Modulation of ryanodine receptor by luminal calcium and accessory proteins in health and cardiac disease

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The cardiac ryanodine receptor (RyR2) is the sarcoplasmic reticulum (SR) Ca^{2+} release channel which is responsible for generation of the cytosolic Ca^{2+} transient required for activation of cardiac contraction. RyR2 functional activity is governed by changes in [Ca^{2+}] on both the cytosolic and luminal phase of the RyR2 channel. Activation of RyR2 by cytosolic Ca^{2+} results in Ca^{2+}-induced Ca^{2+} release (CICR) from the SR. The decline in luminal [Ca^{2+}] following release contributes to termination of CICR and Ca^{2+} signalling refractoriness through the process of luminal Ca^{2+}-dependent deactivation of RyR2. The control of RyR2 by luminal Ca^{2+} involves coordinated interaction of the channel with several SR proteins, including the Ca^{2+}-binding protein calsequestrin (CASQ2), and the integral proteins triadin 1 (TRD) and junctin (JCN). CASQ2 in addition to serving as a Ca^{2+} storage site and a luminal Ca^{2+} buffer modulates RyR2 function more directly as a putative luminal Ca^{2+} sensor. TRD and JCN, stimulatory by themselves, mediate the interactions between CASQ2 and RyR2. Acquired and genetic defects in proteins of this junctional Ca^{2+} signalling complex lead to disease states such as cardiac arrhythmia and heart failure by impairing luminal Ca^{2+} regulation of RyR2.

1. Introduction
Sarcoplasmic reticulum (SR) is a major Ca^{2+} storage reservoir in muscle. Following electrical excitation of cardiac myocytes, Ca^{2+} ions are rapidly released from the SR into the cytosol via Ca^{2+} release channels known as ryanodine receptors (RyRs, of three isoforms, isoform RyR2 is preferentially expressed in the heart). Subsequently, Ca^{2+} is resquested into the SR by ATP-consuming Ca^{2+} pumps (SR Ca^{2+}-ATPase, SERCA). The resulting transitory elevation of cytosolic [Ca^{2+}] ([Ca^{2+}]), the Ca^{2+} transient, is responsible for activation of contractile filaments and hence for the generation of the heart beat. Until recently, the interest in SR Ca^{2+} stores has primarily focused on events occurring on their external surfaces, including, perhaps most importantly, activation of RyR2s by cytosolic Ca^{2+}, a process known as Ca^{2+}-induced Ca^{2+} release (CICR). However, more recently evidence has come to light indicating that Ca^{2+} accumulated inside the SR plays a pivotal role in governing SR-mediated Ca^{2+} signalling in cardiac muscle. In particular, luminal Ca^{2+}-dependent changes in RyR2 gating are involved in Ca^{2+} release termination and release refractoriness, processes essential for normal rhythmic activity of the heart. Instead interacts with elaborate molecular machinery that senses and translates changes in luminal Ca^{2+} to RyR2. The SR luminal proteins cardiac calsequestrin (CASQ2), junctin (JCN), and triadin 1 (TRD) are established parts of this complex. Given the importance of this molecular complex in controlling SR Ca^{2+} release, it is not surprising that the acquired and genetic defects in its components lead to pathological states including cardiac arrhythmia and heart failure (HF). In this review, we intend to summarize the evidence accumulated in the literature regarding the molecular events and players involved in modulation of SR Ca^{2+} release by luminal Ca^{2+} and discuss the mechanisms whereby alterations in luminal Ca^{2+} signalling can lead to cardiac disease.

2. Ca^{2+} in the sarcoplasmic reticulum
The total amount of Ca^{2+} in the SR is determined by Ca^{2+} uptake (via SERCA), Ca^{2+} efflux via RyRs, and by the binding capacity of intra-SR Ca^{2+} binding sites. Most of Ca^{2+} in the SR (50–90%) is bound to low-affinity luminal Ca^{2+} buffering protein CASQ2. The presence of bound Ca^{2+} in the SR lumen allows this organelle to supply sufficient Ca^{2+} for activation of the contractile machinery despite its minuscule luminal space (~3.5% of cell volume, Bers, Table 3). Although the total [Ca^{2+}] in the SR is important

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to the overall functional size of the Ca\(^{2+}\) store, many aspects of Ca\(^{2+}\) store's operation are determined by free rather than total luminal Ca\(^{2+}\). Not only does free [Ca\(^{2+}\)]\(_{\text{SR}}\) ultimately determine the total [Ca\(^{2+}\)] in the SR, but it also sets the maximum thermodynamic gradient that the SR Ca\(^{2+}\)-ATPase can create, establishes the driving force for SR Ca\(^{2+}\) release, and affects RyR2 activity. At steady-state, free [Ca\(^{2+}\)]\(_{\text{SR}}\) is determined by Ca\(^{2+}\) transport mechanisms in the SR membrane and is independent of the presence and abundance of luminal Ca\(^{2+}\)-binding sites. However, these bindings sites influence the dynamics at which steady-state [Ca\(^{2+}\)]\(_{\text{SR}}\) is attained. Using a low-affinity Ca\(^{2+}\) dye (Fluo-5N) loaded in the SR, it has been estimated that the resting (diastolic) free [Ca\(^{2+}\)]\(_{\text{SR}}\) constitutes 1–1.5 mM and becomes only partially depleted (24–63%) during contraction. Local depletion signals in individual SR cisterna during Ca\(^{2+}\) sparks (termed ‘blinks’) have been also measured in myocytes from both normal and diseased hearts. Dynamic changes in [Ca\(^{2+}\)]\(_{\text{SR}}\) provide a basis for luminal Ca\(^{2+}\) signalling.

3. Modulation of sarcoplasmic reticulum Ca\(^{2+}\) release by luminal Ca\(^{2+}\)

Fabiato demonstrated as early as in 1972 that in myocytes in which the surface membrane was removed, increasing SR Ca\(^{2+}\) load leads to the generation of waves of propagating CICR and periodic contractions. A large body of experimental evidence has since accumulated that indicates that increased SR Ca\(^{2+}\) content stimulates Ca\(^{2+}\) release whereas reduced SR Ca\(^{2+}\) content inhibits Ca\(^{2+}\) release from the SR of cardiac myocytes (reviewed by Györke et al.\(^{13}\)). A combination of three mechanisms seems to account for the effects of luminal Ca\(^{2+}\) on SR Ca\(^{2+}\) release: (i) dependency of release flux magnitude on [Ca\(^{2+}\)]\(_{\text{SR}}\); (ii) Ca\(^{2+}\)-dependent activation of RyR2s at the cytosolic side of the channel; and (iii) sensitization of RyR2s to activation by cytosolic Ca\(^{2+}\) at distinct luminal sites.

The direct effects of luminal Ca\(^{2+}\) on RyR2 function have been studied in RyR2 channels reconstituted in planar lipid bilayers, an approach that offers direct access to the luminal side of the channel (reviewed by Györke et al.\(^{13}\)). These studies showed that elevation of Ca\(^{2+}\) on the luminal (trans) side of the channel in the micromolar to millimolar range increases RyR2 open probability (Figure 1A and B). The \(K_d\) value of RyR2 activation with luminal Ca\(^{2+}\) (\(\sim 1\) mM) corresponds to the resting, i.e. diastolic [Ca\(^{2+}\)]\(_{\text{SR}}\) in myocytes (Figure 1B). This means that RyR2 luminal Ca\(^{2+}\) modulation could influence SR Ca\(^{2+}\) release in at least two ways depending on the direction of the [Ca\(^{2+}\)]\(_{\text{SR}}\) change: (i) by reducing RyR2 activity when [Ca\(^{2+}\)]\(_{\text{SR}}\) becomes lowered during and following SR Ca\(^{2+}\) release (until the stores are refilled), and (ii) by stimulating RyR2 activity when diastolic Ca\(^{2+}\) is increased above normal levels. The significance of these effects to normal myocyte Ca\(^{2+}\) cycling and excitation–contraction coupling is discussed below.

3.1 Termination of sarcoplasmic reticulum Ca\(^{2+}\) release

As a mechanism with an intrinsic positive feedback, CICR should be highly unstable and prone to self-perpetuation. Yet, in cardiac myocytes Ca\(^{2+}\) release is tightly graded according to the amplitude of \(I_{\text{Ca}}\) and robustly terminates leaving a substantial Ca\(^{2+}\) reserve in the SR. Based on the

![Figure 1](https://academic.oup.com/cardiovascres/article-abstract/77/2/245/428086/428086)

**Figure 1** Modulation of ryanodine receptor (RyR2) by luminal Ca\(^{2+}\). (A) Representative single-channel recordings illustrating the effects of increasing luminal Ca\(^{2+}\) on RyR2 activity. Openings are downward. (B) RyR2 open probability as a function of luminal [Ca\(^{2+}\)]. The arrow denotes the direction of [Ca\(^{2+}\)]\(_{\text{SR}}\) change during SR Ca\(^{2+}\) Release. (C) Representative images of Ca\(^{2+}\) sparks recorded in control myocytes and myocytes overexpressing or underexpressing the Ca\(^{2+}\)-buffering protein CASQ2. (D) Temporal profiles of averaged Ca\(^{2+}\) sparks presented in C at the spatial peak. Enhanced Ca\(^{2+}\) buffering by CASQ2 prolongs Ca\(^{2+}\) release while reduced buffering shortens Ca\(^{2+}\) release consistent with the role of luminal Ca\(^{2+}\) in Ca\(^{2+}\) release termination.
fact that RyR2 activity diminishes as luminal Ca$^{2+}$ is decreased from millimolar to micromolar concentrations, it has been proposed that termination of SR Ca$^{2+}$ release involves changes in RyR2 activity brought about by the decline of [Ca$^{2+}$]$_{SR}$ during Ca$^{2+}$ release.\textsuperscript{15} Computational simulations using a mathematical model of Ca$^{2+}$ release in which RyR2 activity linearly depended on SR luminal [Ca$^{2+}$] further supported the validity to this hypothesis.\textsuperscript{5} The role of changes in luminal Ca$^{2+}$ in the termination of SR Ca$^{2+}$ release was experimentally tested by loading the SR with extrinsic Ca$^{2+}$ chelators with various Ca$^{2+}$ affinities, including citrate, maleate and acetamidonic acid (ADA).\textsuperscript{2} Buffering or stabilizing [Ca$^{2+}$]$_{SR}$ using these chelators prolonged the duration of Ca$^{2+}$ release during both Ca$^{2+}$ sparks and global Ca$^{2+}$ transients in proportion to the buffering capacity of the chelators, providing direct evidence for the role of luminal Ca$^{2+}$ in Ca$^{2+}$ release termination. Subsequently, similar results were obtained by varying the expression level (increasing or decreasing) of the endogenous Ca$^{2+}$ buffer CASQ2 in cardiac myocytes (Figure 1C and D).\textsuperscript{3} Recently, the role of this mechanism has received further support from the observation that Ca$^{2+}$ sparks terminate at a constant free [Ca$^{2+}$]$_{SR}$ regardless of the extent of [Ca$^{2+}$]$_{SR}$ buffering by CASQ2. Moreover, mutations in CASQ2 that enhance the responsiveness of RyR2s to luminal Ca$^{2+}$ reduced this [Ca$^{2+}$]$_{SR}$ threshold for local Ca$^{2+}$ release termination.\textsuperscript{17}

3.2 Refractoriness of Ca$^{2+}$ signalling

Once RyR2s close due to a shift in their gating mode (inactivation/deactivation), a certain time must pass before they can be activated again. Indeed, such refractoriness of Ca$^{2+}$ release has been demonstrated in several studies at both global and local level.\textsuperscript{18} Furthermore, if termination of Ca$^{2+}$ release is caused by changes in luminal Ca$^{2+}$ then restitution of release from refractoriness must be also dependent on [Ca$^{2+}$]$_{SR}$. A growing body of evidence suggests that this is indeed the case.\textsuperscript{2,4,19} For example, Terentyev et al.\textsuperscript{2,3} showed that introduction of various exogenous buffers as well as overexpression of the endogenous Ca$^{2+}$ buffer CASQ2 prolonged restitution of Ca$^{2+}$ sparks triggered at fixed locations by the RyR2 activating scorpion toxin Imperatoxin A. Similar to the effects on Ca$^{2+}$ release duration, the effects on restitution were proportional to the buffering strength, such that buffers with the highest Ca$^{2+}$ binding capacity prolonged refractoriness to the largest extent. At the same time, reducing CASQ2 accelerated restitution. Thus, substantial evidence supports the concept that changes in luminal Ca$^{2+}$ control not only termination of CICR, but also cause a refractory state that persists until recovery of [Ca$^{2+}$]$_{SR}$.

3.3 Luminal Ca$^{2+}$-dependent leak

A process opposing luminal Ca$^{2+}$-dependent deactivation is the activation of RyR2s by elevated luminal Ca$^{2+}$, which is manifested as an increased frequency of Ca$^{2+}$ sparks, or elevated SR Ca$^{2+}$ leak at an increased SR Ca$^{2+}$ load.\textsuperscript{18,20,21} While deactivation of RyR2s plays an important role in CICR termination and refractoriness, the adaptive value of RyR2 activation by elevated Ca$^{2+}$ is less obvious. It has been speculated that by dynamically linking the SR Ca$^{2+}$ load to RyR2 activity, a luminal-Ca$^{2+}$ controlled SR Ca$^{2+}$ leak could stabilize myocyte Ca$^{2+}$ cycling when either SR Ca$^{2+}$ release or Ca$^{2+}$ uptake is altered.\textsuperscript{20,22} Although the significance of RyR2 stimulation by elevated luminal Ca$^{2+}$ for normal myocyte physiology is unclear, this process has a well-established relationship to various cardiac disease states, including Ca$^{2+}$-dependent arrhythmia and HF (see below).

4. Modulation of ryanodine receptor by sarcoplasmic reticulum luminal proteins

The Ca$^{2+}$ release channel is a macromolecular complex composed of more than a dozen of proteins centred around the RyR2.\textsuperscript{6,7,23,24} Although the bulk of the RyR2 homotetramer is situated on the cytosolic side of the SR membrane (seen on EM micrograph as the foot), a significant portion of the protein extends to the SR lumen. On the luminal side, the RyR2 is complexed with a number of proteins, including TRD, JCN, and CASQ2. (Figure 2). Together these proteins form the core of the Ca$^{2+}$ release channel complex and compose a network of interacting proteins at the luminal face of the junctional SR (JSR).\textsuperscript{6} Based on RyR2 hydrophyplot, a minimum of four putative transmembrane segments (TM1-4) per monomer are predicted to span the SR membrane, such that residues connecting TM1 and TM2 form the first intraluminal loop and residues TM3 and TM4 contribute to the second intraluminal loop. The second RyR2 loop is highly conserved and is enriched in charged amino acids.\textsuperscript{25} In addition to being involved in ion conduction and

![Figure 2](https://academic.oup.com/cardiovascres/article-abstract/77/2/245/428086/729245)
selectivity, this region is also presumed to be a site of interaction with the luminal domains of TRD and JCN. TRD and JCN interact with each other and with the RyR2 and at the same time link CASQ2 to the RyR2 channel complex.

4.1 Calsequestrin

4.1.1 Ca²⁺ binding and structure

Calsequestrin was identified in skeletal muscle (CASQ1) by MacLennan and Wong in 1971 as a major Ca²⁺ binding protein with an estimated molecular weight of 44 kDa which localized to the interior of the SR membrane. Both skeletal and cardiac CASQ (CASQ2) are highly acidic proteins with more than 37% of the total number of amino acids presented by either Asp or Glu concentrated in the C-terminal tails. According to Jones and coworkers, both isoforms can bind up to 800 nM of Ca²⁺ per mg of protein (~40 ions per molecule) with a Kd of about 1 mM at normal ionic strengths. However, others reported that the cardiac isoform binds only half as much of Ca²⁺ (~20 ions per molecule; e.g. Park et al.) as its skeletal counterpart. Upon Ca²⁺ binding, CASQ undergoes major changes in structure. Notably, skeletal and cardiac CASQ is present in soluble form at low Ca²⁺, however, increasing Ca²⁺ concentration causes protein precipitation that leads to fibrils or needle shape crystals. Under such conditions, two thirds of the total bound Ca²⁺ is associated with Ca²⁺-CASQ aggregates, while one third is associated with the soluble form of CASQ. Thus, formation of Ca²⁺-protein complexes appears to be required for high capacity Ca²⁺ binding by CASQ. Cloning CASQ2 did not reveal any distinct Ca²⁺ binding structures such as the EF hand motif characteristic of typical Ca²⁺ binding proteins such as troponin C or calmodulin. It was concluded that rather than relying on the presence of multiple discrete Ca²⁺ binding sites, Ca²⁺ binding to CASQ involves electrostatic attraction to negatively charged residues of CASQ. Wang et al. took advantage of the ability of CASQ1 to form rectangular crystals at high ionic strengths to derive the crystal structure of the protein. These studies provided further clues to the mechanisms of Ca²⁺ binding by CASQ. Unexpectedly, in contrast to sequence analysis that provided no indication of structural repeats, the structure analysis revealed that this protein is made up of three nearly identical tandem domains, I, II, and III, each with a topology similar to that of bacterial thioredoxin. Individual monomers stack in the crystal lattice into ribbon-like polymers. In the crystal, two topologically distinct dimerization contacts have been determined: front-to-front and back-to-back. Both of these dimerization interfaces carry large numbers of acidic residues. Ca²⁺ binding occurs through largely filling the electro-negative pockets formed in the dimerization contacts and absorption to the negatively charged surface of the protein.

4.1.2 Polymerization

Park et al. used a series of truncation mutants to determine the role of Ca²⁺-dependent formation of front-to-front and back-to-back contacts in CASQ2 polymerization. Conformation and polymerization states were determined in vitro by intrinsic fluorescence, circular dichroism, and Ca²⁺ binding. The following model of calsequestrin folding and polymerization has been proposed based on these results (Figure 2). In the absence of Ca²⁺, CASQ is largely unfolded due to charge repulsion. As [Ca²⁺] is increased, CASQ thioredoxin domains are formed and come together as the charge repulsion is shielded. As Ca²⁺ concentration is further increased, the front-to-front interaction occurs first because the N-terminal region involved in this contact has relatively few acidic residues. The back-to-back interaction occurs last because more Ca²⁺ ions are required to shield the negatively charged amino acids in regions involved in formation of this contact, such as the Asp/Glu rich C-terminal tail. A linear polymer is eventually formed as the front-to-front and back-to-back dimers assemble at [Ca²⁺] > 5 mM. At the Ca²⁺ and protein concentrations within the SR, CASQ is expected to be present as a mixture of monomers, dimers, and various size multimers. Indeed electron microscopy reveals the presence of electron dense fibrous matrices in the SR of skeletal and cardiac muscle, which are made by CASQ polymers and multimers. Additionally, chemical cross-linking studies showed that most of the CASQ in the SR exists in a wide range of high-molecular-mass clusters. An important question that remains to be resolved is whether changes in CASQ polymerization status can occur dynamically or whether CASQ remains polymerized during a release-uptake cycle. Dynamic Ca²⁺-dependent interconversions between monomeric and polymeric forms of CASQ2 could provide an attractive mechanism for facilitated dissociation of Ca²⁺ from CASQ2 in the SR during excitation-contraction coupling.

4.1.3 Physical association with other junctional proteins

Besides interacting with itself to form oligomers, Ca²⁺ binding protein calsequestrin interacts with other components of the junctional complex, including TRD, JCN, and Protein concentrations. Consistent with the involvement of the N-terminal region of CASQ2 in binding to the RyR2-TRD complex, a naturally occurring mutation in its N-terminus (R33Q) has been shown to disrupt functional interactions of CASQ2 with the RyR2 channel. However, another mutation (D307H) that is harboured in the third thioredoxin domain of CASQ2 has been also shown to reduce CASQ2 binding to TRD. These results suggest either that the CASQ2 mutations affect CASQ2 binding to TRD indirectly by altering the conformational state of the protein or that the TRD binding site is formed by several separate domains in the linear sequence of the protein.

4.1.4 Calsequestrin function

CASQ2 appears to play at least two different roles in cardiac myocytes: as a Ca²⁺ storage reservoir in the SR and as an active modulator of the Ca²⁺ release process. As a Ca²⁺ storage molecule and a Ca²⁺ buffer CASQ2 supplies the bulk of Ca²⁺ required for contractile activation. Given its localization in SR, in addition to simply storing Ca²⁺, CASQ2 concentrates Ca²⁺ near the points of release consequently influencing the release process by controlling free Ca²⁺ dynamics near the luminal regulatory sites of the RyR2 complex. As a modulator of Ca²⁺ release, CASQ2...
controls RyR2 open probability (via protein–protein interactions involving TRD and JCN) in a luminal Ca\(^{2+}\)-dependent manner potentially serving as luminal Ca\(^{2+}\) sensor for RyR2. The function of CASQ2 has been studied using various approaches including genetic mouse models, acute expression studies in myocytes, and in vitro reconstitution experiments. The results of these studies are briefly summarized below.

4.1.4.1 Calsequestrin overexpression and ablation in genetic mice models
Murine or canine CASQ2 has been overexpressed 10 to 20-fold in mouse hearts and shown to cause hypertrophy in genetic mice models.

4.1.4.2 Acute changes in calsequestrin expression in myocytes
CASQ2 function has been studied in myocytes following acute overexpression or knockdown of CASQ2 protein levels using adenoviral constructs (sense and antisense, respectively). Three to four-fold overexpression of CASQ2 in rat myocytes results in a proportional increase in the SR Ca\(^{2+}\) content and increases the amplitude and rise time of both global \(I_{Ca}\)-induced Ca\(^{2+}\) transients and Ca\(^{2+}\) sparks. These effects are similar to those observed previously with exogenous Ca\(^{2+}\) buffers (e.g. citrate, maleate) loaded into the SR. They are similarly attributable to stabilized free [Ca\(^{2+}\)]\(_{SR}\) at the luminal phase of the RyR2 delaying luminal Ca\(^{2+}\) depletion-mediated deactivation of RyR2s, and prolonging SR Ca\(^{2+}\) release. Increased CASQ2 abundance also slows the restitution of RyR2s from a luminal Ca\(^{2+}\)-dependent refractory state by slowing the recovery dynamics of free intra-SR [Ca\(^{2+}\)] during Ca\(^{2+}\) re-uptake. In contrast, CASQ2 underexpression myocytes (30% of control) display shorter Ca\(^{2+}\) sparks but accelerated restitution of Ca\(^{2+}\)-release sites associated with arrhythmogenic Ca\(^{2+}\) oscillations and delayed afterdepolarizations (DADs). These results show that CASQ2 is a key determinant of the SR Ca\(^{2+}\) release function. As a Ca\(^{2+}\) buffer, CASQ2 not only supplies Ca\(^{2+}\) for release but also governs Ca\(^{2+}\)-release duration and release site refractoriness by controlling free [Ca\(^{2+}\)]\(_{SR}\) dynamics near the luminal side of the RyR2 channel. While clearly supporting the Ca\(^{2+}\) buffering role of CASQ2, these studies are not inconsistent with direct modulatory effects of CASQ2 on RyR2 channel function. Since CASQ2 has been shown to inhibit RyR2s, one would expect, for example, that knockdown of CASQ2 expression would enhance Ca\(^{2+}\) spark occurrence, which was not observed. A possible explanation for this apparent discrepancy is that CASQ2 is expressed at very high level so that even after its suppression to 30% of control, a sufficient number of copies of the proteins remain to fulfill its role as a RyR2 modulator, even though its buffering capacity is reduced. Consistent with this explanation, expression of certain CASQ2 mutants (e.g. R33Q) alters SR Ca signalling in a dominant-negative manner by displacing WT CASQ2 from the RyR2 complex.

4.1.4.3 Effects of calsequestrin on ryanodine receptor channel in lipid bilayers
The direct functional effects of CASQ2 on the RyR2 channel have been studied using an in vitro reconstitution approach, i.e. lipid bilayers. In these studies, CASQ2 has been found to inhibit RyR2s complexed with TRD and JCN but had no effect on purified RyR2s stripped of TRD and JCN. The inhibition was luminal Ca\(^{2+}\)-dependent such that it occurred at low (20 \(\mu\)M) but not at high luminal Ca\(^{2+}\) (2 mM). The luminal Ca\(^{2+}\) dependency of the modulatory effects of CASQ2 raises the possibility that CASQ2 may serve as a luminal Ca\(^{2+}\) sensor for RyR2. In support of this possibility, RyR2s lost their ability to respond to luminal Ca\(^{2+}\) following purification. Moreover, purified RyR2s regained their responsiveness to luminal Ca\(^{2+}\) following re-association with all three proteins (TRD, JCN, and CASQ2) but not with TRD and JCN only. Qualitatively similar results have been obtained for skeletal muscle in which skeletal CASQ2 also inhibited RyR2 in a Ca\(^{2+}\) dependent manner.

4.2 Triadin
4.2.1 Biochemistry and structure
Triadin was first identified as a 95 kDa integral membrane protein in the junctional SR vesicles isolated from skeletal muscle by Caswell et al. and Knudson et al. Structurally, it is composed of a short cytoplasmic N-terminal segment (47 residues); a single membrane spanning domain (20 residues), and a highly charged C-terminal region localized in
the SR lumen which comprises the bulk of the protein. Subsequently, three cardiac-specific isoforms have been found (35, 40, and 75 kDa) of which TRD-1 (40 kDa) is the most abundant form, comprising more than 95% of the total amount of TRD in cardiac myocytes. Since the protein is partially glycosylated, it is present as a doublet of 35 and 40 kDa molecular weight proteins on SDS–PAGE. All TRD isoforms are splice variants of the same gene. They share identical sequences from residues 1–264 and the divergence after residue 264 is largely due to variations in the length of the C-terminal tail. According to in vitro binding studies, the sites for interaction of TRD with its binding partners, i.e. RyR2, CASQ2, and JCN, reside in its charged C-terminal tail. This portion of TRD is characterized by the frequent occurrence of long stretches of alternating positively and negatively charged residues known as KEKE motifs. These segments are considered to be protein–protein-binding domains. The CASQ2-binding domain of TRD-1 has been localized to a single KEKE motif comprised of 15 residues (210–224). Within this domain, eight even numbered, mostly charged amino acids [Lys(210)–Lys(224)] are critical for CASQ2 binding. This structure is consistent with a model that involves positively charged amino acids of TRD interacting directly with the negatively charged residues of CASQ2 to form a polar zipper that links the two proteins together. Whereas TRD binding to CASQ2 is Ca\(^{2+}\)-dependent such that the proteins dissociate at high Ca\(^{2+}\) (>5 mM), its binding to RyR2 is independent of Ca\(^{2+}\).

### 4.2.2 Triadin 1 function

Two possibilities have been considered in the literature concerning TRD function in cardiac muscle. One possibility is that TRD simply plays an anchoring role by concentrating CASQ2 near the junctional phase of the SR. In this scaffolding capacity, TRD is thought to facilitate SR Ca\(^{2+}\) release indirectly by permitting CASQ2 to buffer Ca\(^{2+}\) in the vicinity of the release sites. The other possibility is that TRD directly regulates the activity of the RyR2 Ca\(^{2+}\) release channel. The function of TRD has been examined in transgenic mice with cardiac overexpression of TRD. Five-fold overexpression of TRD is accompanied with hypertrophy and selective downregulation of JCN and RyR2 but not CASQ2. Cardiac myocytes from TRD overexpressing mice exhibit altered Ca\(^{2+}\) handling including slowed relaxation and Ca\(^{2+}\) transient decay and depressed contractile strength only at low stimulation frequencies. Although demonstrating that TRD is an integral part of the Ca\(^{2+}\) handling process, these results are not easily interpretable in terms of TRD’s intrinsic function due to adaptive and pathological changes accompanying chronic protein overexpression. The role of TRD in cardiac EC coupling has been investigated using acute overexpression of this protein in cardiac myocytes. By measuring local and global Ca\(^{2+}\) transients and recordings from single RyR2 channels, it has been found that elevated TRD enhances the activity of the RyR2 Ca\(^{2+}\) release channel resulting in increased spark mediated SR Ca\(^{2+}\) leak, reduced SR Ca\(^{2+}\) content, arrhythmogenic spontaneous releases, and membrane depolarizations, similar to those observed in myocytes expressing CPVT1-linked CASQ2 mutants. These effects of TRD require a complete primary structure including the luminal tail region of the protein encompassing amino acids 200–224, as expression of a mutant protein missing this domain failed to produce any of the effects observed with its WT counterpart. Consistent with these TRD overexpression studies, re-association of purified RyR2 with TRD increases RyR2 channel open probability in lipid bilayers. Interestingly, these effects of TRD and JCN could be reversed by CASQ2 in a Ca\(^{2+}\)-dependent fashion. Specifically, CASQ2 inhibited the RyR2/TRD/JCN complex at low and intermediate luminal Ca\(^{2+}\) (<5 mM) but not at high Ca\(^{2+}\) (>5 mM). Based on these results, it has been suggested that a dual regulation of RyR2s by TRD (or JCN) and CASQ2 accounts for the ability of RyR2s to sense and respond to changes in the SR luminal Ca\(^{2+}\).

### 4.3 Junctin

Junctin has been identified as a 26 kDa CASQ-binding protein in cardiac and skeletal muscle junctional SR membranes. It is localized strictly to SR and is absent from other SR compartments including the corbular SR. Junctin has been purified and cloned and shown to be expressed equally in skeletal and cardiac muscle. Although smaller than TRD, JCN has a similar domain structure composed of an N-terminal cytosolic segment, a membrane spanning domain, and a highly charged C-terminal tail projected into the SR. Similar to TRD, the luminal domain of JCN contains several KEKE motifs, and putative sites for protein interaction. In contrast to TRD, JCN does not appear to have a single discrete CASQ2 binding domain as deletion of any of the several KEKE motifs results in reduced CASQ2 binding. In lipid bilayers, luminal JCN appears to stimulate RyR2 activity similar to TRD. At the same time, acute adenoviral-mediated overexpression of JCN depresses myocyte shortening and Ca\(^{2+}\) transient amplitude, whereas downregulation of JCN causes an increase in these parameters. Although the specific mechanisms of altered cardiomyocyte contractility by manipulation of JCN are to be determined, JCN may directly regulate SR Ca\(^{2+}\) cycling by affecting SR Ca\(^{2+}\) load and modulating RyR2 Ca\(^{2+}\) release. Cardiac-specific 5 to 10-fold overexpression of canine JCN in transgenic mice results in reduced expression of CASQ2, TRD and sodium-calcium exchanger (NCX), and hypertrophy. Overexpression of JCN also leads to altered myocyte Ca\(^{2+}\) handling, including reduced SR Ca\(^{2+}\) content, prolonged decay of Ca\(^{2+}\) transients, and decreased Ca\(^{2+}\) spark frequency. Notably, JCN-overexpressing myocytes exhibit marked changes in SR structure, consisting of narrowing of SR cisternae, compaction of content i.e. CASQ2, and extension of JCN domains to non-junctional regions (i.e. ‘frustrated’ SR). Thus, JCN not only plays a role in Ca\(^{2+}\) signalling but is also involved in SR morphogenesis and in assembling of the molecular components of the Ca\(^{2+}\) release machinery in SR. Recently, a genetic mouse model with cardiac specific ablation of JCN has been generated. Ablation of JCN is associated with enhanced cardiac function but the JCN-deficient animals exhibit DAD-induced arrhythmias and premature mortality under conditions of physiological stress. At the myocyte level, ablation of JCN results in selective upregulation of NCX expression and function, as well as increases in SR Ca\(^{2+}\) content, I\(_{Ca}\)-induced Ca\(^{2+}\) transients and sparks. Future studies will have to determine how the lack of JCN leads to enhanced Ca\(^{2+}\) cycling and increased SR Ca\(^{2+}\) load as well as the role of potential compensatory mechanisms.
in these changes. Similarly, it will be interesting to see how ablation of JCN affects SR structure.

4.4 Other sarcoplasmic reticulum proteins

There are several other intra-SR proteins that might interact with the RyR2 complex and modulate SR Ca\(^{2+}\) release, including sarcalumenin, calreticulin, and a histidine-rich Ca\(^{2+}\)-binding protein.\(^{23}\) The specific roles of these minor SR proteins remain to be determined. Interestingly, myocytes from CASQ2 knockout mice showed a compensatory increase in the Ca\(^{2+}\)-binding protein calreticulin in an apparent attempt to preserve SR Ca\(^{2+}\) storing function.\(^{58}\)

5. Abnormal ryanodine receptor luminal regulation and cardiac disease

As discussed in previous sections, RyR2 luminal Ca\(^{2+}\) regulation is an important check point involved in maintaining the ordered myocyte Ca\(^{2+}\) signalling required for normal cardiac function. Thus, it is not surprising that alterations in this mechanism lead to cardiac disease states. In general, defective luminal Ca\(^{2+}\) signalling can arise in two ways: (i) in a variety of conditions that lead to increased SR Ca\(^{2+}\) load (i.e. ‘Ca\(^{2+}\) overload’), sensitization of RyR2 by elevated luminal Ca\(^{2+}\) contributes to generation of spontaneous Ca\(^{2+}\) releases thus leading to DADs and triggered arrhythmia; (ii) acquired and genetic defects in RyRs which enhance the responsiveness of RyR2 to luminal Ca\(^{2+}\) such that the channel becomes hyperactive even at reduced SR Ca\(^{2+}\) content. This state of ‘perceived’ Ca\(^{2+}\) overload can also lead to Ca\(^{2+}\)-dependent arrhythmias as shown both in certain familial tachycardias associated with mutations in RyR2 or CASQ2, and in arrhythmias during HF. Additionally, when RyR2 activity is excessively high, the SR becomes leaky resulting in depleted SR Ca\(^{2+}\) stores as it may happen in HF.

5.1 Ca\(^{2+}\) overload and triggered arrhythmia

Excess intracellular Ca\(^{2+}\) is a feature characteristic of various pathological states such as metabolic inhibition, ischaemia/reperfusion, and digitalis poisoning. Ca\(^{2+}\) overload causes triggered arrhythmias, which can initiate sustained tachyarrhythmias. The causal relation between Ca\(^{2+}\) overload and triggered activity has been relatively well established (reviewed by Pogwizd and Bers\(^^{59}\) and Ter Keurs and Boyden\(^^{60}\)); it is thought to involve the following sequence of events: (i) increased [Ca\(^{2+}\)]\(_{SR}\) sensitizes RyRs leading to generation of spontaneous Ca\(^{2+}\) release from the SR; (ii) elevated Ca\(^{2+}\) induces depolarizing membrane currents that give rise to DADs;\(^{3}\) DADs activate ectopic APs and these ectopic APs can then initiate tachyarrhythmias. Although DADs have been best characterized in isolated myocytes, evidence exists that links DADs to triggered arrhythmia in cardiac muscle using simultaneous ECG and AP measurements\(^{61}\) and high-resolution optical mapping of intracellular Ca\(^{2+}\) and transmembrane potential.\(^{62}\)

5.2 Catecholaminergic polymorphic ventricular tachycardia

Catecholaminergic polymorphic ventricular tachycardia (CPVT) is a familial arrhythmogenic disease characterized by syncope and sudden death following exercise and emotional stress.\(^{63}\) Two genetic forms of CPVT have been described: a recessive form associated with mutations in CASQ2\(^{64,65}\) and a dominant form linked to mutations in RyR2s. Since CPVT linked to RyR2 mutations have been reviewed elsewhere,\(^{66,67}\) we will focus primarily on the molecular, subcellular, and cellular mechanisms of malignant arrhythmia associated with mutations in CASQ2. Seven mutations in CASQ2 linked to CPVT have been identified so far (Figure 3B).\(^{38,64,65,68}\) How do these defects result in arrhythmia? Studies with adenoviral expression of CPVT CASQ2 mutants in cardiac myocytes (Figure 3B and C) have suggested that various CASQ2 mutations can act through at least two different mechanisms converging on a common pathological pathway to induce irregular Ca\(^{2+}\) transients and electrical activity at the myocyte level (Figure 4). One of these mechanisms involves alteration in the Ca\(^{2+}\) storing and buffering function of CASQ2 and the other involves changes in CASQ2’s role as a RyR2 modulator. In vivo these mechanisms are likely to act in parallel to cause arrhythmia. Genetic defects that either compromise CASQ2 expression (e.g. R33X; 532+1 G→A; and 62delA\(^{64}\)) or impair its Ca\(^{2+}\) binding activity can act by reducing intra-SR Ca\(^{2+}\) buffering which in turn alters the dynamics of free [Ca\(^{2+}\)]\(_{SR}\) sensed by the RyR2 channel complex. Early [Ca\(^{2+}\)]\(_{SR}\) recovery results in premature and more complete restitution of Ca\(^{2+}\) signalling from a luminal Ca\(^{2+}\)-dependent refractory state, thus, leading to DADs and cellular arrhythmia.\(^{3,69}\) Interestingly, expression of certain CASQ2 mutants (Del.339–354 and D307H) produces dominant negative effects manifest in reduced SR Ca\(^{2+}\) content and cytosolic Ca\(^{2+}\) transients. These effects have been attributed to disrupted CASQ2 polymerization and impaired ability of CASQ2 to bind Ca\(^{2+}\).

Genetic defects can also alter the sensitivity of the RyR2 channel to luminal Ca\(^{2+}\) by affecting interactions of CASQ2 with the RyR2 complex, as demonstrated with the CPVT CASQ2 mutation R33Q and possibly D307H.\(^{38,39}\) In this case, the Ca\(^{2+}\) binding capacity of CASQ2 may not be necessarily compromised, but the altered modulation of RyRs by luminal Ca\(^{2+}\) (i.e. compromised ability of CASQ2 to deactivate the RyR2) leads again to premature recovery of Ca\(^{2+}\) release from refractoriness. Thus, mutations in CASQ2 can exert their deleterious effects through at least two different mechanisms involving the two primary functions of the protein: as SR Ca\(^{2+}\) storage site and a modulator of the RyR2 channel activity. The common point of convergence is premature restitution of RyR2 channels from a luminal Ca\(^{2+}\)-dependent refractory state. Whether premature restitution is due to accelerated recovery dynamics of [Ca\(^{2+}\)]\(_{SR}\) near the luminal side of the channel or caused by sensitization of the channel by luminal Ca\(^{2+}\), the final effect is the same—generation of spontaneous extrasystolic Ca\(^{2+}\) releases and DADs. Considering the described relationship between abnormal luminal Ca\(^{2+}\) modulation and arrhythmia, a common mechanism can be envisioned in which CPVT is caused by genetic defects in any component of the luminal Ca\(^{2+}\) signalling pathway, including: (i) control and sensing of free [Ca\(^{2+}\)]\(_{SR}\) in the vicinity of the RyR2; (ii) transmitting the [Ca\(^{2+}\)]\(_{SR}\) signal to RyR2; and (iii) changes in RyR2 interdomain interactions ultimately linked to gating conformations, resulting in premature restitution, spontaneous Ca\(^{2+}\) releases and DADs. Consistent with this unifying view,
CPVT-linked mutations in RyR2 have been reported to alter the luminal Ca\(^{2+}\) sensitivity of the RyR2.\(^{70}\) Interestingly, a similar sensitization of RyR2 to activation by luminal Ca\(^{2+}\) has been shown to occur in HF,\(^ {11}\) suggesting that the abnormal luminal Ca\(^{2+}\) sensing is a common feature in cardiac disease.

A question to be considered is how does SR Ca\(^{2+}\) leak cause arrhythmia given the tendency of elevated leak to self correction. Indeed, increased open RyR2 probability is expected to lower SR Ca\(^{2+}\) load and in turn reduce SR Ca\(^{2+}\) leak and spontaneous RyR2 activity.\(^ {71}\) This issue is referred to as the ‘Ca\(^{2+}\) overload paradox’ and its solution appears to be in the specific nature of RyR2 abnormality, i.e. abnormal modulation of RyR2 by luminal Ca\(^{2+}\).\(^ {72}\) Sensitization to luminal Ca\(^{2+}\) produces a state homologous to Ca\(^{2+}\) overload in which predisposition to arrhythmia arises not as a result of [Ca\(^{2+}\)]\(_{SR}\) reaching elevated threshold for generation of spontaneous Ca\(^{2+}\) release but rather as a consequence of the threshold decreasing to a much lower [Ca\(^{2+}\)]\(_{SR}\) (‘perceived Ca\(^{2+}\) overload’). SERCA functional capacity is clearly also an important factor in arrhythmogenesis. It determines to what extent the SR is able to preserve a sufficient SR Ca\(^{2+}\) load required for Ca\(^{2+}\) release even in the presence of elevated leak. Consistent with this notion, arrhythmias induced by mutations in RyR2 and CASQ2 rely on elevated circulating catecholamines,\(^ {58}\) which stimulate SR Ca\(^{2+}\) uptake through phosphorylation of phospholamban.

5.3 Abnormal ryanodine receptor luminal Ca\(^{2+}\) regulation and heart failure

HF is a disease state in which the muscle of the heart becomes too weak to adequately pump blood through the body. Additionally, HF is accompanied by increased risk of malignant arrhythmia. Although HF is very complex,\(^ {73-75}\) abnormal RyR2 function is increasingly recognized as a potential contributor to the pathophysiology of this disease.\(^ {11,24,76,77}\) Evidence obtained using different models of HF and in human suggests that RyR2s become excessively active in HF. Uncontrolled RyR2s gating is expected to result in increased diastolic SR Ca\(^{2+}\) leak causing a reduction of the SR Ca\(^{2+}\) content, thus, limiting the ability of cardiac muscle to contract. The exact role and rate of SR Ca\(^{2+}\) leak has been difficult to quantify due to the presence of competing Ca\(^{2+}\) fluxes, including those mediated by SERCA and NCX. By using a canine model of chronic HF and directly measuring both RyR2-mediated Ca\(^{2+}\) loss from the SR and SERCA-dependent SR Ca\(^{2+}\) uptake, we have recently shown that enhanced

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**Figure 3** Mutations in cardiac calsequestrin gene (CASQ2) associated with CPVT and alterations in Ca\(^{2+}\) handling caused by ectopic expression of some of these CASQ2 mutants in cardiac myocytes. (A) Locations of CPVT mutation sites within the CASQ2 sequence. (B) Representative immunoblots detecting CASQ2 and its variants in cardiac myocytes infected with several different adenoviral constructs for the expression of the protein and its variants. (C) Line-scan images along with averaged temporal profiles of [Ca\(^{2+}\)] changes acquired in a control myocyte and in a myocyte underexpressing CASQ2 and in myocytes expressing two different CPVT-linked CASQ2 mutants all stimulated at 2 Hz in the presence of 0.5 μM isoproterenol in the bathing solution.
RyR2-mediated Ca^{2+} leak is a major factor in determining the reduced SR Ca^{2+} content and the slowed Ca^{2+} transients in HF myocytes. In this model, intrinsic activities of SERCA and NCX showed no or only a relatively small change, respectively, and the reduced Ca^{2+} content could be normalized by blocking the RyR2s with ruthenium red. Future studies utilizing similar techniques will have to determine whether these findings are specific to this particular model or whether they similarly apply to other models and stages of HF.

Although it is easy to understand how elevated SR Ca^{2+} leak can lead to reduced SR Ca^{2+} content, the existence of a sustained accelerated SR Ca^{2+} leak at reduced [Ca^{2+}]_{SR} presents a paradox (i.e. 'Ca^{2+} overload paradox') given the established positive relationship between RyR2 activity and SR Ca^{2+} content. Indeed, when stimulated by a RyR agonist such as caffeine, SR Ca^{2+} leak increases only temporarily and then subsides due to a decline in the SR Ca^{2+} content. It has been proposed that local regions with elevated SR Ca^{2+} content exist in HF myocytes despite the decrease in the cell-average SR Ca^{2+} content and that this regional inhomogeneity in [Ca^{2+}]_{SR} accounts for the overall leaky SR by producing areas with hyperactive RyR2s. However, direct spatially resolved [Ca^{2+}]_{SR} measurements with an SR-entrapped Ca^{2+} indicator demonstrated that [Ca^{2+}]_{SR} is lowered throughout the entire SR network in HF myocytes. An alternative solution to the Ca^{2+} overload paradox is that the sensitivity of the RyR2s to stimulation by luminal Ca^{2+} is enhanced in HF.

The underlying biochemical causes of HF-related abnormalities in RyR2 function are a subject of intense debate. Marks and co-workers have argued that excessive phosphorylation of RyR2 by PKA (on Ser 2809) followed by dissociation of FKBP12.6 causes RyR2 to become hyperactive, i.e. leaky in HF. However, various aspects of this particular scenario have been questioned by others (e.g. Bers et al.). More recently, Bers and colleagues presented data suggesting that RyR2 phosphorylation by CAMKII (on Ser 2815) accounts for the leaky RyR2s in HF (but see).

Another possibility is that enhanced RyR2 activity is caused by modification of the channel protein by reactive oxygen and/or nitrogen species generated in the failing hearts. Finally, altered composition of the RyR2 channel complex due to altered expression and/or targeting of components of this complex (including CASQ2, TRD, and RyR2 itself), may also contribute to altered RyR2 function in HF. To identify the molecular causes of HF-related changes in RyR2 gating, it will be important to investigate which of the potential biochemical mechanisms, e.g. altered RyR2

![Figure 4](https://academic.oup.com/cardiovascres/article-abstract/77/2/245/428086)
phosphorylation, free radical modification, etc., is associated with altered RyR2 luminal Ca\(^{2+}\) sensitivity in failing hearts. In conclusion, increased sensitivity to luminal Ca\(^{2+}\) might be a common pathological alteration involved in various disease states, including HF and CPVT and may provide a therapeutic target to treat cardiac dysfunction.

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**References**


