Calsequestrin 2 and arrhythmias

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Faggioni M, Knollmann BC. Calsequestrin 2 and arrhythmias. Am J Physiol Heart Circ Physiol 302: H1250–H1260, 2012. First published December 23, 2011; doi:10.1152/ajpheart.00779.2011.—Calsequestrin is the most abundant Ca-binding protein of the specialized endoplasmic reticulum found in muscle, the sarcoplasmic reticulum (SR). Calsequestrin binds Ca with high capacity and low affinity and importantly contributes to the mobilization of Ca during each contraction both in skeletal and cardiac muscle. Surprisingly, mutations in the gene encoding the cardiac isoform of calsequestrin (Casq2) have been associated with an inherited form of ventricular arrhythmia triggered by emotional or physical stress termed catecholaminergic polymorphic ventricular tachycardia (CPVT). Despite normal cardiac contractility and normal resting ECG, CPVT patients present with a high risk of sudden death at a young age. Here, we review recent new insights regarding the role of calsequestrin in genetic and acquired arrhythmia disorders. Mouse models of CPVT have shed light on the pathophysiological mechanism underlying CPVT. Casq2 is not only a Ca-storing protein as initially hypothesized, but it has a far more complex function in Ca handling and regulating SR Ca release channels. The functional importance of Casq2 interactions with other SR proteins and the importance of alterations in Casq2 trafficking are also being investigated. Reports of altered Casq2 trafficking in animal models of acquired heart diseases such as heart failure suggest that Casq2 may contribute to arrhythmia risk beyond genetic forms of Casq2 dysfunction.

catecholaminergic polymorphic ventricular tachycardia; cardiac arrhythmia; calcium handling

Introduction

In cardiac muscle, excitation-contraction coupling is regulated by voltage-gated Ca influx through the sarcolemmal L-type Ca channels (8). The resulting increase in the cytosolic Ca concentration triggers the opening of cardiac ryanodine receptor (RyR2) Ca release channels located in the terminal cisternae of the sarcoplasmic reticulum (jSR), the main intracellular Ca-storage compartment (Fig. 1A) (37). Tethered to RyR2 Ca release channel complex (108) are polymers of the cardiac isoform of calsequestrin, calsequestrin 2 (Casq2), which is a low-affinity high-capacity Ca-binding protein thought to provide much of the Ca released from the SR during each heart beat (Fig. 1A) (66).

The released Ca binds to troponin C on the myofilaments starting the contraction. Relaxation occurs when Ca release ceases and the cytosolic Ca concentration returns to diastolic values. Ca is removed from the cytosol by three independent mechanisms: 1) reuptake of Ca into the SR via a Ca-ATPase pump (SERCA); 2) extrusion from the cell via the Na/Ca exchanger (NCX), and to a lesser degree by the sarcolemmal Ca-ATPase pump; and 3) mitochondrial uptake via the Ca uniporter. Together, these mechanisms effectively reduce the cytosolic Ca ions promoting Ca dissociation from the myofilaments (7).

Arrhythmogenic SR Ca release occurs when RyR2 Ca release channels open spontaneously without being triggered by voltage-gated Ca influx. As shown in Fig. 1B, the spontaneous SR Ca release activates the electrogenic NCX and produces a delayed afterdepolarization (DAD; Ref. 36). Premature DAD-triggered action potentials can result in life-threatening ventricular tachycardia and have been implicated as the underlying mechanism responsible for familial arrhythmia disorders caused by mutations in the genes encoding RyR2 or Casq2 (50, 75). This review focuses on Casq2 properties and physiological functions and on the role of Casq2 in the pathophysiology of genetic as well as acquired arrhythmia disorders.

Properties of Casq2 and Its Role in Ca Handling

Protein conformation and characteristics. Calsequestrin is a glycoprotein isolated for the first time in the early 1970s from the SR of rabbit skeletal muscle (63). A few years later the cardiac-specific isoform Casq2 was identified (11). Amino acid sequencing and crystal structure studies have shed light on the conformation of this molecule: Casq2 is composed of three domains each with negatively charged thioredoxin-like folds with four α-helix surrounding a β-sheet core. The center of
each domain is hydrophobic due to high aromatic acid content, whereas the exterior domain surfaces have an electronegative potential due to a large number of acidic residues. The interdomain space and connecting loops also contain many acidic residues, rendering the core of the molecule overall hydrophilic (100). Therefore, to stabilize the molecular core, a divalent cation such as Ca is required, inducing at the same time conformational changes that further increase its Ca-binding capability (100). Formation of a Casq2 dimer creates additional negatively charged pockets that can absorb more Ca than two single monomers (100). Initially, the intermolecular binding occurs by front to front dimer formation through the N-terminal arms of Casq2. At higher Ca concentrations, back to back binding occurs through acidic C-terminal tails, producing tetramers. Salt bridges stabilize the union. Deletion of either the N terminal or C terminal of the monomers results in the inability of the molecule to form linear polymers (5). Polymerization can also be inhibited by K ions that compete with Ca ions in binding to Casq2, but being a monovalent cation, K fails to produce cross bridges with other monomers (4, 70). Ca-induced dimerization starts at ion concentrations in the range of 0.5–1 mM, and at 3 mM Ca there is a detectable tetramer population (69). Once the polymeric state is reached ~40 Ca ions are bound to the molecule (66).

**Cellular localization and function.** Electron microscopy images of muscle fiber thin sections show that Casq2 is localized in the terminal cisternae and is connected to the jSR membrane (24). Casq2 binds to the ryanodine receptor Ca channel (RyR2) in the SR through two proteins, triadin and junctin (Fig. 1), which, extending from the junctional face of the membrane into the lumen of the SR, form a trimeric complex with Casq2 and are involved in the regulation of Ca releases (84).
Arrhythmogenesis

Genetic and Clinical Evidence for Casq2 in Arrhythmogenesis

**Table 1. Human CASQ2 mutations**

<table>
<thead>
<tr>
<th>Nucleotide Change (Reference No.)</th>
<th>Amino Acid Change</th>
<th>Mutation Type</th>
<th>Mutation Effect In Vitro</th>
<th>Zygosity</th>
<th>Mouse Model</th>
</tr>
</thead>
<tbody>
<tr>
<td>1038 G&gt;C (53)</td>
<td>D307H</td>
<td>Missense</td>
<td>Impairment of Ca-buffering activity</td>
<td>Homozygous</td>
<td>Present</td>
</tr>
<tr>
<td>97 C&gt;T (75)</td>
<td>R33X</td>
<td>Nonsense</td>
<td>Premature stop codon with complete absence of Casq2</td>
<td>Homozygous</td>
<td>Present</td>
</tr>
<tr>
<td>532 + 1 G&gt;A (75)</td>
<td>—</td>
<td>Splicing</td>
<td>Premature stop codon with complete absence of Casq2</td>
<td>Homozygous</td>
<td>Present</td>
</tr>
<tr>
<td>62delA (75)</td>
<td>L23fs + 14X</td>
<td>Deletion</td>
<td>Premature stop codon with complete absence of Casq2</td>
<td>Homozygous</td>
<td>Present</td>
</tr>
<tr>
<td>98 G&gt;A (94)</td>
<td>R33Q</td>
<td>Missense</td>
<td>Impaired interactions of Casq2 with the RYR2 channel. Altered regulation of RYR2 by luminal Ca. Altered Casq2 polymerization</td>
<td>homozygous</td>
<td>Present</td>
</tr>
<tr>
<td>339-354 del (21)</td>
<td>G112 + 5X</td>
<td>Deletion</td>
<td>Reduced Ca-binding properties</td>
<td>Homozygous</td>
<td>—</td>
</tr>
<tr>
<td>500 T&gt;A (21)</td>
<td>L167H</td>
<td>Missense</td>
<td>Reduction of SR Ca release and Ca content</td>
<td>Compound heterozygous</td>
<td>—</td>
</tr>
<tr>
<td>164A&gt;G (20)</td>
<td>Y55C</td>
<td>Missense</td>
<td>Cumulative effect with other mutations</td>
<td>Compound heterozygous</td>
<td>—</td>
</tr>
<tr>
<td>923C&gt;T (20)</td>
<td>F308L</td>
<td>Missense</td>
<td>Likely reduction of Ca-binding capacity or its interaction with RYR2</td>
<td>Compound heterozygous</td>
<td>—</td>
</tr>
<tr>
<td>(61)</td>
<td>F189L</td>
<td>Missense</td>
<td>Altered Ca-binding capacity of Casq2 domain II</td>
<td>Heterozygous</td>
<td>—</td>
</tr>
<tr>
<td>529G&gt;C (105)</td>
<td>E177Q</td>
<td>Missense</td>
<td>Altered Ca-binding capacity of Casq2 domain II</td>
<td>Heterozygous</td>
<td>—</td>
</tr>
<tr>
<td>196A&gt;G (105)</td>
<td>T66A</td>
<td>SNP</td>
<td>no change</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>226G&gt;A (105)</td>
<td>V76M</td>
<td>SNP</td>
<td>Reduced Ca-binding, altered Casq2 polymerization</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>1185 C&gt;T (105)</td>
<td>D395D</td>
<td>SNP</td>
<td>Altered Ca-binding capacity and aggregation state</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>618 A&gt;C (46)</td>
<td>K206N</td>
<td>Missense</td>
<td>Altered Ca-binding capacity and aggregation state</td>
<td>Heterozygous</td>
<td>—</td>
</tr>
</tbody>
</table>

Casq2, cardiac isoform of calsequestrin; SNP, single-nucleotide polymorphism; SR, sarcoplasmic reticulum.

probable, likely through its interaction with triadin and junctin (28, 95).

**Genetic and Clinical Evidence for Casq2 in Arhythmogenesis**

**Genetics.** Catecholaminergic polymorphic ventricular tachycardia (CPVT) is an inherited arrhythmogenic condition with an estimated prevalence in the population of 1 in 10,000. An autosomal-dominant form of CPVT was initially mapped to the chromosomal locus 1q42-q43 where the RYR2 gene was colocalized (93). A later study (78) confirmed that mutations in the RYR2 gene are in fact responsible for a dominant inherited CPVT phenotype and suggested that impaired Ca handling is the underlying mechanism of this disease. In the same year, an autosomal recessive form of CPVT was observed in 7 related families of a Bedouin tribe with 9 cases of sudden cardiac death and 12 other children with recurrent syncope and seizures starting at the age of 6 ± 3 yr. The disorder was mapped, once again, on chromosome 1 but this time on the short arm, 1p13–21. Compared with individuals with RYR2 mutations described by Swan et al. (93), the recessive form of CPVT in Bedouins was more severe with an earlier onset, higher penetrance, and younger age at death (54). Responsible for this severe form of CPVT was a missense mutation in an highly conserved region of the CASQ2 gene that changed aspartic acid to the positively charged histidine at position 307 of the protein (55).

Since then, 14 other mutations in the CASQ2 gene have been associated with CPVT: seven missense mutations like the D307H described by Lahat et al. (55), three single-nucleotide polymorphisms and four nonsense, deletion, or frameshift mutations that are predicted to cause a loss of CASQ2 protein in the SR (Table 1).

Since CASQ2-linked CPVT accounts for 3–5% of all CPVT cases (1), one can estimate the overall prevalence of these homozygous Casq2 mutations in the general population to be between 1:400,000 and 1:200,000 [similar to the prevalence of aortic arch interruption (106) and ~20 times lower than LQT syndrome (83)]. The frequency of heterozygous mutation carriers is much higher (1:20,000) and may contribute as added risk in combination with other disorders that cause RYR2 dysfunction (e.g., heart failure).

Despite the many different CASQ2 mutations leading to CPVT, the clinical presentation of these patients appears to be more homogeneous than that of RYR2-linked CPVT. A possible explanation is provided by studies in knockin mice (discussed below), which showed that all mutations lead to either severe reduction or complete loss of CASQ2 protein (80, 88).

**Clinical features.** Subjects affected by CPVT experience life-threatening ventricular arrhythmia induced by emotional or physical stress in the absence of any structural heart disease. These patients are frequently diagnosed at a young age (9 ± 4 yr) following single or recurrent syncope and/or seizures (75). Other symptoms are light-headedness, dizziness, and palpitations (56). The age at the onset of the first symptoms may vary, and it appears to be related to the severity of the phenotype, with early onset being associated with worse long-term prognosis. The diagnosis of CPVT is not easy, as the physical examination is negative and the resting ECG is normal with a QT interval in the physiologic range. A slight bradycardia is often the only finding observed in these subjects (76). However, that is not specific and CPVT should be suspected in individuals with a personal and/or family history of syncope, seizures, and unexplained sudden cardiac deaths triggered by physical or emotionally stressful activity. The diagnosis can be made with 24-h Holter ECG recording or exercise stress test.
During exercise, patients develop sporadic premature ventricular beats that turn into bigeminal patterns and eventually ventricular tachycardia with higher heart rates (56, 77). A typical ECG finding during exercise or acute emotional stress is bidirectional ventricular tachycardia (BVT), characterized by a beat-to-beat 180° rotation of the QRS complex (Fig. 1D). However, it must be noted that BVT is not pathognomonic of CPVT, because BVT also occurs in acquired conditions such as digitalis toxicity, ischemic heart disease, and myocarditis (10, 27, 89).

**Treatment.** Early identification and treatment of CPVT patients are very important because of the high mortality of untreated subjects by the time they reach 20 to 30 yr of age (32). The cornerstone of pharmacological treatment of CPVT are β-adrenergic receptor blockers (76). Sequential exercise tests are used to assess the dose and to monitor the efficacy of the treatment. Ca channel blockers, such as verapamil can be used as a second-line treatment in place of a β-blocker or in addition to it in patients who are still symptomatic with the maximum tolerated dose of β-blockers (81, 92). Unfortunately β-blockers and Ca channel blockers are not effective in all the patients, and implantable cardioverter defibrillators (ICD) are frequently used as an additional measure to prevent sudden death. However, ICDs are not always protective because defibrillation shocks can cause catecholamine release and electrical storm, leading to more arrhythmic events; episodes of sudden cardiac deaths in CPVT patients with ICDs have been reported (65, 73, 76). Hence, the need for new pharmacological approaches to CPVT led to the recent observation that the class I antiarrhythmic drug flecainide effectively prevents exercise-induced CPVT in transgenic mice and patients (101), probably because of a dual effect of the drug on Na channels and RyR2 channels that reduces SR spontaneous Ca release and triggered beats (25, 59). In 2011, a clinical case series (97) of 33 CPVT patients unresponsive to conventional drug therapy showed additional antiarrhythmic benefit from adding flecainide to β-blockers. Propafenone, another class I antiarrhythmic medication that also inhibits RyR2 channels, was proposed as a possible alternative drug to flecainide after treatment of a 22-yr-old subject with CPVT drastically reduced ICD shocks during a 12-mo observation period (35). Most recently, the β-blocker carvedilol was found to also inhibit RyR2 channels and was superior to other β-blockers in a transgenic mouse model of CPVT (109). Compounds such as S107 and JTV519 reportedly increase the binding of the modulatory protein calsequestrin to the RyR channels and prevent SR Ca leak in experimental models (57, 58), although JTV519 was ineffective in vivo in a CPVT mouse model (85). Nevertheless, despite these promising results, drug efficacy in CPVT is currently based only on observational studies, hence the need for prospective clinical trials (48). An S107 derivative is currently in early phase clinical trials for CPVT in Europe. A placebo-controlled prospective trial of flecainide in CPVT is open for enrolment in the U.S. and Europe (ClinicalTrials.gov: NCT01117454).

**Calsequestrin 2 and arrhythmias**

**Experimental Evidence from Casiq2 Mouse Models**

*Casiq2* gene-targeted mouse models of CPVT. In recent years, studies in transgenic mouse models of *CASIq2*-linked CPVT have clarified the pathophysiological mechanisms underlying the ventricular arrhythmia as well as identified new therapeutic approaches. In contrast to mouse models of *RYR2*-linked CPVT, all *Casiq2* mutant mouse models consistently reproduce the major aspects of human CPVT: exercise and/or emotional stress-induced polymorphic ventricular tachycardia, sinus bradycardia, normal contractility, and normal ECG parameters (76). Table 2 summarizes key findings from the five mouse models currently available: Casiq2 homozygous knockout mice (50), Casiq2 heterozygous knockout mice (14), Casiq2<sup>D307H</sup>, Casiq2<sup>ΔE9</sup> (88), and Casiq2<sup>R33Q</sup> knockin mice (80). Interestingly, all the mutations studied in knockin mice result in either complete loss or severe reduction of Casiq2 protein in the jSR. For example, Casiq2<sup>ΔE9</sup> is a frame shift mutation that causes a premature stop codon that leads to a truncated, nonfunctional mRNA and loss of Casiq2 protein. On the other hand, Casiq2<sup>D307H</sup> knockin mice have normal mRNA levels. However, mutant Casiq2<sup>D307H</sup> protein is almost completely absent (>95% reduction) in the hearts of adult mice, suggesting that the D307H mutation causes enhanced protein degradation or impairs cellular trafficking in vivo (49). In vitro (34) studies suggest that the single amino acid change between the second and third domain of the molecule disrupts the formation of properly oriented dimers and impairs the interactions with triadin and junctin, either of which might contribute to reduced retention of the mature protein in the jSR. Similarly, in Casiq2<sup>R33Q</sup> knockin mice, the mutant protein reaches full maturation but does not form Ca-dependent polymers at physiological Ca concentrations (5, 45). The defective Casiq2<sup>R33Q</sup> molecule is more susceptible to proteolysis (80), which may explain the drastically reduced levels of mutant Casiq2 protein found in this model (Table 2).

*Casiq2* overexpression mouse models of CPVT. A different approach that gives further insight on the role of Casiq2 in CPVT comes from transgenic overexpression of Casiq2 carrying the CPVT-linked D307H point mutation using an α-myosin heavy chain promoter either in wild-type or Casiq2 knockout mice. In the first report, a chronic increase (from 2- to 6-fold) of mutant Casiq2<sup>D307H</sup> protein in wild-type mice that also have wild-type Casiq2 protein produces intracellular changes and in vivo phenotype of catecholamine-induced ventricular arrhythmia (23). This seems to suggest a negative dominant behavior of this mutated protein but is at odds with the human data showing that heterozygous carriers of the D307H mutant are asymptomatic. An explanation may be provided by results obtained by overexpressing Casiq2<sup>D307H</sup> in Casiq2 knockout mice that lack Casiq2 protein, which models the situation in homozygous human carriers of the D307H mutation. Contrary to the complete loss of mature protein observed in the D307H knockin mice, in this transgenic overexpression model Casiq2<sup>D307H</sup> protein is present at approximately twofold higher concentration than wild-type Casiq2 found in nontransgenic littermates. This result suggests that a strong transgenic promoter (α-myosin heavy chain) may be sufficient to overcome the increased protein destruction rate. The mature Casiq2<sup>D307H</sup> displays a folding pattern similar to wild-type Casiq2 protein and is also successfully targeted to the jSR cisternae (39). Surprisingly, when expressed at such high levels, the mutant Casiq2<sup>D307H</sup> not only restores the SR ultrastructure compromised in the Casiq2 knockout mice but also prevents spontaneous Ca release events in myocytes and almost completely rescues the CPVT phenotype of Casiq2 knockout mice (40).
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Table 2. Mouse models of Casq2

<table>
<thead>
<tr>
<th>Mouse Model (Reference No.)</th>
<th>In Vivo Phenotype</th>
<th>Cellular Phenotype</th>
<th>Casq2 Protein Expression</th>
</tr>
</thead>
<tbody>
<tr>
<td>Homozygous knockout (50)</td>
<td>Slight increase in heart weight and left-ventricular wall thickness with normal contractile function: severe CPVT phenotype.</td>
<td>Near absence of the Casq2-binding proteins triadin-1 and junctin. Increased SR volume with preserved Ca content. Normal Ca-induced SR Ca releases, increased spontaneous SR Ca release during catecholaminergic stress.</td>
<td>Absent</td>
</tr>
<tr>
<td>Heterozygous knockout (14)</td>
<td>Normal cardiac morphology. Mild CPVT phenotype with 3-fold higher rates of ventricular ectopy under stress compared with wild-type littermates.</td>
<td>No significant change in the expression of other SR protein. Field-stimulated Ca transients, cell shortening, L-type Ca current, and SR volume not significantly different compared with wild-type myocytes. Increased SR Ca leak at the same free intra-SR Ca concentrations.</td>
<td>25% reduction</td>
</tr>
<tr>
<td>Homozygous knockin (D309 ΔE9) (88)</td>
<td>Structurally normal hearts at young age with stress-induced ventricular arrhythmias; aging produces cardiac hypertrophy and reduced contractile function.</td>
<td>Reduced total SR Ca load. Increased calreticulin and RyR2 levels. Prolonged Ca release, and delayed SR Ca re-uptake. Stress-induced diastolic Ca release.</td>
<td>95% reduction</td>
</tr>
<tr>
<td>Homozygous knockin (R33Q) (80)</td>
<td>Structurally normal hearts. Ventricular ectopy at rest and on exposure to environmental stress.</td>
<td>Unchanged SR volume. Significant reduction of triadin and junctin. Reduced SR Ca content. Adrenergically induced delayed (DADs) and early (EADs) afterdepolarizations leading to triggered activity.</td>
<td>&gt;50% reduction</td>
</tr>
<tr>
<td>Casq2 overexpression (38)</td>
<td>Severe cardiac hypertrophy, with a 2-fold increase in heart mass and cell size.</td>
<td>Downregulation of RyR2, triadin, and junctin. Normal levels of Ca-ATPase and phospholamban. Severe reduction of Ca-mediated SR Ca releases and frequency of spontaneous Ca sparks.</td>
<td>10-fold increase</td>
</tr>
<tr>
<td>Casq2 overexpression (82)</td>
<td>Increased heart-to-body weight ratio. Mild hypertrophy in the left ventricular free walls and intraventricular septa, with depressed rates of contraction and relaxation.</td>
<td>Normal levels of RyR2, triadin and junctin. Increased expression of SR Ca-ATPase, phospholamban, and calreticulin. Increased SR Ca storage capacity but reduced Ca-induced Ca releases and Ca transient amplitude (45%) and reduced L-type Ca density.</td>
<td>20-fold increase</td>
</tr>
<tr>
<td>Casq2D307H overexpression in wild-type background (23)</td>
<td>Structurally normal hearts. Mild arrhythmogenic phenotype: few spontaneous ventricular arrhythmias at rest and nonsustained polymorphic ventricular tachycardia during stress</td>
<td>Normal levels of RyR2, triadin, SERCA, and L-type Ca channel. Diminished Ca-induced Ca transient amplitude and duration. Increased Ca spark frequency.</td>
<td>2- to 6-fold increase</td>
</tr>
<tr>
<td>Casq2D307H overexpression in Casq2 knockout background (39, 40)</td>
<td>Structurally normal hearts. Partial rescue of Casq2 knockout CPVT phenotype.</td>
<td>Expression of mutant Casq2 targeted to junctional SR. Partial recovery of SR ultrastructure compared with Casq2 knockout mice (e.g., terminal cisternae contain Casq2D307H and triadin levels are partially restored). Normal levels of calreticulin and SERCA. Altered protein conformation and increased proteolysis compared with wild type.</td>
<td>2-fold increase</td>
</tr>
</tbody>
</table>

RyR2, ryanodine receptor 2; SERCA, sarco(endo)plasmic reticulum Ca$^{2+}$-ATPase; CPVT, catecholaminergic polymorphic ventricular tachycardia.

Taken together, these results are consistent with the emerging paradigm that in homozygous carriers of Casq2 point mutations, the severe reduction or complete loss of Casq2 is due to altered trafficking and/or increased protein degradation and is the primary underlying mechanism responsible for the CPVT phenotype. In heterozygous carriers, enough wild-type Casq2 is present to compensate for the high degradation rate of the mutant Casq2 mRNA or protein; hence heterozygous individuals are asymptomatic. These results also suggest that the dominant-negative effects of the mutant Casq2 protein observed in overexpression experiments, using mice or isolated myocytes, may not be relevant for the pathogenesis of human CASQ2-linked CPVT. However, this conclusion has been recently challenged by a study of induced pluripotent stem cells derived from a homozygous carrier of the CASQ2-D307H mutation, which showed that mutant D307H protein was indeed present in induced pluripotent stem cell-derived cardiomyocytes (68). Yet, it is still not known if mutant D307H protein is present in the human heart. Thus the definitive answer can only be provided by a cardiac muscle biopsy from a patient with the D307H mutation. Until one becomes available, the exact role of mutant Casq2-D307H protein in the pathogenesis of CPVT remains speculation.

How Does Casq2 Dysfunction Cause Ventricular Arrhythmia?

Cellular arrhythmia mechanisms. Cardiac contractility and systolic Ca releases from the SR appear normal in Casq2 mutant mice at rest. The higher arrhythmogenic risk during stress can be attributed to spontaneous Ca releases from the SR that occur during diastole. The rise in diastolic Ca concentra-
tion activates the Na/Ca exchanger, causing an inward Na current that can result in DAD of the cell membrane and triggered activity (Fig. 1B). Although all cardiomyocytes have the potential to generate a DAD, it has been proposed that DADs occurring in the Purkinje fibers (PF) of the specialized conduction system are the source of the triggered ventricular arrhythmia in CPVT. PF have several characteristics that make them particularly prone for spontaneous Ca releases: decreased T-tubular density (22), the presence of inositol 1,4,5-trisphosphate-sensitive Ca\(^{2+}\) channels (33), and susceptibility to Ca\(^{2+}\) overload (60, 98). An indirect support to this hypothesis is the observation that the drug flecainide, which is effective clinically in CPVT, preferentially suppresses arrhythmogenic Ca waves in isolated PF compared with ventricular myocytes (42).

The PF as the cellular origin of CPVT is supported by experimental evidence in vivo showing conversion of bidirectional VT to monomorphic VT in RyR2 mutant mice after chemical ablation of the right ventricular branch of the conduction system (12). Furthermore, a recent computer model of CPVT reproduced the characteristic ECG pattern of bidirectional VT with a simple “ping pong” mechanism of reciprocating bigeminy between the right and left branches of the conduction system (3). However, based on the computer model, the origin of the bidirectional VT is not restricted to the bundle branches and could also originate from more distal PF or the working myocardium. This is illustrated by recent data from our group, where isolated Casq2 knockout hearts exhibit alternating ventricular activation that originated from the right and left ventricle at sites distinct from the bundle breakthrough points during normal sinus rhythm (Fig. 1C). The resulting bidirectional QRS pattern on the ECG record of the isolated heart (Fig. 1C) was similar to that recorded in vivo using telemetry (Fig. 1D). Thus while the PF are the likely culprit, the exact cellular origin of the bidirectional ventricular arrhythmias remains unknown and is an area of active investigation.

Theoretically, the increased rate of spontaneous Ca releases in Casq2 knockout hearts could produce stochastic membrane DADs that can induce intercellular discordant electrical alternans: when neighboring groups of cells are electrically uncoupled, different repolarization gradients are created in adjacent areas of the heart and they can be a substrate for reentry especially at elevated heart rates (71, 104). However, we (51) recently demonstrated that Casq2 knockout hearts are protected against tachycardia-induced ECG T-wave alternans, which is a marker for susceptibility to reentrant ventricular arrhythmia in animal models and in clinical studies (99). Thus the results from the Casq2 knockout mice suggest that loss of Casq2 causes an increased risk for triggered arrhythmia but may paradoxically protect against reentrant-type arrhythmia.

**Subcellular and molecular arrhythmia mechanisms.** At the subcellular level, we propose at least three independent mechanisms that can explain how reduced or dysfunctional calsequestrin causes the spontaneous SR Ca release events that can trigger ventricular arrhythmia: 1) loss of Ca buffering in the jSR, 2) loss of RyR2 regulation by Casq2, and 3) remodeling of SR structure and proteins. These three mechanisms are discussed in more detail next.

**LOSS OF CA BUFFERING IN THE JSR.** Casq2 is the main Ca-buffering protein of the SR. Both acute as well as chronic overexpression of Casq2 causes a large increase in the SR Ca content (38, 82). Surprisingly, Casq2 homozygous knockout mice display a preserved SR Ca content. Studies on isolated myocytes show that loss of Casq2 Ca-buffering activity was compensated for by a significant (~50%) increase in the SR volume (50). Although the total SR luminal Ca content seems to be preserved, a recent report shows that the kinetics of free Ca in the lumen of the SR are different in Casq2 knockout mice. Loss of Casq2-mediated Ca buffering causes a faster rise in luminal free Ca after each beat (51). The resulting faster rise of Ca near the pore of the RyR2 Ca channels can be expected to have two effects: 1) an increased releasable amount of Ca early during the cardiac cycle, and 2) a faster recovery from SR release refractoriness. Both factors would promote accelerated recovery of SR Ca release, which has recently been confirmed in experiments with intact hearts from Casq2 null mice (51), as shown in Fig. 2. Another consequence of the faster recovery of Ca release in hearts lacking Casq2 is a protection against tachycardia-induced Ca transient alternans and T-wave alternans on ECG, which was significantly reduced in Casq2 knockout mice compared with wild-type hearts (51). Thus Casq2 likely plays a key role in the genesis of Ca transient alternans and T-wave alternans.

**LOSS OF RyR2 REGULATION BY CASQ2.** Regulation of the RyR2 channel permeability appears to be very complex. Both cytosolic and SR luminal free Ca concentration affect the open probability of this channel and influence the duration of the local Ca releases from the SR (52). A large body of evidence supports the concept that SR Ca leak rate is proportional to SR luminal Ca concentration in a nonlinear fashion (62, 86). Loss of Casq2 in Casq2 knockout myocytes makes this relationship much steeper (Fig. 3): in other words, in the absence of Casq2, the SR Ca load threshold at which spontaneous Ca release

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**Fig. 2.** Casq2 regulates Ca release refractoriness. In wild-type mouse hearts (Casq2\(^{+/+}\)), Ca release is strongly suppressed for very premature beats. This phenomenon is known as Ca release refractoriness. Ca release refractoriness is almost completely lost in Casq2 knockout hearts (Casq2\(^{-/-}\)). Ca fluorescent records are from the epicardial surface of isolated perfused hearts loaded with rhod-2 AM at 37 °C (51).
SR releases (43).

stimulation lead to an increase in SR Ca load and spontaneous protein kinase A-mediated effects of the strain on the intracellular Ca handling in CPVT patients. The for any given luminal free Ca concentration (Fig. 3).

net effect is an increased probability of spontaneous Ca release of Casq2 itself or of the proteins mediating Casq2 binding to whether this loss of RyR2 regulation is attributable to reduction the RyR2 channel complex (91). In addition, it is uncertain threshold for luminal or cytosolic activation of Ca release from Ca-induced and spontaneous Ca release despite the much pressing Casq2 that show a significant reduction of both single RyR2 channel experiments in artificial lipid bilayers direct inhibitory action of Casq2 on RyR2 is also supported by which is independent of Casq2’s action as a Ca chelator. The direct inhibitory action of Casq2 on RyR2 is also supported by single RyR2 channel experiments in artificial lipid bilayers (79), and results from studies (38) in cardiomyocytes overexpressing Casq2 that show a significant reduction of both Ca-induced and spontaneous Ca release despite the much higher total SR Ca pool available.

Controversy remains as to whether lack of Casq2 lowers the threshold for luminal or cytosolic activation of Ca release from the RyR2 channel complex (91). In addition, it is uncertain whether this loss of RyR2 regulation is attributable to reduction of Casq2 itself or of the proteins mediating Casq2 binding to RyR2 channels (28). Regardless of the exact mechanism, the net effect is an increased probability of spontaneous Ca release for any given luminal free Ca concentration (Fig. 3).

In this context, any catecholaminergic stress puts further strain on the intracellular Ca handling in CPVT patients. The protein kinase A-mediated effects of the β-adrenergic receptor stimulation lead to an increase in SR Ca load and spontaneous SR releases (43).

REMODELING OF SR PROTEINS AND SR ULTRASTRUCTURE. Almost all Casq2 mutant mouse models studied to date display a significant reduction of the Casq2 binding proteins triadin and junctin (Table 2). Downregulation of triadin and junctin has also been reported in a mouse model of Casq2 overexpression, indicating a possible interdependence of Casq2 and its binding protein expression both in physiologic and pathologic conditions (38). Moreover, gene-targeted deletion of triadin or junctin also generates an arrhythmogenic phenotype in the absence of Casq2 mutations (17, 107). Loss of triadin leads to profound structural modification of the SR (50% reduction of RyR2, Casq2, and junctin) and of the terminal cisternae (up to 50% reduction in the contacts between jSR and t tubules) causing impaired excitation-contraction coupling (17). On the other hand, SR protein expression is not altered in junctin knockout mice although junctin ablation is associated with altered Ca-cycling parameters, and isoproterenol induced DADs (107). Taken together, these findings suggest that the reduction of triadin and junctin may independently contribute to the arrhythmia risk associated with Casq2 mutations (47). Increased protein expression of RyR2, calreticulin, and histidine-rich Ca-binding protein has also been reported in some but not in all of the mouse models (29, 66, 88). However, the relevance of these protein expression changes for the pathogenesis of CPVT remains to be determined. Casq2 knockout mice also compensate for the loss of Casq2 Ca buffering by an increase in SR volume but without significant changes in the jSR cisternae and dyad morphology (50). To what extent the associated changes in SR ultrastructure (e.g., SR volume increase in Casq2 null mice; Ref. 50) contribute to the arrhythmogenic Ca release is not known.

Potential Role of Casq2 in Acquired Arrhythmias

So far, we described the role of Casq2 in CPVT, an inherited arrhythmogenic condition caused by mutations in the Casq2 gene. However, secondary Casq2 dysfunction may contribute to an increased arrhythmia risk in other inherited and acquired cardiac diseases characterized by Ca-handling abnormalities: heart failure, muscular dystrophy, mitochondrial dysfunctions, and drug cardiotoxicity.

Casq2 in heart failure. Heart failure is associated with profound alterations in intracellular Ca handling (9, 102). One consistent finding is an increased SR Ca leak probably facilitated by increased open probability of RyR2 Ca release channels, which, similar to CPVT, increases the risk for spontaneous Ca release under conditions of β-adrenergic stimulation (9, 102). Once spontaneous Ca releases occur, a DAD might be triggered. However, due to an upregulation of the Na/Ca exchanger in failing myocytes, any rise in intracellular Ca results in a greater arrhythmogenic inward current, reducing the quantity of Ca release required to trigger a DAD (74). Oxidative, PKA and/or CaMKII-dependent modifications of the RyR2 channels during the disease progression have also been implicated in the arrhythmogenic potential of failing hearts by reducing the ability of the RyR2 channels to become refractory after a Ca release (6, 102). Interestingly, Casq2 protein levels are usually unchanged in cardiac tissue from humans and animal models of heart failure. However, investigators have identified posttranscriptional modifications of the Casq2 protein in pacing-induced heart failure models, with a significant change in the mannose content of glycans in Casq2 molecules compared with controls. The structure of the glycan residues observed in this model differs from the one expected in the jSR of healthy cells, suggesting a possible alteration in the metabolism or in the trafficking of Casq2 protein through the secretory compartments (44). These results suggest that Casq2 might be altered by posttranscriptional modifications and never reaches its subcellular target in the jSR (64). Consistent with this hypothesis, mass spectrometry analysis of Casq2 suggests that higher amounts of Casq2 protein may be
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retained in the rough ER of the perinuclear cisternae of cardiac myocytes during disease states such as heart failure (44, 64). Furthermore, the reduction of triadin and junctin levels, observed in failing hearts, might also play a role in a subcellular redistribution of Casq2 in the myocyte (8, 26). Taken together, these results raise the intriguing possibility that in failing hearts, even in the presence of a normal intracellular concentration of Casq2, the protein may be differently distributed in the cellular organelles, with reduced levels in the proximity of the RyR2, where Casq2 plays its pivotal role in the regulation of RyR2-mediated Ca releases and SR Ca release refractoriness (51).

Casq2 in muscular dystrophy. Duchenne (DMD) and Becker’s muscular dystrophy are X-linked inherited diseases resulting from mutations in the dystrophin gene. They are characterized by high susceptibility to contraction-induced muscle damage that leads to severe progressive muscle deterioration (18, 96). Although the skeletal muscle is primarily affected, cardiac involvement is frequently reported especially in subjects with DMD (96). Dilated cardiomyopathy with extensive fibrosis is usually diagnosed during the second decade of life (90). Mouse models reproducing the human phenotype of DMD display impaired Ca handling mostly due to reduction of two proteins: CaM and Casq2. Before any histological sign of cardiomyopathy can be detected in these mice, SR protein levels are still in the physiological range. As the condition progresses, CaM and Casq2 are downregulated with a net loss of Ca buffering activity. Investigators (72) have suggested that the consequent rise in the intracellular free Ca levels could activate proteases and profibrotic signals promoting structural remodeling.

Casq2 in mitochondrial dysfunction. Although the precise pathophysiological mechanism is still not clear, subjects with mitochondrial alterations can develop cardiomyopathy with an abnormal Ca-handling function. Impairment in the fatty acid oxidation pathway, as observed in individuals with mutations in the very long-chain acyl-CoA dehydrogenase gene, puts individuals at high risk for cardiac disease and sudden death. Mice with the same mutation display increased incidence of polymorphic VT during isoproterenol challenge. The L-type Ca current characteristics were not different compared with controls but the SR function was altered with an increase in the SR Ca load (up to 48%) and Ca transients amplitude, similar to the mouse models of Casq2 overexpression. Protein assays of the very long-chain acyl-CoA dehydrogenase mutant mice show an upregulation of RyR2, Casq2, and phospholamban (103).

Mitochondrial dysfunction can also be observed during oxidative stress. Mouse models of reactive oxygen species-induced mitochondrial damage have been obtained by administration of carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone, an uncoupler of oxidative phosphorylation. This oxidative damage eventually leads to a mitochondrial cardiomyopathy associated with a reduction of Casq2 protein levels and alterations in the SR function. Coexposure of the myocytes to both carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone and a reactive oxygen species scavenger such as N-acetylcysteine attenuates the Casq2 downregulation and improves Ca handling (31).

Casq2 as target for drug binding. Cardiotoxicity can be a serious side effect of commonly used drugs such as tricyclic antidepressants, antipsychotic drugs, and anthracyclins. In vitro studies (41) suggest that many of these drugs bind to Casq2 and reduce its Ca-buffering capacity. One of the first experimental evidence suggesting an involvement of Ca-handling abnormalities in the pathogenesis of the drug-induced cardiac damage comes from a rabbit model of doxorubicin-induced cardiomyopathy. After 8 wk of chronic treatment, these animals have reduced levels of SR proteins including RyR2 and Casq2 in the presence of normal levels of Ca ATPase and Na/Ca exchanger (2). In support of this hypothesis is the recent observation that acute administration of anthracyclines causes a severe reduction in SR Ca releases most likely through an interaction with Casq2 and oxidation of RyR2 (13, 30).

The tricyclic antidepressant amitriptyline binds to Casq2 at low micromolar concentrations and causes increased spontaneous Ca releases and consequent depletion of the SR Ca content. However, the contribution of drug binding to Casq2 is probably minimal, since the RyR2 hyperactivity results from direct interaction with the RyR2 channel and can be reproduced in myocytes lacking Casq2 (16, 110). Whether amitriptyline binding to Casq2 contributes to cardiotoxicity during long-term exposure is yet to be determined.

Conclusions and Future Developments

The last decade brought a huge progress in our understanding of Casq2-linked CPVT. Basic and translational research on CPVT mouse models have provided information about the intracellular Ca handling and have identified potential drugs for CPVT treatment that have recently been tested in patients with promising results. However, further investigations are needed to clarify the complex interactions among SR proteins that play a central role not only in inherited but also in acquired arrhythmogenic conditions. Future studies will also focus on how cardiac cells subtypes (Purkinje cells and ventricular myocytes in particular) are affected by impaired Ca handling and new treatments developed that specifically target the cell type responsible for CPVT initiation. Finally, the contribution of Casq2 alterations (e.g., altered protein expression levels, genetic variants, and haploinsufficiency in heterozygous carriers of Casq2-null mutations) to arrhythmia risk in ischemic heart disease and/or heart failure will be explored.

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DISCLOSURES

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