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Review article

Ryanodine receptor and calsequestrin in arrhythmogenesis: What we have learnt from genetic diseases and transgenic mice

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ABSTRACT

The year 2001 has been pivotal for the identification of the molecular bases of catecholaminergic polymorphic ventricular tachycardia (CPVT): a life-threatening genetic disease that predisposes young individuals with normal cardiac structure to cardiac arrest. Interestingly CPVT has been linked to mutations in genes encoding the cardiac ryanodine receptor (RyR2) and cardiac calsequestrin (CASQ2): two fundamental proteins involved in regulation of intracellular Ca^{2+} in cardiac myocytes. The critical role of the two proteins in the heart has attracted interests of the scientific community so that networks of investigators have embarked in translational studies to characterize in vitro and in vivo the mutant proteins. Overall in the last seven years the field has substantially advanced but considerable controversies still exist on the consequences of RyR2 and CASQ2 mutations and on the modalities by which they precipitate cardiac arrhythmias. With so many questions that need to be elucidated it is expected that in the near future the field will remain innovative and stimulating. In this review we will outline how research has advanced in the understanding of CPVT and we will present how the observations made have disclosed novel arrhythmogenic cascades that are likely to impact acquired heart diseases.

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Contents

1. Introduction

The pivotal role of Ca^{2+} handling for the maintenance of the physiological properties of the heart is highlighted by the evidence

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that derangements in Ca^{2+} flow through the sarcolemma and the sarcoplasmic reticulum associated with diseases such as heart failure, arrhythmias and cardiac hypertrophy [1,2]. As a consequence, advancements in the understanding of the pathophysiology of Ca^{2+} handling may be critical for the development of novel therapeutic strategies for heart diseases. Catecholaminergic polymorphic ventricular tachycardia (CPVT) is an inherited disease characterized by adrenergically mediated polymorphic ventricular tachycardia leading to syncope and sudden cardiac death [3]. Since 2001, the identification of mutations in genes encoding the cardiac ryanodine receptor (RyR2) and calsequestrin (CASQ2) in CPVT patients [4,5], stimulated a broad interest in elucidating the arrhythmogenic mechanisms underlying the disease. These functional studies are contributing to the understanding of CPVT and are also helping to unravel the mechanisms of arrhythmias related to abnormal Ca^{2+} handling in acquired heart diseases. In the present review, we will discuss the advancements in the understanding of CPVT achieved in the last seven years as well as the unresolved controversies.

2. Clinical profile of CPVT

The typical CPVT clinical presentation encompasses exercise- or emotion-induced syncopal events and a distinctive pattern of reproducible, stress-related, bidirectional VT in the absence of both structural heart disease and a prolonged QT interval [6]. Two genetics form of CPVT have been reported: the autosomal dominant form linked to mutations in cardiac RyR2 and the autosomal recessive form associated with CASQ2 mutations [4,5,7,8]. In 2007 Bhuiyan et al. identified a novel autosomal recessive form of CPVT and mapped on chromosome 7p14–22 [9], however the gene remains unknown. With the exception of beta-blockers, no pharmacologic therapy of proven effectiveness is available, unfortunately up to 30% of patients undergoing therapy with beta-blockers experience recurrence of cardiac arrhythmias and eventually require an ICD implantation to prevent cardiac arrest [6]. Recently Wilde and Schwartz reported 3 patients in whom left cardiac sympathetic denervation was an effective addition to beta blockers to prevent tachyarrhythmias [10].

3. Molecular and functional abnormalities related to mutations in the RyR2 gene

In vitro characterization of RyR2 mutations has generated a variety of hypotheses to link protein abnormalities to arrhythmogenesis. Such hypotheses reflect different theories on the function and regulation of RyR2 and therefore contribute to the understanding of the physiology of intracellular Ca^{2+} release and to the comprehension of arrhythmogenesis in CPVT. The hope to develop new therapeutic strategies to cure CPVT patients is rooted in the characterization of RyR2 mutants to define whether it is possible to devise "mutation-specific" therapies for the disease.

3.1. In vitro functional characterization of mutations in the cardiac ryanodine receptor

To date, more than 70 RyR2 mutations have been reported (see: http://www.fsm.it/cardmoc/). Most of RyR2 mutations identified in CPVT are single-base-pair substitutions leading to the replacement of highly conserved amino acids. About 20 RyR2 mutations have been characterized in vitro. In 2002, Jiang et al. [11] elucidated the functional properties of the R4497C mutation identified in humans by expressing in HEK293 cells the equivalent mutation R4496C engineered in mouse RyR2. This study showed that the mutation results in enhanced sensitivity of the channel to the activation by Ca^{2+} and by caffeine. Subsequently, in experiments performed in lipid bilayers and in HL1-cardiomyocytes, other investigators confirmed that RyR2 mutations produce a "loss of normal function" and cause diastolic Ca^{2+} "leakage" from the endo/sarcoplasmic reticulum(ER/SR) in condition of sympathetic activation [12–14]. Several other studies characterized in vitro RyR2 mutations identified in CPVT patients showing very similar findings and

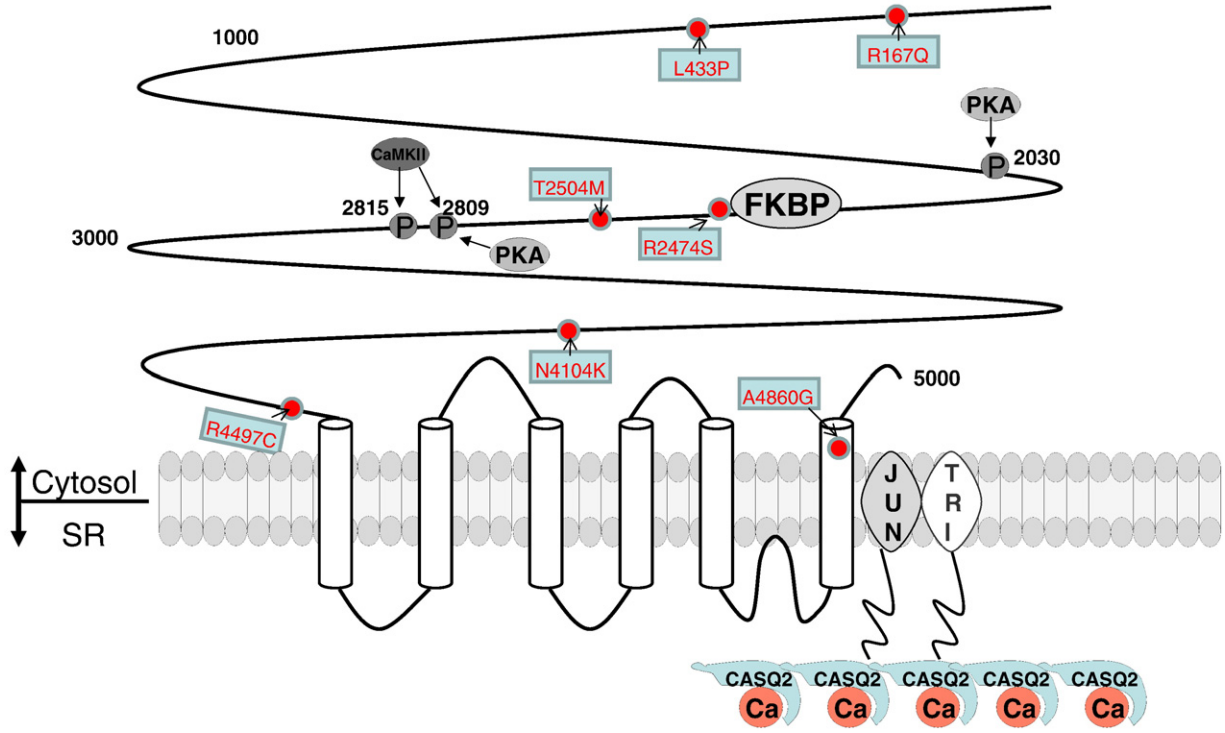

Fig. 1. Schematic cartoon showing the predicted structure of the cardiac ryanodine receptor, RyR2, including the sites of interaction with key ancillary proteins and the phosphorylation sites. Calsequestrin (CASQ2), junction (JUN), and triadin (TRI), proteins interacting with ryanodine receptor in the SR, are also depicted. P, phosphorylation sites; CaMKII, calmodulin-dependent protein kinase II; PKA, protein kinase A. Circle in red: mutation.

pointing to the fact that, independently from the position of the mutation, the electrophysiological abnormalities are remarkably similar [15], but the molecular mechanisms by which RyR2 mutations alter the physiological properties of RyR2 in CPVT remain highly controversial (see below).

3.2. Debate on the role of FKBP12.6 disassociation from mutant RyR2 in the pathogenesis of CPVT

FKBP12.6 is one of several proteins that are part of the RyR2 macromolecular complex (Fig. 1) and it acts as a "stabilizer" that preserves the RyR2 channel closed during diastole [14]. In an elegant series of studies, Marks and colleagues supported the hypothesis that abnormal Ca^{2+} leak from SR is facilitated by the dissociation of FKBP12.6 from RYR2 that occurs when the ryanodine receptor is phosphorylated by PKA. The authors initially proposed that this mechanism is implicated in the genesis of triggered arrhythmias in heart failure [16–18]. Subsequently they extended the hypothesis linking FKBP12.6 dissociation from RyR2 to arrhythmogenesis in CPVT (Fig. 2) when they reported that mutant RyR2 already presents a reduced binding affinity to FKBP12.6 under basal conditions and that this defect is further exaggerated after PKA phosphorylation of RyR2, i.e. in a situation similar to adrenergic activation that elicits arrhythmias in CPVT patients. In support of their hypothesis the authors also demonstrated that, a benzothiazepine derivative compound called K201 (formerly called JTV519) enhances the binding of FKBP12.6 to RyR2 and therefore is able to rescue the function of mutant RyR2 by impairing FKBP12.6 dissociation [12,14]. Subsequently, this group developed an FKBP12.6 knock-out heterozygous mouse model that develops polymorphic VT upon adrenergic stimulation and suggested that this observation is the proof in support of the hypothesis that FKBP12.6 dissociation is the common final dysfunction of mutant RyR2 identified in CPVT. Interestingly the drug K201 was able to prevent life-threatening VT in the FKBP12.6 knock-out heterozygous mouse, thus supporting the view that promoting FKBP12.6 binding to RyR2 may have a therapeutic value for CPVT [19]. More recently, the group of Marks also developed a RyR2 R2474S knock-in mouse and demonstrated that FKBP12.6 binding to RyR2 R2474S is significantly reduced under adrenergic stimulation and also provided evidence that a novel benzothiazepine derivative compound, S107 was able to stabilize the $RyR2^{R2474S/WT}$ channel in the closed state by increasing FKBP12.6 binding and to prevent stress-induced VT and sudden cardiac death in $RyR2^{R2474S/WT}$ mice [20].

Albeit Marks' hypothesis is attractive and is fully supported by all the different experimental models used by the authors, it has been challenged by studies performed by different groups. George et al. and Jiang et al. reported that mutant RyR2 channels show a normal RyR2/FKBP12.6 interaction under both resting condition and upon

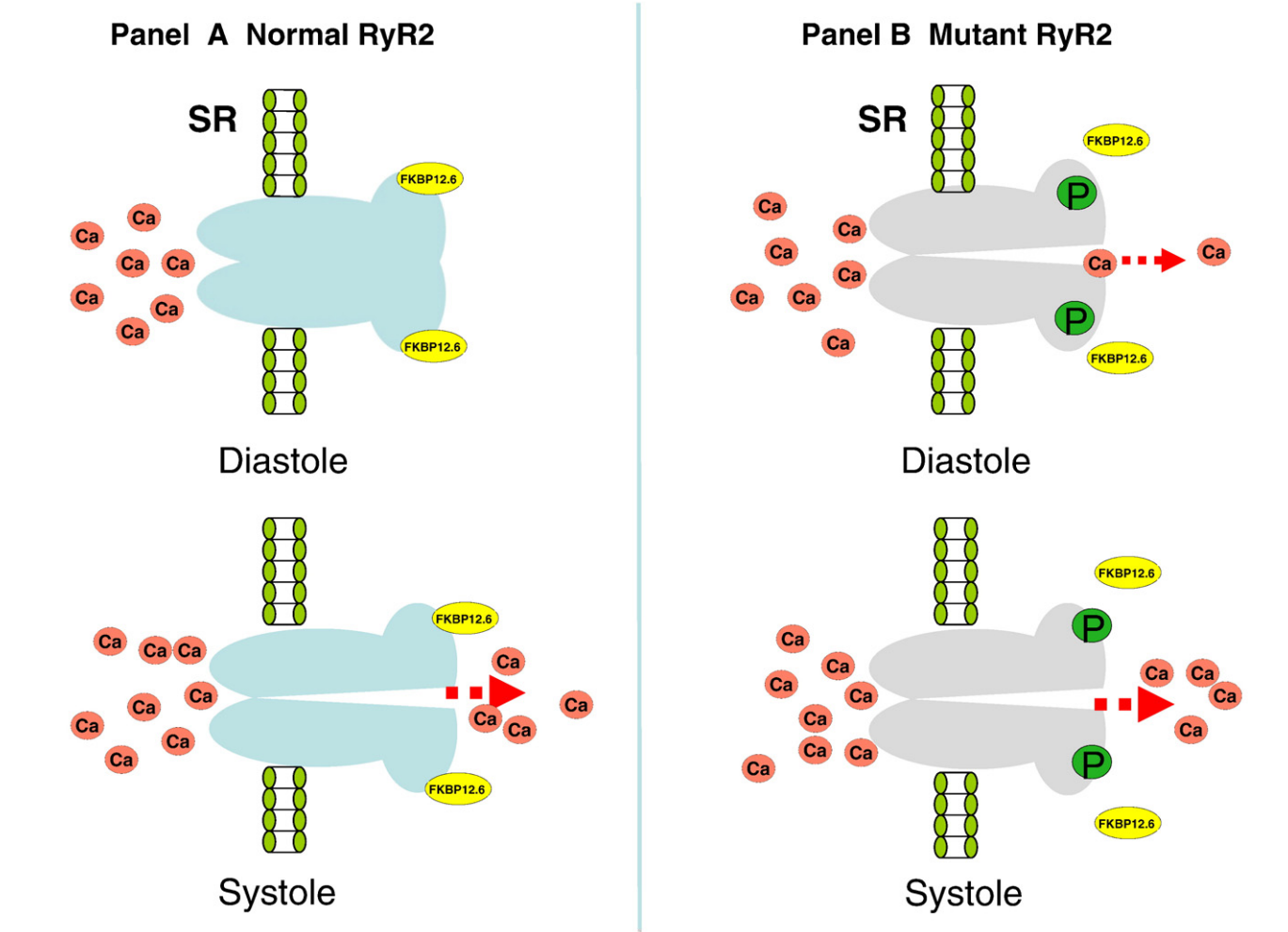

Fig. 2. Panel A shows that in diastole the wild type ryanodine receptor (RyR2) is closed (top panel) while during systole it opens (bottom panel). Panel B shows that the mutant RyR2 opens during systole and is leaky during diastole. FKBP12.6 (yellow ovals) acts as a "stabilizer" that preserves the RyR2 channel closed during diastole. It has been hypothesized that some mutant RyR2 presents a reduced binding affinity to FKBP12.6 under basal conditions and this defect is further exaggerated after PKA phosphorylation of RyR2, which leads abnormal Ca^{2+} leak from SR during diastole (see text for details).

catecholamine stimulation [13,21]. Hunt et al. [22] found that K201 suppresses spontaneous Ca^{2+} release and [3H] ryanodine binding to RyR2 irrespective of FKBP12.6 association and suggested that the drug exerts its antiarrhythmic action through a different mechanism. In lipid bilayer experiments, Marks group has demonstrated that S2246L, R2474S, and R4497C impaired FKBP12.6 binding to RyR2 thus causing the CPVT phenotype [14]. However when we tested the efficacy of K201 in RyR2R4496C/WT knock-in mice (equivalent to the R4497C mutations identified in CPVT families) we failed to demonstrate its protective effect against catecholamine induced DADs in vitro and against adrenergically mediated ventricular arrhythmias in vivo supporting the view that RyR2 R4496C mutation does not disrupt FKBP12.6 interaction [23].

Is it possible to reconcile the discrepancies observed among the different models? It is obviously not easy to answer this question recently, however finding an explanation to the conflicting results obtained by different laboratories may profoundly influence the development of cures for CPVT patients. At present time treatment options for CPVT patients are limited and far from optimal: beta blockers are the mainstay treatment in CPVT and they are only partially effective in preventing malignant arrhythmias. The use of drugs like K201 or S107 might therefore represent a real opportunity for improving CPVT patients' outcome. However more robust experimental evidence should be gathered before the investigational use of the two compounds is attempted in CPVT patients.

Recent data have advanced a stimulating hypothesis to account for discrepancies of observations concerning arrhythmogenesis in CPVT. Initially Yamamoto et al. [24] demonstrated that the binding site of K201 is located at domain 2114–2149 of RyR2, furthermore they demonstrated that this portion of RyR2 binds the central domain of the protein (2234–2750) and showed that interruption of this domain-interaction by the drug mediates stabilization of RyR2. Subsequently Tateishi et al. demonstrated that K201 can correct defective inter-domain interaction caused by RyR2 mutation in central domains but not the mutations located in C-terminal (cytoplasmic) region [25]. Overall it is intriguing to think that properties of individual mutations may influence the response to drugs in CPVT similarly to what happens in other genetic conditions such as in LQT3 where the response to sodium channel blockers is determined by the biophysical properties of individual mutations [26].

3.3. RyR2 mutations may alter the sensitivity to luminal or cytoplasmic Ca^{2+}

Chen and colleagues observed that several RyR2 mutations primarily increase the channel sensitivity to luminal Ca^{2+}, but not to cytosolic Ca^{2+} and lower the threshold for Ca^{2+} release by the endoplasmic reticulum [21,27]. These RyR2 mutations are distributed in the discrete domains of RyR2 channel, so this group proposed the "store overload-induced Ca^{2+} release" (SOICR) model to explain the occurrence of diastolic Ca^{2+} leak from the SR in the presence of mutant RyR2 (Fig. 3). According to this hypothesis selected RyR2 mutations confer hypersensitivity to Ca^{2+} within the SR facilitating Ca^{2+} release. Thomas et al. [28] proposed that RyR2 mutations R176Q/T2504M and L433P impair the calcium-dependent channel inhibition thus enhancing the process of activation of RyR2 by cytosolic Ca^{2+}. Recently Chen and colleagues reported that the RyR2 A4860G mutation displays a loss of luminal Ca^{2+} activation [29]. Overall when data from different groups are considered it seems that different CPVT mutations may alter the response to the luminal or cytosolic Ca^{2+} in a specific and

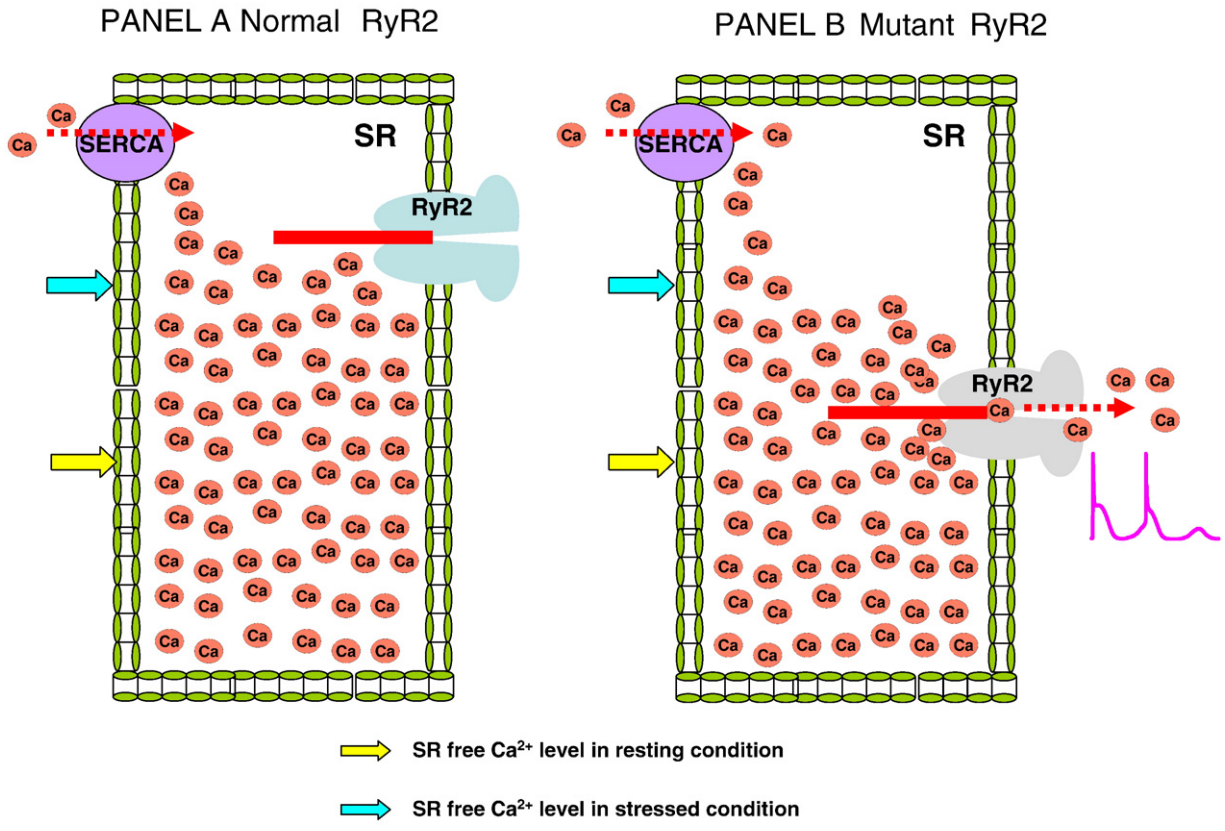

Fig. 3. This cartoon describes the "store overload-induced Ca^{2+} release" (SOICR) hypothesis to explain why under adrenergic activation mutant RyR2 releases calcium (see text). Panel A shows normal RyR2 and Panel B shows Mutant RyR2. In each panel the green rectangle depicts the SR and its key calcium regulatory proteins SERCA and RyR2. The yellow arrow and the light blue arrow indicate respectively levels of SR-free Ca^{2+} at resting conditions or stressed conditions. In normal RyR2 both resting and stressed levels of free calcium are below the SOICR threshold indicated by the red bar next to RyR2. On the contrary in mutant RyR2 the SOICR threshold falls below the levels of free SR calcium thus leading to SR Ca^{2+} spillover, and to generation of delayed afterdepolarization and triggered activity.

unexpected way. At present the mechanisms underlying the altered sensitivity to luminal or cytosolic Ca^{2+} in mutant RyR2 [30,31] and their role in the arrhythmogenesis of CPVT remain to be resolved.

3.4. Defective intramolecular domain interactions in RyR2 mutations

As briefly mentioned in Section 2, the intramolecular interaction between discrete RyR2 domains is necessary for the proper folding of the channel and therefore folding abnormalities are emerging as an important mode of channel autoregulation and response to signals that reach the cytoplasmatic portion of RyR2 (Fig. 4) [32]. Oda et al. [33] reported that DP10, a synthetic peptide corresponding to Gly2460-Pro2495 of RyR2, mimics mutant channels present in CPVT patients (such as RyR2 R2474S) by acting competitively to reduce stabilizing interactions between the N-terminal and central domains. Recently this group further demonstrated that different peptides that mimic mutations located in N-terminal and C-terminal regions of RyR2 (DP163-195 and DP4090-4123, which mimic RyR2 mutations R176Q and N4104K respectively) can cause defective inter-domain interaction in RyR2 and result in increased spontaneous Ca^{2+} release from SR [25]. These findings highlight different molecular mechanisms leading to defective intramolecular domain interactions in RyR2 mutations. More direct evidence that RyR2 mutations can impair the intramolecular domain interactions came from the study of George et al. [34] who used high-resolution confocal microscopy and fluorescence resonance energy transfer (FRET) analysis to demonstrate that aberrant intramolecular domain interactions in mutant RyR2 may be the structural substrate for pathogenetic RyR2 mutations. More interestingly, these authors reported that different RyR2 mutations present distinct patterns of conformational instability in Ca^{2+} handling.

4. RyR2 knock-in mouse models

4.1. Arrhythmogenesis in RyR2 knock-in mouse models

The hypothesis that arrhythmias in CPVT are initiated by delayed afterdepolarizations (DADs) and triggered activity had been advanced based on the observation that the bidirectional VT recorded in CPVT patients closely resembles digitalis-induced arrhythmias [3]. It is well known that digitalis-induced intracellular Ca^{2+} overload leads to the activation of sodium-calcium exchanger that, in turn, generates a net inward current (the so-called "transient inward" current, I_{Ti}). I_{Ti} underlies diastolic membrane depolarizations, DADs, that may reach the voltage threshold for sodium channel activation and trigger abnormal beats. This mechanism for arrhythmias initiation is called "triggered activity" (TA).

The hypothesis that DADs elicit arrhythmias in CPVT received the first experimental support through the electrophysiological characterization of RyR2 R4496C knock-in mouse model. Upon epinephrine and caffeine administration, 50% of $RyR2^{R4496C/WT}$ mice develop polymorphic VT and/or bidirectional VT, which mimics the typical onset and morphology of arrhythmias observed in patients with CPVT (Fig. 5) [35]. Upon recording of action potential in isolated $RyR2^{R4496C/WT}$ myocytes, we showed that the administration of isoproterenol fosters development of DADs and TA (Fig. 6) [23]. Subsequently, these findings were confirmed in RyR2 R176Q and RyR2 R2474S knock-in mouse models (Table 1) [20,36–38]. Interestingly under physiological "unstimulated" conditions, DADs were observed in 63% and TA developed in 12% of $RyR2^{R4496C/WT}$ myocytes but never in wild type myocytes. Interestingly $RyR2^{R176Q/WT}$ myocytes and $RyR2^{R2474S/WT}$ myocytes [20,36,37] also presented a similar behaviour that is in agreement with the in vitro findings of Jiang et al. [11] suggesting that RyR2 mutations enhance abnormal Ca^{2+} release even in the absence of adrenergic stimulation. To provide even further support to the view that triggered activity is the pivotal arrhythmogenic mechanism in CPVT, Paavola et al. [39] used monophasic action potentials and were able to record DADs in CPVT patients carriers of *RyR2* mutations under baseline conditions.

Bidirectional VT is almost a "trademark" for CPVT and derives its name from the peculiar electrocardiographic morphology of the tachycardia that shows 180° rotation of the QRS axis on a beat-to-beat basis [6]. Bidirectional VT is often hemodynamically tolerated but it may degenerate into rapid polymorphic VT and ventricular fibrillation. It is known that Purkinje fibers are more susceptible to

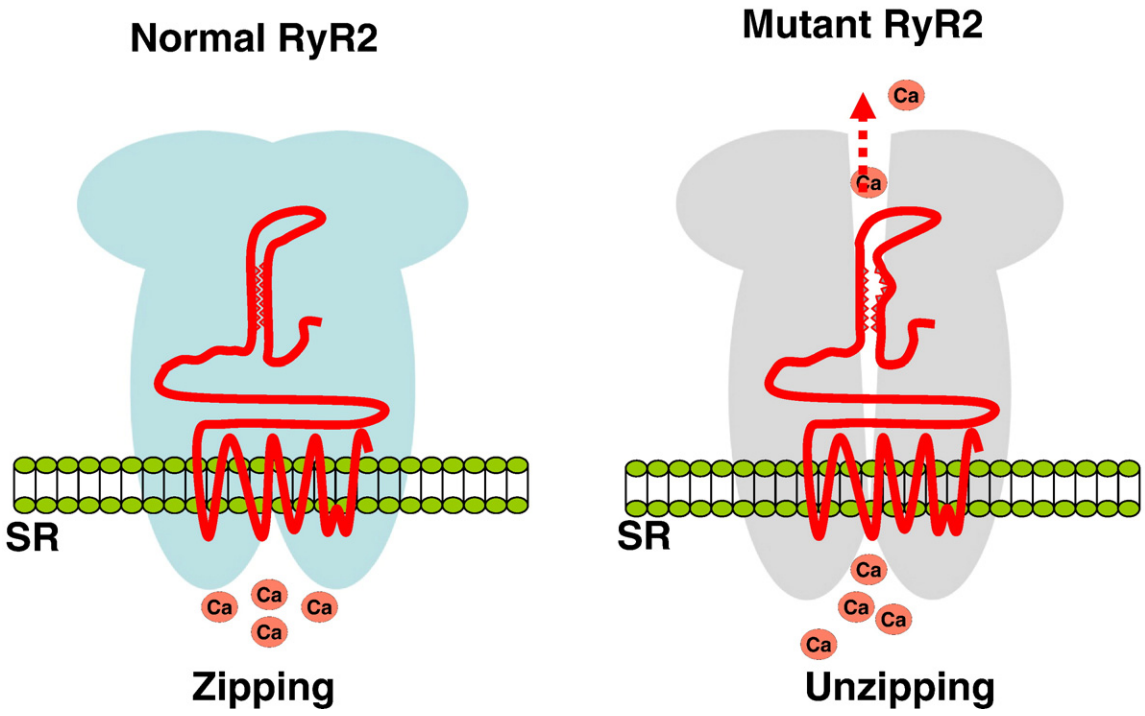

Fig. 4. The N-terminal domain and the central domain interact with each other to function as a regulatory switch for RyR2 channel gating, with a tight "zipping" of the interacting domains serving to stabilize the channel. A mutation in either domain weakens the interdomain interaction (unzipping), which causes activation and leakiness of RyR2 channel.

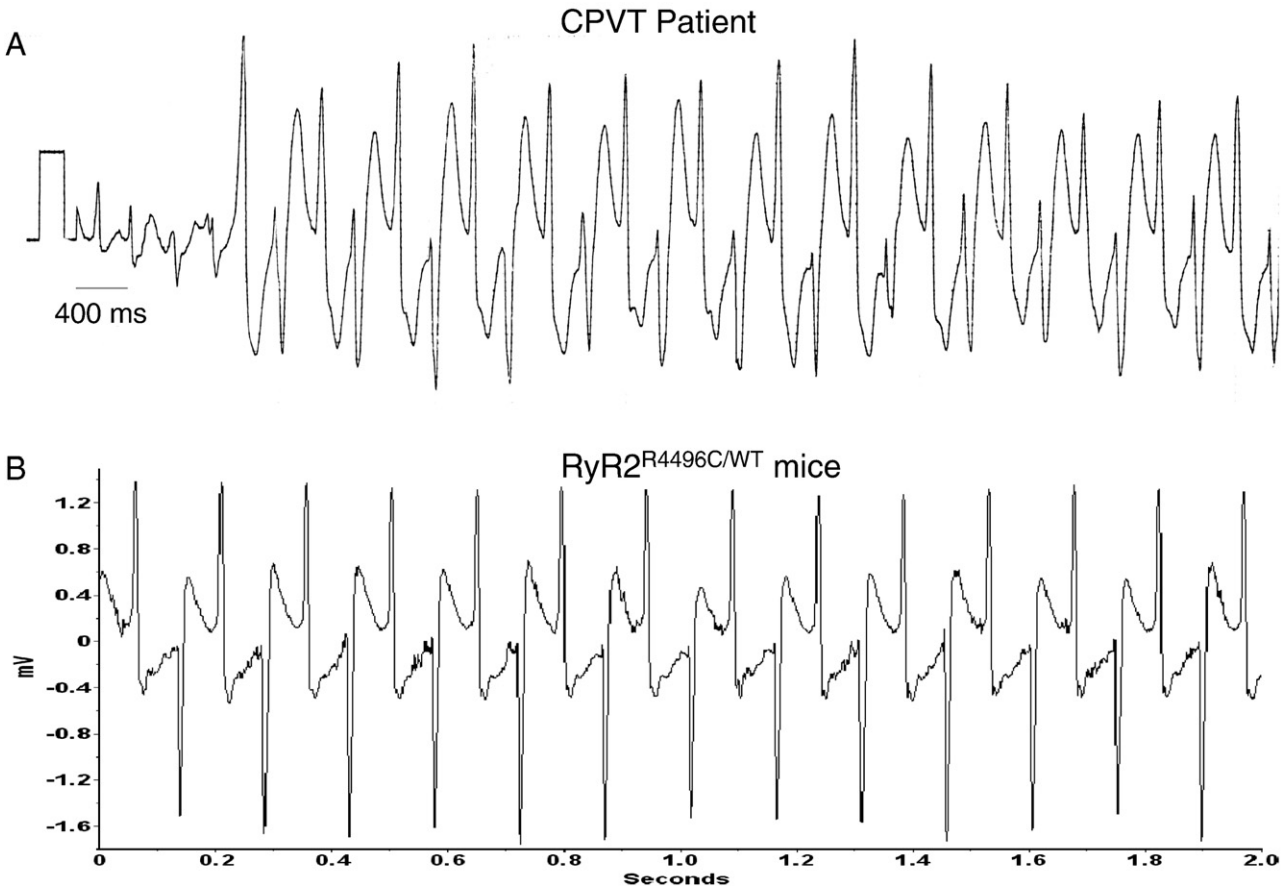

Fig. 5. (A) ECG recording of a sustained bidirectional VT in a CPVT patient. (B) ECG recording of a sustained bidirectional VT in a RyR2$^{R4496C/WT}$ mouse.

Ca^{2+} overload than ventricular muscle possibly because of their greater sodium load and longer action potential duration [40]. It has been hypothesized that bidirectional VT in CPVT may be initiated by triggered activity in the Purkinje fibers and then evolve into polymorphic VT. As adrenergic activation persists in the patient, DADs also develop in ventricular myocytes thus creating an "irregularly irregular" VT that may progressively accelerate and degenerate into VF. A collaborative study performed with the group of Jalife demonstrated that Purkinje fibers are an important source of focal arrhythmias in RyR2$^{R4496C/WT}$ knock-in mouse model [41]. Endocardial optical mapping with voltage sensitive dyes showed that the arrhythmic foci in this model do originate within the specialized conduction system and selective chemical ablation of the right ventricular Purkinje network turned the bidirectional VT into

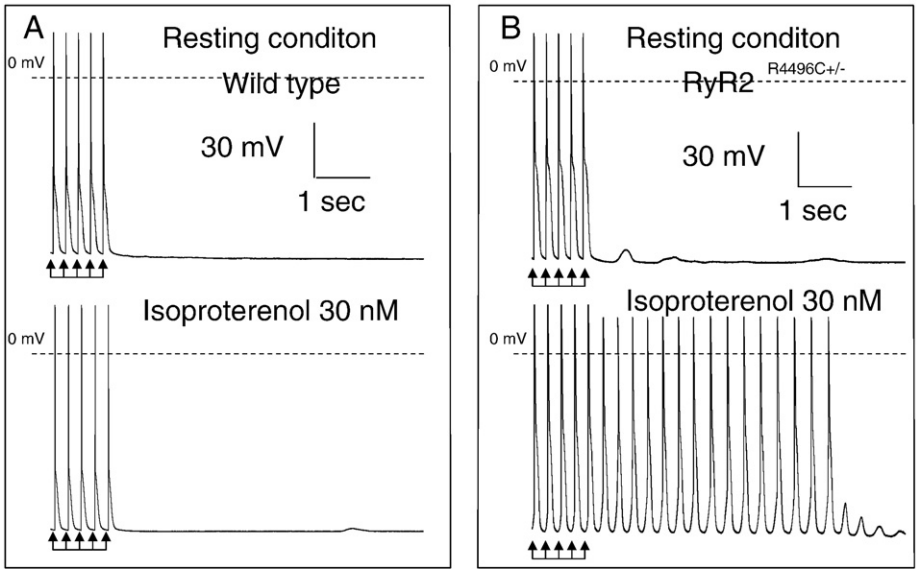

Fig. 6. Action potential recording from a WT myocyte (panel A) and a RyR2$^{R4496C/WT}$ myocyte (panel B) in the absence (top panel) and in the presence (bottom panel) of isoproterenol (30 nM). Arrows indicate the last five paced action potentials.

Table 1
Transgenic CPVT mouse models

Mutation	Clinical diagnosis	Transgenic methods	Phenotypes in vivo	Phenotypes in isolated myocytes	Cardiac morphology	Reference
Autosomal dominant CPVT transgenic mouse models						
$RyR2^{R4496C}$	CPVT	Knock-in	No spontaneous arrhythmias, bidirectional and/or multiple VT induced by adrenergic and caffeine stimulation in heterozygous mice	DADs and TA developed in resting condition and increased TA upon adrenergic stimulation.	Normal	Cerrone et al. [35], Liu et al. [23]
$RyR2^{R176Q}$	ARVD and CPVT	Knock-in	No spontaneous arrhythmias, bidirectional and/or multiple VT induced by adrenergic and/or caffeine stimulation in heterozygous mice.	Spontaneous Ca^{2+} oscillations in resting condition and increased non-evoked Ca^{2+} transients upon adrenergic stimulation	Normal	Kannankeril et al. [36]
$RyR2^{R2474S}$	CPVT	Knock-in	Bidirectional VT in all heterozygous mice during or after treadmill test.	Increased Ca^{2+} sparks in heterozygous myocytes.	Normal	Kobayashi et al. [37], Uchinoumi et al. [38]
$RyR2^{R2474S}$	CPVT	Knock-in	Sustained VT and sudden cardiac death in heterozygous mice during treadmill test.	Increased Ca^{2+} oscillations in heterozygous myocytes upon adrenergic stimulation.	None report	Lehnart et al. [20]
Autosomal recessive CPVT transgenic mouse models						
$CASQ2^{D307H}$	CPVT	Overexpression	No spontaneous arrhythmias, VT induced by adrenergic and caffeine stimulation, normal cardiac structure.	Spontaneous Ca^{2+} transient and TA upon adrenergic and caffeine stimulation	Normal	Dirksen et al. [48]
CASQ2		Knock-out (homozygous)	VT induced by adrenergic stimulation in homozygous mice	Decreased SR Ca^{2+} content, spontaneous Ca^{2+} release and TA upon adrenergic stimulation	Cardiac hypertrophy	Knollmann et al. [55]
CASQ2		Knock-out (heterozygous)	No spontaneous arrhythmias, ventricular ectopies and VT induced by adrenergic stimulation and programmed stimulation respectively in heterozygous mice.	Normal SR Ca^{2+} content in resting condition, increased Ca^{2+} leak and triggered beats upon adrenergic stimulation	Normal	Chopra et al. [57]
$CASQ2^{D307H}$	CPVT	Knock-in	Spontaneous arrhythmias in unstressed homozygous mice, adrenergic stress aggravates arrhythmias.	Decreased SR Ca^{2+} content, spontaneous Ca^{2+} release and TA upon adrenergic stimulation	Normal in young mice, cardiac hypertrophy in aged mice.	Song et al. [50]
$CASQ2^{\Delta E9}$		Knock-in	Spontaneous arrhythmias in unstressed homozygous mice, adrenergic stress aggravates arrhythmias.	Decreased SR Ca^{2+} content, spontaneous Ca^{2+} release and TA upon adrenergic stimulation	Normal in young mice, cardiac hypertrophy in aged mice.	Song et al. [50]
$CASQ2^{R33Q}$	CPVT	Knock-in	Spontaneous arrhythmias in unstressed homozygous mice, adrenergic stress aggravates arrhythmias further.	Decreased SR Ca^{2+} content, spontaneous Ca^{2+} release and TA upon adrenergic stimulation	Normal in young mice.	Rizzi et al. [58]

monomorphic VT with wide QRS. Interestingly these experiments demonstrated that the bidirectional morphology of VT is caused by alternating firing of triggered activity from the right and left bundle (Fig. 7).

4.2. RyR2 knock-in mouse models fail to show structural cardiac abnormalities suggestive of arrhythmogenic right ventricular cardiomyopathy (ARVC)

Shortly after the demonstration that RyR2 mutations cause CPVT, the group of Danieli claimed that they had identified RyR2 mutations as the cause of ARVC in few families diagnosed with an atypical form of right ventricular cardiomyopathy [42,43], defined as ARVC2, that combines mild or nearly absent macroscopic structural abnormalities of the right ventricle and stress-induced polymorphic VT. However, other groups failed to confirm this finding thus questioning the view that RyR2 mutations cause ARVC2. So far none of the RyR2 knock-in mouse models that have been successfully engineered presented structural abnormalities consistent with the diagnosis of ARVC. Kannankeril et al. [36] developed a mouse model carrier of the RyR2 R176Q mutation, that in humans had been associated with ARVC2, but failed to observe the diagnostic markers of ARVC (i.e. fibrosis and fibro-fatty replacement in the right ventricle) in the knock-in animals. We and Uchinoumi et al. also failed to demonstrate structural or histological abnormalities in the heart of $RyR2^{R4496C/WT}$ and $RyR2^{R2474S/WT}$ knock-in mice [35,38]. Taken together, the data reinforce the concept that the presence of one *RyR2* missense mutation predisposes the murine heart to VT in response to adrenergic stimulation (CPVT), but it fails to induce ARVC.

5. Molecular mechanisms of CASQ2 mutation

5.1. Role of CASQ2 in cardiac Ca^{2+} handling

The CASQ2 protein serves as the major Ca^{2+} reservoir within the SR of cardiac myocytes. CASQ2 exists in monomeric and polymeric forms, CASQ2 polymerization occurs at high SR Ca^{2+} content and polymeric CASQ2 has a high Ca^{2+} binding capacity. It has been hypothesized that the functional interactions between triadin, junctin and calsequestrin modulate responsiveness of RyR2 to luminal Ca^{2+}. According to the proposed model, binding of CASQ2 to triadin and junctin inhibits the activity of the RyR2 channel at low luminal Ca^{2+} concentrations. When luminal Ca^{2+} increases, this inhibition is gradually relieved as the Ca^{2+} binding sites on CASQ2 become progressively occupied by Ca^{2+}, leading to weakened interactions between CASQ2 and triadin and/or junctin thus increasing open probability of the channel. So it is commonly believed that CASQ2 acts not only as a local intra-SR Ca^{2+} buffer but as a calcium-dependent regulator of RyR2 channel function [44].

5.2. Molecular and functional abnormalities related to mutations in the CASQ2 gene

Given the importance of CASQ2 in the regulation of Ca^{2+} handling, it is not surprising that genetic alterations of this proteins lead to cardiac disease. Homozygous mutations in CASQ2 are found approximately in 3% of CPVT patients. To date seven recessive mutations in the *CASQ2* gene have been linked to CPVT (see: http://www.fsm.it/cardmoc/). Four of these mutations (a nonsense R33X, a splicing 532+1 G>1, a 1 base pare deletion, 62delA and a 16 bp deletion at position 339–354

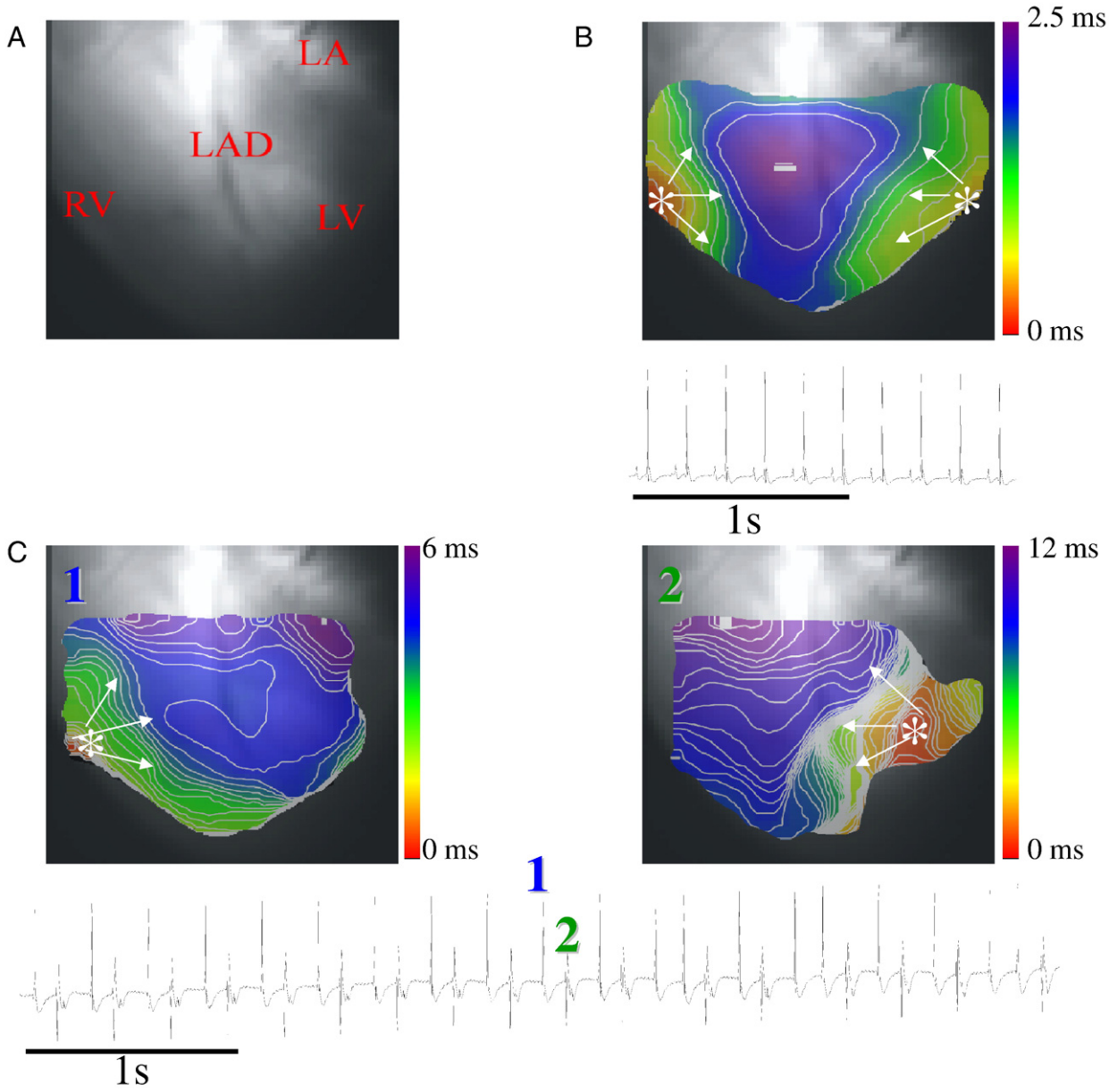

Fig. 7. Bidirectional VT in RyR2$^{R4496C/WT}$ heart. (A) Heart image. (B) Epicardial activation map and ECG in sinus rhythm. (C) Epicardial activation maps of 2 consecutive ventricular beats with an origin that changed from right ventricle (beat 1) to left ventricle (beat 2). Bottom, ECG in bidirectional VT (2.7 mM Ca^{2+}; 100 nM isoproterenol). LAD indicates left anterior descendent coronary; LA, left atrium. From reference 36.

leading to a frameshift and a stop codon after 5aa, G112+5X) induce premature stop codons generating truncated forms of the protein and resulting in lowered CASQ2 levels. Terentyev et al. [45] proposed that a reduction in CASQ2 leads to a shortening of the time required for functional recharging of SR Ca^{2+} store, thus rendering RyR2 prone to premature activation and leading Ca^{2+} leak from RyR2 during diastole (Fig. 8). According to this theory all mutations leading to haploinsufficiency share a common mechanism for precipitating VT thus creating the rationale to assume that a knock-out CASQ2 mouse model would phenocopy CPVT (see below).

The additional three mutations identified in recessive CPVT are single nucleotide replacements leading to an amino acid substitution and therefore their functional consequences may be different from that of the mutations leading to a truncated protein. The first point mutation converts a negatively charged aspartic acid into a histidine in a highly conserved region of CASQ2 (D307H) identified in seven consanguineous families from a Bedouin tribe in the north of Israel [5]. Houle et al. [46] reported that the D307H mutation affects the calcium-dependent conformation of the protein and the ability of CASQ2 to interact with junctin and triadin. Since the D307H mutation is localized to a putative Ca^{2+} binding region between the second and third thioredoxin-like domains of the protein, it was suggested that it may lead to CPVT by influencing CASQ2 affinity for Ca^{2+}. Accordingly Kim et al. [47] showed that D307H mutation results in the production of monomers that are unable to form a properly oriented dimers thus impairing Ca^{2+} binding capacity. In isolated rat cardiomyocytes overexpressing CASQ2^{D307H} protein and in the cardiac-specific expression of the CASQ2^{D307H} transgenic myocytes, the SR Ca^{2+} store capacity is compromised despite the presence of endogenous WT CASQ2 [48,49]. Interestingly, as described in details in the section dedicated to knock-in mouse models, the CASQ2$^{D307H/WT}$ mice present an unexplained reduction in the content of CASQ2 [50].

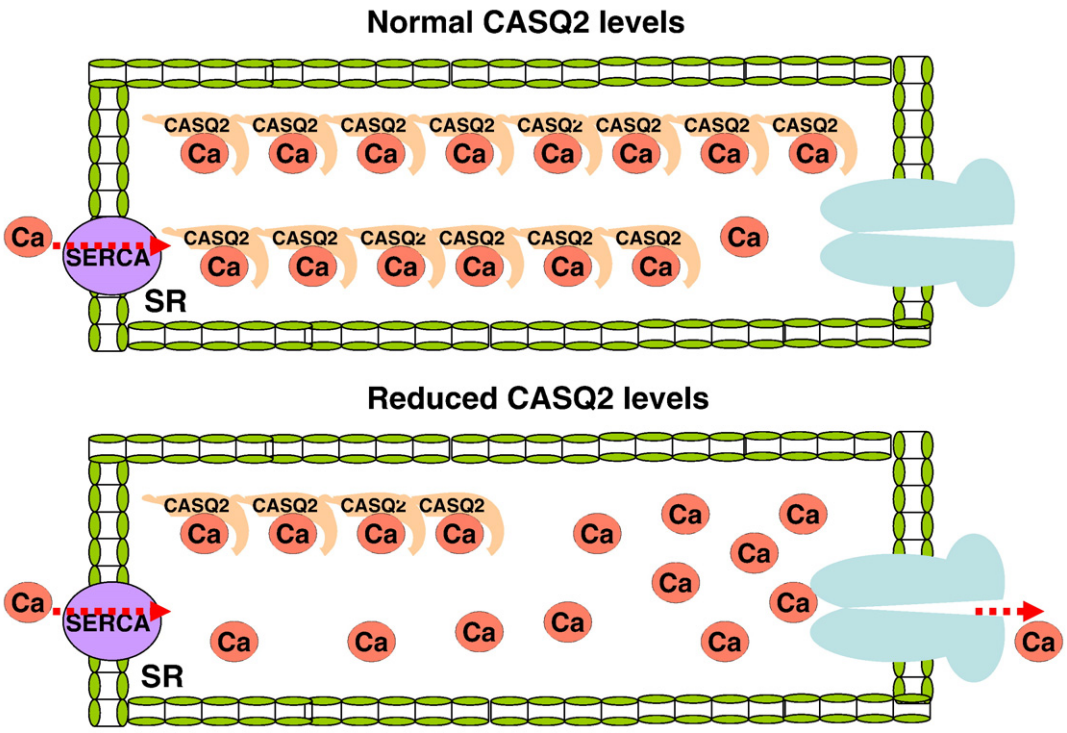

Fig. 8. SR Ca^{2+} store capacity is determined by the level of CASQ2. RyR2 function is regulated by SR free luminal Ca^{2+}. Reduced CASQ2 levels shorten the time for functional recharging of SR Ca^{2+} store, thus rendering RyR2 prone to premature activation and leading Ca^{2+} leak from RyR2 during diastole.

The second point mutation (R33Q) was identified by us in a CPVT patient with history of syncope and a brother who died suddenly at young age. Terentyev et al. showed that R33Q overexpression in rat cardiac myocytes promotes abnormal spontaneous diastolic Ca^{2+} release without impairing the SR Ca^{2+} store capacity. Recently Kim et al. [47] proposed that R33Q impairs dimerization of the protein but this findings are at variance with those of Volpe et al. who showed that R33Q is able to form dimers and polymers but at higher concentrations of Ca^{2+} [51]. In two independent studies performed in planar lipid bilayers, Gyorke and Fill suggested that the pivotal abnormality of $CASQ^{R33Q}$ is its reduced ability to inhibit RyR2 at low luminal Ca^{2+} [52,53].

The last CASQ2 mutation that has been characterized in vitro is the L167H that was identified in a young patient presenting with stress-induced ventricular arrhythmia and cardiac arrest who also carried the deletion G112+5X thus being the first patient with compound heterozygous CASQ2 mutations described [54]. In the report by di Barletta et al. overexpression of L167H mutant does not improve the SR Ca^{2+} store capacity in rat cardiomyocytes. Biochemical studies showed that L167H mutant cannot respond to Ca^{2+} [47] and almost completely inhibits back-to-back dimer interactions [51]. Based on single channel analysis, Qin et al. [53] demonstrated that L167H mutant is unable to reduce RyR2 opening at sub-millimolar concentrations of Ca^{2+}. Overall data from different groups concur to the view that L167H is essentially a functionally inert protein that most likely leads to a functional knock-out phenotype.

6. CASQ2 mouse models

6.1. Arrhythmogenesis in CASQ2 mouse models

After the successful engineering of the first $RyR2^{R4496C/WT}$ mouse model that proved the concept that it is possible to elicit CPVT phenotype in the heart of mice, the confidence in the development of transgenic mice to investigate arrhythmogenic mechanisms of CPVT extended to the recessive variant of the disease. Knollmann et al. [55] described $CASQ2^{-/-}$ knock-out mice that respond to isoproterenol exposure with typical CPVT phenotype. The most important contribution of this model does not relate to the genetic disease, rather it provides novel information on the role of CASQ2 in the heart. Interestingly SR Ca^{2+} content was largely preserved in $CASQ2^{-/-}$ myocytes and the compensatory mechanism seems to be the unexpected compensatory increase in SR volume and the post-transcriptional reduction of junctin and triadin, suggesting that the triad "calsequestrin, junctin and triadin" is regulated to retain a physiological stochiometry, however no insights on how such the equilibrium is maintained are so far available. In the CASQ2 knock-out model the arrhythmogenic mechanism leading to CPVT is not substantially different than the one hypothesized based on the in vitro experiments: the absence of CASQ2 increases the diastolic SR Ca^{2+} leak and causes premature spontaneous SR Ca^{2+} release and triggered beats resulting in arrhythmias. The results indicate that CASQ2 is not essential to maintain the SR Ca^{2+} store, given the fact that the system has a back-up compensatory response based on the expansion of SR volume [55]. A matter of controversy remains the identification of the mechanism by which the absence of CASQ2 causes SR Ca^{2+} leak. In this respect the evidence provided by bilayer experiments demonstrated that removal of CASQ2 increases RyR2 open probability [56] at fixed intraluminal Ca^{2+} suggests that CASQ2 influences the open probability of RyR2. Interestingly data derived from the heterozygous $CASQ2^{WT}/^{-}$ showed that even a modest decrease of CASQ2 protein (25%) is able to induce an increased diastolic SR Ca^{2+} leak in the absence of changes in triadin, junctin level and SR volume [57].

Song et al. [50] developed two transgenic mouse models: the homozygous knock-in for the missense mutation D307H ($CASQ2^{D307H/D307H}$) and the homozygous carrier of the deletion ΔE9 ($CASQ2^{\Delta E9/\Delta E9}$) that resulted in novel and interesting information. Both models develop stress-induced polymorphic VT and bidirectional VT and as expected the $CASQ2^{\Delta E9/\Delta E9}$ mice lack calsequestrin being therefore similar to the CASQ2 knock-out mice, unexpectedly however the $CASQ2^{D307H/D307H}$ mice also present a 95% reduction of CASQ2 protein. In both transgenic animals the protein levels of RyR2

and calreticulin (CRT) increased through an undefined mechanism that is not clarified by the authors. Since CRT is an SR Ca^{2+} binding protein that is expressed during fetal life and disappears in adult healthy heart. The authors speculated that absence of CASQ2 results in high CRT levels as a compensatory response that provides SR Ca^{2+} binding capacity. It remains however unclear why the increase in CRT and RyR2 is not present in the CASQ2 knock-out model and by which mechanism it represents a common response to the two mutations investigated by the authors.

Recently we generated a knock-in mouse model carrier of the R33Q point mutation [58]. The homozygous mice exhibited stress-induced polymorphic VT and bidirectional VT. In vitro studies showed that the $CASQ2^{R33Q/R33Q}$ myocytes develop DADs as well as early afterdepolarizations and that $CASQ2^{R33Q/R33Q}$ myocytes decrease the SR Ca^{2+} content. Interestingly the $CASQ2^{R33Q/R33Q}$ mice showed a 50% reduction of CASQ2 protein in the presence of normal CASQ2 mRNA. We attempted to clarify the mechanisms by which $CASQ2^{R33Q}$ is reduced and demonstrated that the mutant protein is more susceptible to trypsin digestion [51,58]. Moreover the changes in the protein levels of CRT and RyR2 were not observed thus supporting the findings of Knollman et al. [55] as compared to data reported by Song et al. [50].

Overall reduction in CASQ2 seems to be the common characteristics of all transgenic mouse models and may therefore be a pivotal aspect in the pathogenesis of autosomal recessive CPVT (Table 1). It remains still unclear which is the critical decrease in CASQ2 that triggers the arrhythmogenic phenotype. This issue has a clinical relevance considering that at variance with heterozygous mice [57], heterozygous individuals carriers of premature truncations of CASQ2 are asymptomatic: whether comparable levels of CASQ2 depletion are needed in mice and in humans to trigger the phenotype of CPVT is unknown.

7. Conclusions

In the last seven years, impressive advancements have occurred in the understanding of the arrhythmogenic mechanisms of RyR2 and CASQ2 related CPVT. Although CPVT is an uncommon genetic disorder, it is likely to play an important role for the understanding of pathophysiology of abnormalities of intracellular Ca^{2+} handling. The data collected from in vitro functional characterization of mutant RyR2 and CASQ2 and investigations carried out in transgenic mouse models have provided novel insights into calcium-mediated arrhythmogenesis that are likely to impact our understanding of arrhythmogenesis in acquired heart diseases associated with dysfunctional intracellular Ca^{2+} handling, such as heart failure. The unexpected complexity of the multiple interactions among different proteins has attracted to the investigators with different expertise and this field is already making important steps forward in the translation of novel understanding into devising novel therapeutic targets and novel pharmacological strategies to rescue the pathologic phenotypes.

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