model of CPVT. Finally, Kifuensine, an inhibitor of mannosidase-I, was used to successfully rescue expression of calsequestrin and reduce CPVT occurrence in triadin-KO mice by preventing proteasomal degradation of misfolded proteins (Cacheux *et al.* 2019).

Conclusion

The goal of this topical review was to update the field on the current molecular and tissue mechanisms of CPVT and highlight therapeutic approaches. At a molecular level, six genes that affect calcium handling have been found to cause CPVT: *RYR2*, *CASQ2*, *TRDN*, *CALM1*, *CALM2* and *CALM3*. More work is needed to understand exactly how mutations disrupt protein function and cause pathological calcium release at the cellular level. At a tissue level, current research suggests that sinus node dysfunction contributes mechanistically to the development of exercise-induced ventricular ectopy and could be targeted therapeutically by increasing the sinus heart rate. Future studies are needed to definitively answer whether the ectopic beats originate in the ventricular conduction system (i.e. Purkinje cells) or in the ventricular working myocardium. Insights from studying the molecular and tissue mechanisms extend beyond CPVT, because RyR2 hyperactivity and abnormal calcium handling is also a common feature of structural heart diseases such as ischaemic cardiomyopathy or non-ischaemic heart failure, which are the leading cause of sudden death in the developed world. Hence, CPVT can serve as an important paradigm for studying calcium-related arrhythmia mechanisms and developing novel therapeutics that prevent ventricular arrhythmia and sudden death.

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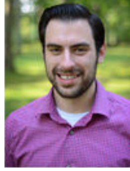
Biographies



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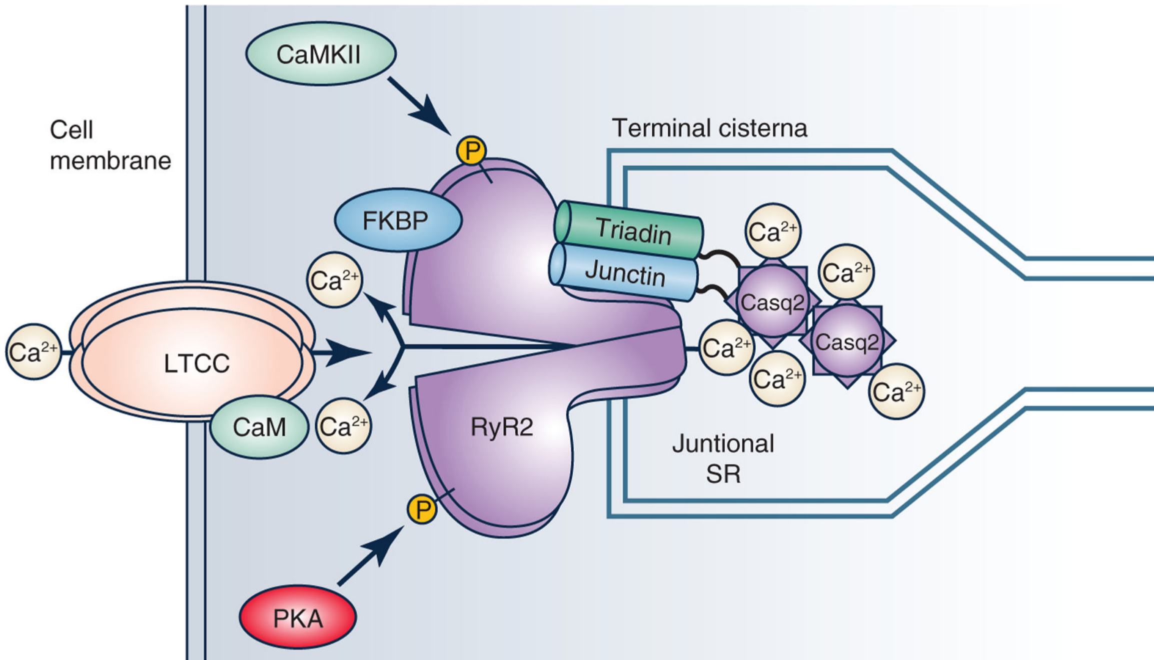

Figure 1. The sarcoplasmic reticulum (SR) calcium release complex in cardiac muscle

Pictured above are the proteins that are involved in the regulation of calcium release from the SR during excitation-contraction coupling. The ryanodine receptor type 2 (RyR2) is a large conductance calcium channel located in the junctional SR membrane that is gated by calcium influx via the L-type calcium channels (LTCC) in the cell membrane. RyR2 open probability is regulated by post-translational modifications (e.g. phosphorylation by calcium-calmodulin kinase II, CaMKII, protein kinase A, PKA), by cytosolic RyR2 binding proteins (calmodulin [CaM], immunophillins such as FK506 binding protein [FKBP]) and SR luminal proteins (calsequestrin [Casq2], triadin, junction). Casq2 forms polymers that are anchored to RyR2 and the junctional SR by triadin and junctin. CaM bound to LTCC mediates calcium-dependent inactivation of LTCC.

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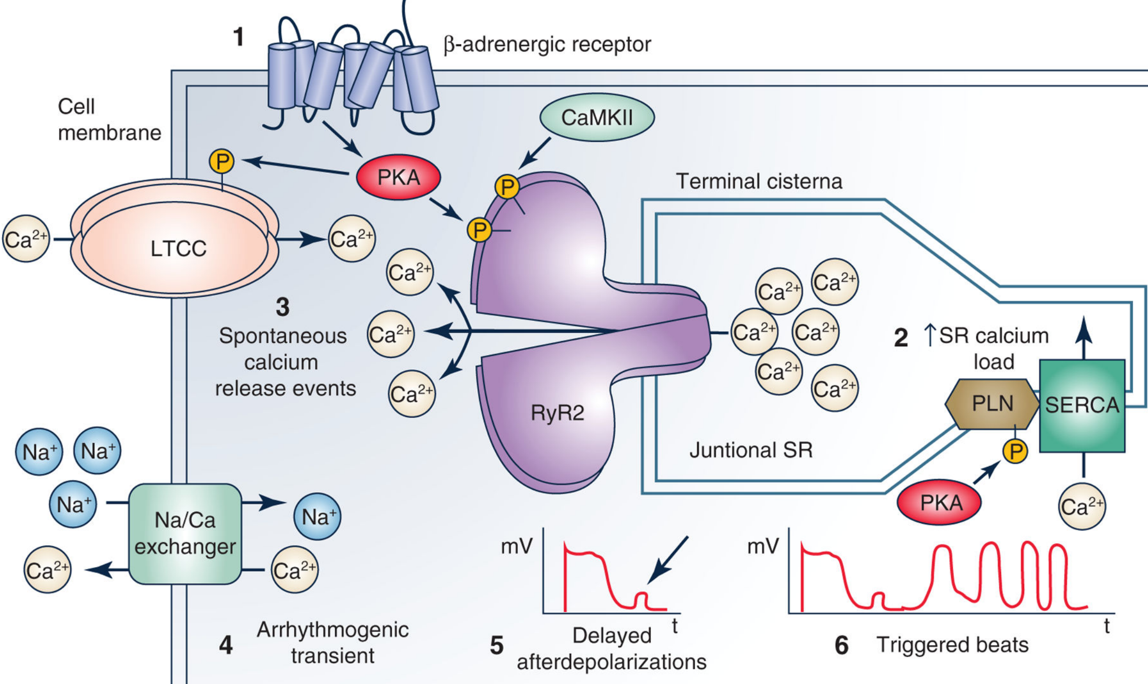

Figure 2. Cellular pathogenesis of catecholaminergic polymorphic ventricular tachycardia (CPVT)

The cartoon illustrates cellular mechanisms underlying CPVT caused by the loss of calsequetrin. 1, catecholamines released during stress or exercise activates *β*-adrenergic receptor signalling, leading to cardiomyocyte calcium loading and enhanced sarcoplasmic reticulum (SR) Ca uptake. 2, the increased SR calcium load is a physiological response necessary for increasing cardiac output during the physiological fight or flight response (Bers, 2001). Normally, ventricular myocytes can handle the increased SR calcium load. 3, if a CPVT mutation is present, RyR2 SR calcium release channels open spontaneously during late diastole, causing unregulated 'pathological' SR calcium release termed 'spontaneous calcium release events' (SCR). 4, the rise in cytosolic calcium during the SCR activates the electrogenic sodium calcium exchanger, which generates an arrhythmogenic transient inward current. 5, this induces a cell membrane depolarization termed 'delayed afterdepolarizations' (DADs). 6, DADs are a well-established cellular mechanism that can then cause triggered beats that lead to ventricular arrhythmias (Priori & Corr, 1990).

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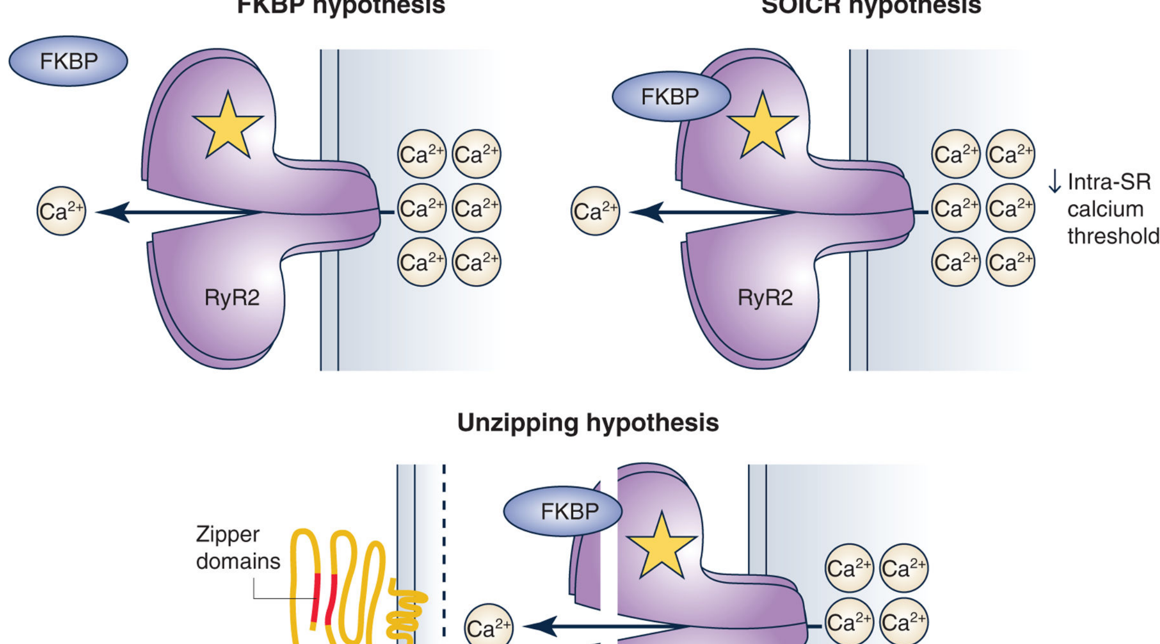

Figure 3. Summary of current hypotheses for how RyR2 mutations could lead to catecholaminergic polymorphic ventricular tachycardia

The first theory states that mutations prevent FKBP binding to RyR2. The second theory states that a mutation can lower the intra-sarcoplasmic reticulum (SR) calcium threshold needed RyR2 to open during diastole, termed 'store overload-induced calcium release' (SOICR). Finally, the 'unzipping' theory stems from the observation that the N-terminal and central domain of RyR2 interact with one another forming a tight seal. Mutations in *RYR2* can affect the interaction and lead to an unzipping of the protein, making RyR2 more prone to open spontaneously.

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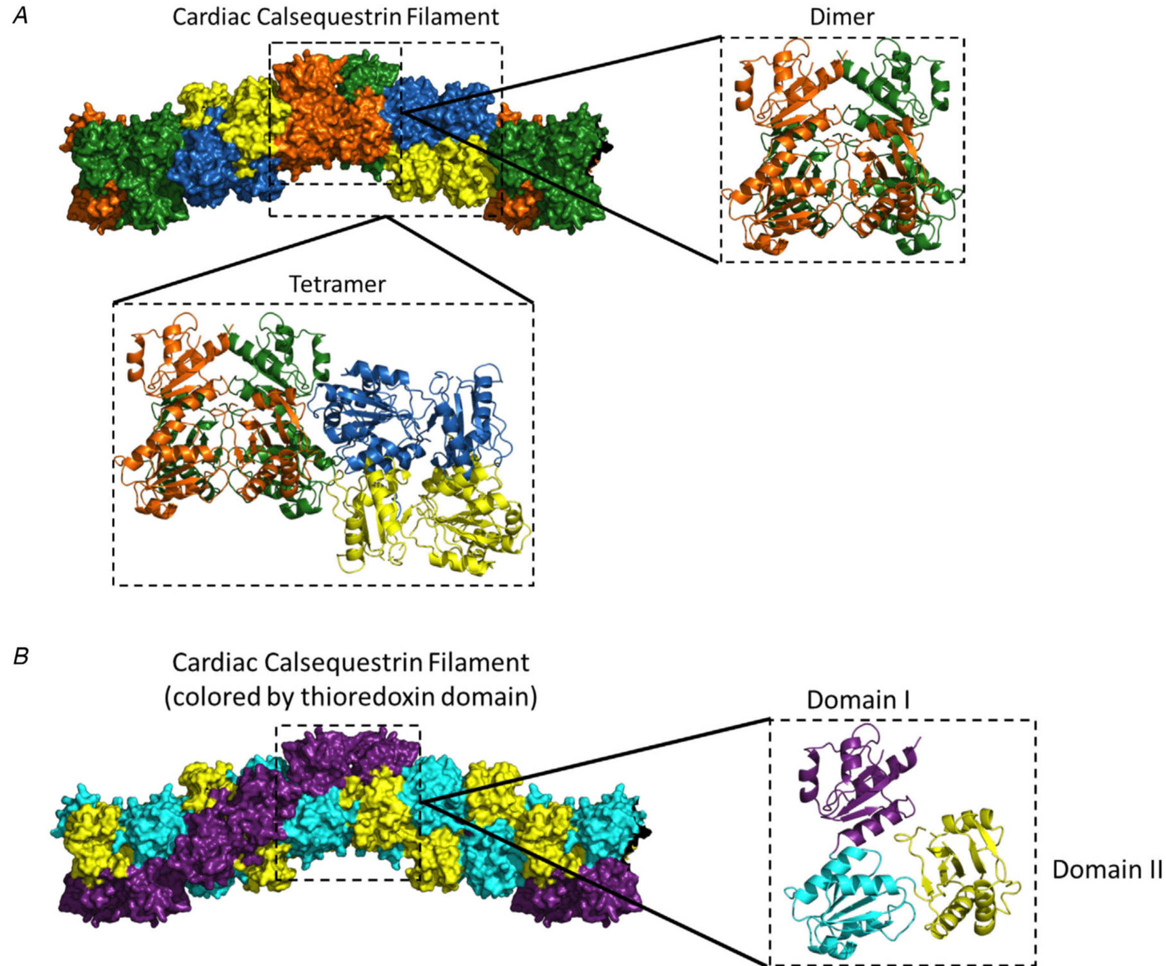

Figure 4. The new cardiac calsequestrin filament (Titus *et al*. 2019, with permission)

Pictured above is the putative structure of the cardiac calsequestrin filament. a, putative calsequestrin filament including its dimeric and tetrameric assembly. b, the filament exhibits a helical structure at the domain level. For simplicity, calsequestrin monomers are coloured by thioredoxin domain (domain I, purple; domain II, yellow; domain III, cyan). The filament is formed by an inner thioredoxin double helix (domains II and III) with an outer thioredoxin single helix (domain I) wrapped around the double helical core. Right side: The monomers are translated but remain in their dimer-forming orientation.

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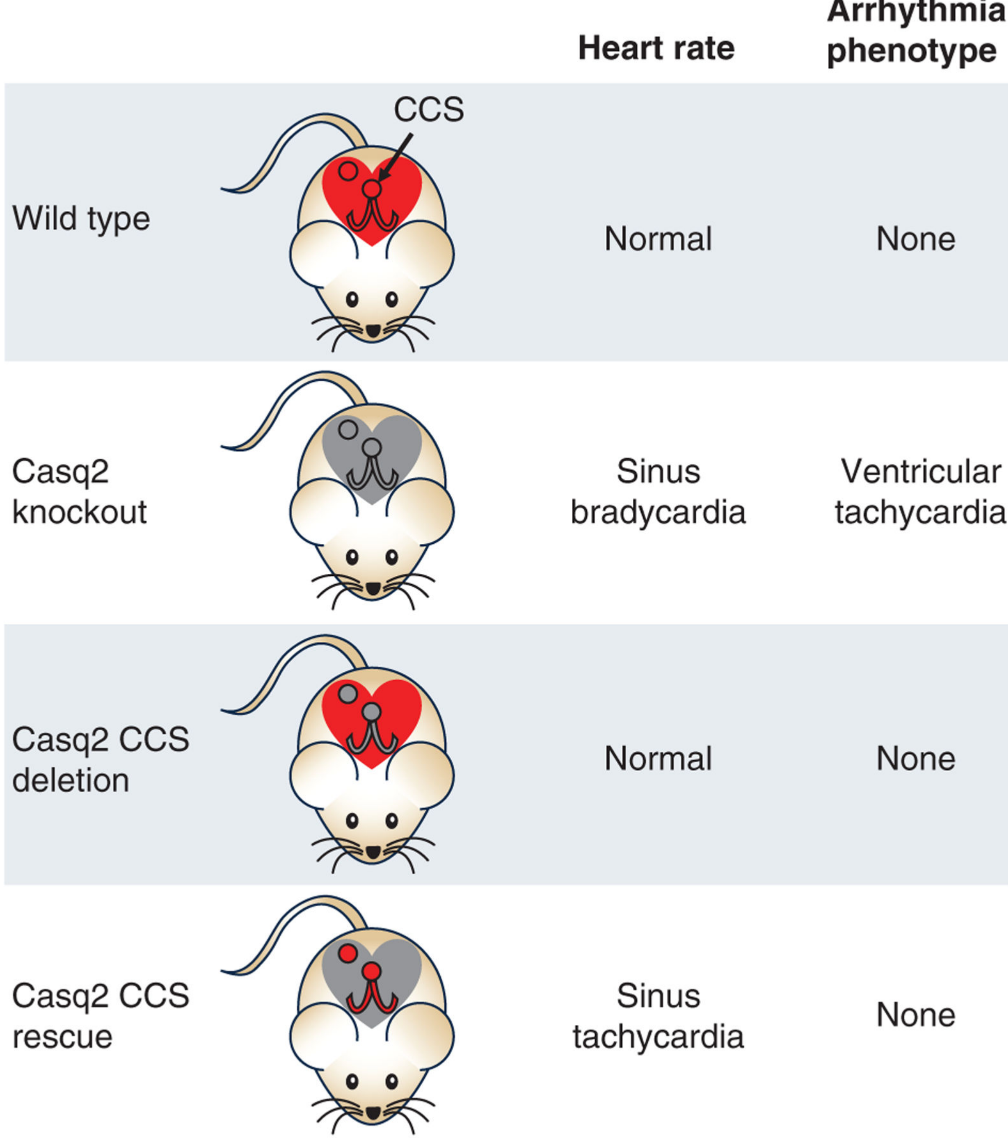

Figure 5. Cardiac conduction system (CSS) targeted *CASQ2* gene deletion or rescue

Cartoon showing the heart rate and ventricular arrhythmia phenotype of mice with conditional deletion or rescue of calsequestrin in the CCS. Red colour indicates functional *CASQ2*.