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REVIEW

Ca^{2+} signaling in striated muscle: the elusive roles of triadin, junctin, and calsequestrin

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Abstract This review focuses on molecular interactions between calsequestrin, triadin, junctin and the ryanodine receptor in the lumen of the sarcoplasmic reticulum. These interactions modulate changes in Ca^{2+} release in response to changes in the Ca^{2+} load within the sarcoplasmic reticulum store in striated muscle and are of fundamental importance to Ca^{2+} homeostasis, since massive adaptive changes occur when expression of the proteins is manipulated, while mutations in calsequestrin lead to functional changes which can be fatal. We find that calsequestrin plays a different role in the heart and skeletal muscle, enhancing Ca^{2+} release in the heart, but depressing Ca^{2+} release in skeletal muscle. We also find that triadin and junctin exert independent influences on the ryanodine receptor in skeletal muscle where triadin alone modifies excitation–contraction coupling, while junctin alone supports functional interactions between calsequestrin and the ryanodine receptor.

Keywords Triadin · Junctin · Calsequestrin · Ryanodine receptors

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Introduction

The Ca^{2+} signals that lead to contraction in striated muscle depend on the capacity of the sarcoplasmic reticulum (SR) to maintain a store of Ca^{2+} within the muscle fiber and to rapidly release that Ca^{2+} into the cytoplasm in response to the surface membrane depolarization associated with an action potential. The process of excitation–contraction (EC) coupling links the action potential to a tightly controlled release of Ca^{2+} from the SR. The action potential is detected by the voltage sensitive Ca^{2+} channel (the dihydropyridine receptor, DHPR) in the surface membrane and communicated to the ryanodine receptor (RyR) Ca^{2+} release channel that is resident in the adjacent SR membrane. This communication is thought to occur through a physical coupling between the DHPR and RyR in skeletal muscle, while the RyR in the heart is activated by Ca^{2+} ions that flow through the DHPR from the extracellular space. The RyR in all muscle types forms the hub of a large multiprotein complex which contains extracellular, cytoplasmic and SR luminal components that are linked by transmembrane entities (Fig. 1). Thus the RyR complex is able to sense the environment (including Ca^{2+} concentration) in each of the three compartments (extracellular, cytoplasmic and SR lumen) and appropriately regulate intracellular Ca^{2+} release. Proteins which associate with the RyR in the lumen of the SR, including calsequestrin (CSQ, the major Ca^{2+} binding protein) and triadin and junctin (co-proteins that link CSQ to the RyR, and themselves dictate RyR activity), also play a role in controlling Ca^{2+} release from the RyR. These luminal proteins are increasingly recognized as important players in both the regulation of Ca^{2+} store load and Ca^{2+} release through the RyR.

This review focuses on molecular interactions between CSQ, triadin, junctin and the RyR which occur in the lumen of the SR which may facilitate changes in Ca^{2+} release in

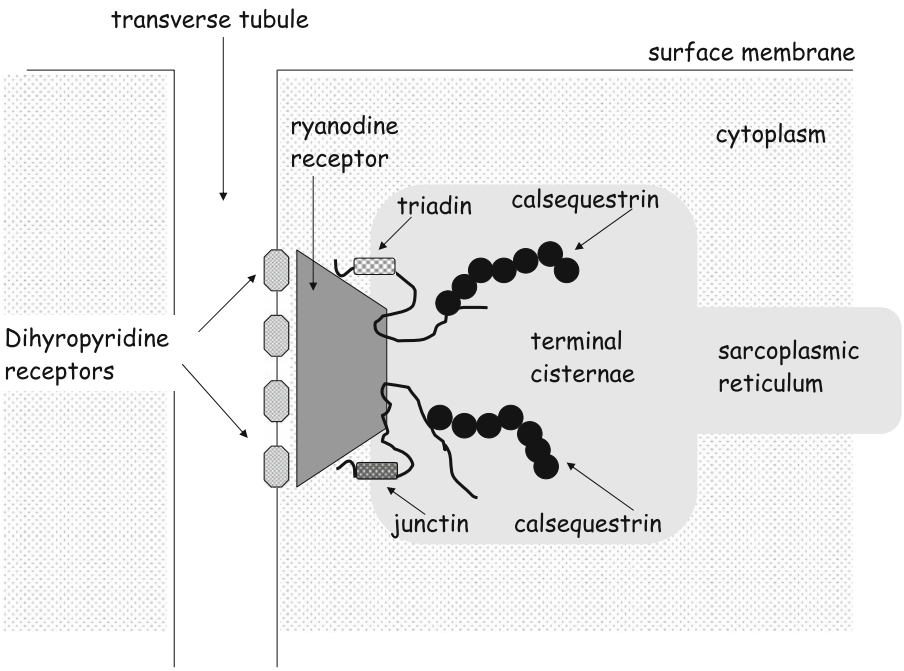

Fig. 1 The arrangement of the surface and its transverse (t-) tubule invaginations and its junction with the terminal cisternae of the sarcoplasmic reticulum in skeletal muscle fibres. The relationship between the dihydropyridine receptor in the t-tubule membrane, the ryanodine receptor, triadin and junctin in terminal cisternae membrane and calsequestrin in the lumen of the terminal cisternae is shown

response to changes in the Ca^{2+} load within the SR store (Beard et al. 2005; Gyorke et al. 2004; Qin et al. 2008; Wei et al. 2006, 2009b). These interactions are of fundamental importance to Ca^{2+} homeostasis in muscle, since there are massive changes in protein expression, membrane geometry, Ca^{2+} store load and Ca^{2+} release when expression of CSQ, triadin or junctin is manipulated in the heart or skeletal muscle (Kirchhefer et al. 2001, 2006; Knollmann et al. 2006; Paolini et al. 2007; Rezgui et al. 2005; Terentyev et al. 2005), while RyR knockout is lethal. Mutations in CSQ in the heart, or its absence, lead to severe functional changes including catecholaminergic polymorphic ventricular tachycardia (CPVT) which can be fatal (Knollmann et al. 2006; Valle et al. 2008). Our recent studies show that the two small "anchoring" proteins, triadin and junctin which are embedded in the SR membrane not only bind to the RyR and to CSQ, but also exert separate influences on the RyR (Wei et al. 2009a). The molecular interactions underlying communication between CSQ and the RyR and the roles of triadin and junctin are not well understood in either skeletal muscle or cardiac myocytes. However, the body of work described in the following sections has defined the overall interactions between the proteins, so that a picture of the function of each of the molecules and the residues that facilitate their communication is emerging.

Calsequestrin in the heart and skeletal muscle

The general properties of CSQ have been reviewed previously (Beard et al. 2004) and are similar in the heart and skeletal muscle. Briefly, CSQ is a very efficient Ca^{2+} storage protein with a high capacity and low affinity for Ca^{2+} ions which facilitate an increase in the total SR calcium to amounts in excess of 20 mM, while maintaining a free Ca^{2+} concentration of 1 mM. The conformation and Ca^{2+} binding capacity of CSQ are highly dependent both on Ca^{2+} concentration and on ionic strength. CSQ has the greatest Ca^{2+} binding capacity when it forms a polymer in the presence of Ca^{2+} concentrations of $\geq$1 mM. The C-terminal tail of the CSQ protein contains the highest density of acidic residues and is constrained to form a Ca^{2+}-binding pocket when CSQ monomers self associate to form a polymer.

Two isoforms of CSQ are expressed in striated muscle and are encoded for by different genes (Fliegel et al. 1990; Scott et al. 1988). CSQ1 or "skeletal" CSQ is the only isoform expressed in fast-twitch skeletal muscle and is the major isoform in slow-twitch skeletal muscle (Biral et al. 1992; Damiani and Margreth 1994). CSQ2, "cardiac" CSQ, is the sole CSQ isoform expressed in cardiac muscle and is a minor transcript in slow-twitch skeletal muscle. The two isoforms of CSQ differ mainly in their C-terminal region with CSQ2 having an extended highly acidic C-terminal tail. CSQ1 and CSQ2 can act as luminal Ca^{2+} sensors for the skeletal isoform of the RyR (RyR1) and cardiac isoform of the RyR (RyR2), respectively (Beard et al. 2004; Gyorke et al. 2004; Qin et al. 2008; Wei et al. 2006). There is no available information on interactions between CSQ (1 or 2) and the third isoform of the RyR (RyR3) which is expressed in many tissues, either alone or with RyR1 and/or RyR2.

Regulation of RyR1 channels by CSQ1: the role of CSQ1 as a Ca^{2+} sensor for RyR1

Molecular interactions between CSQ and the RyR can be studied only in simplified isolated systems. The lipid bilayer single channel setup provides a unique opportunity to evaluate the functional effects of CSQ on the kinetics of RyR channel opening and closing under a variety of tightly controlled cytoplasmic and luminal conditions. SR vesicles containing native RyR1 channels (with endogenous CSQ attached) incorporate into a planar lipid bilayer with the cytoplasmic side facing the solution to which the vesicles were added (*cis* solution). The luminal side of the channel faces the opposite (*trans*) side of the bilayer (Fig. 2). The ionic strength and Ca^{2+} concentration in solutions on either side of the bilayer (and hence the channel) can be changed and drugs or proteins added or removed by perfusion as desired. Changes in RyR activity are recorded when endogenous CSQ1 is dissociated by appropriate changes in luminal (*trans*) Ca^{2+} concentration or ionic strength. Conversely, the effects of re-association with exogenous CSQ1 added to the luminal solution can be evaluated (Beard et al. 2002; 2005; Gyorke et al. 2004; Qin et al. 2008; Wei et al. 2006). Experiments in our laboratory and others using a variety of protein chemistry techniques have allowed us to predict the conformation of CSQ and its association with triadin and junctin and the RyR under the same ionic conditions used in the bilayer experiments and conditions similar to those encountered in vivo (Park et al. 2004; Slupsky et al. 1987; Wang et al. 1998; Wei et al. 2006). Using data obtained in these studies, we have constructed the following model of CSQ1 structure and the way in which CSQ1 regulates RyR1 (Wei et al. 2006, 2009b).

(1) In the physiological range of ionic strength (150–250 mM), CSQ1 is a monomer when the Ca^{2+} concentration is 100 nM–100 μM. The protein polymerizes when free intraluminal Ca^{2+} approaches the physiological level of 1 mM and the polymer becomes super compacted when the Ca^{2+} exceeds 3 mM.
(2) CSQ1 binding to triadin and junctin has an opposite Ca^{2+}-dependence. When the luminal Ca^{2+} concentration is <1 mM, CSQ1 or two monomers bind most strongly to triadin and junctin, while the supercompacted CSQ1 that is favored when luminal Ca^{2+} is ≥3 mM is unable to bind to triadin or junctin. Therefore, when luminal Ca^{2+} is increased from 1 mM to ≥3 mM, the supercompacted CSQ1 polymer dissociates from triadin and junctin.
(3) CSQ1 inhibits RyR1 only when it binds to junctin and only when the luminal Ca^{2+} concentration is 1 mM and CSQ1 is in a polymer form. This inhibition is not seen after exposure to lower Ca^{2+} concentrations (≤100 μM) lasting more than 2–3 min because CSQ1 becomes depolymerised with only a non-functional monomer remaining associated with the anchoring proteins. Similarly, the inhibition is not seen after 3–5 min exposure to higher Ca^{2+} (≥3 mM), when CSQ1 becomes super compacted and dissociates from triadin and junctin.
(4) Since dissociation of CSQ1 from RyR1 is time-dependent, the association between CSQ1 and RyR1 as well

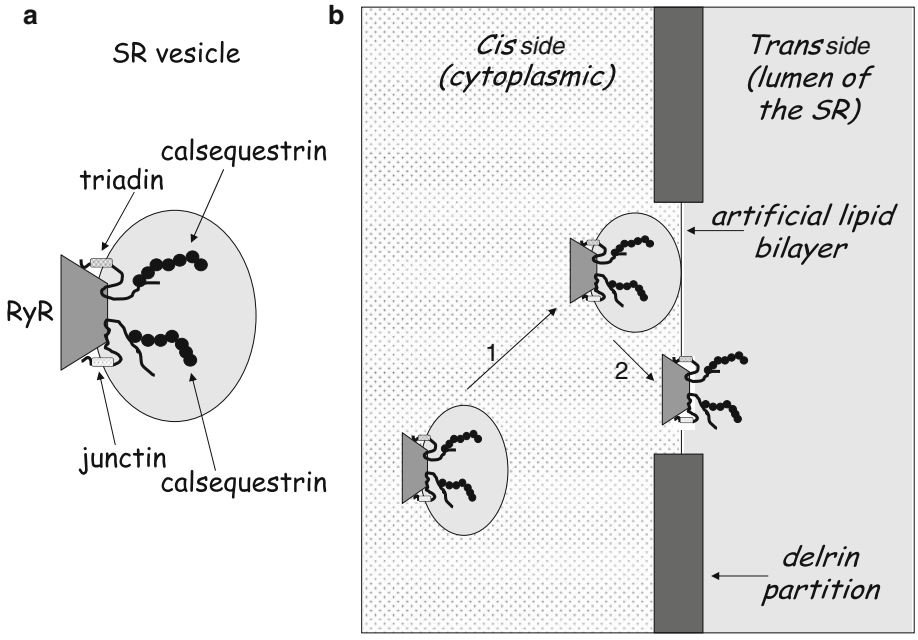

Fig. 2 Microsomal vesicles formed from the terminal cisternae and their incorporation into artificial lipid bilayers. **a** A terminal cisternae vesicle containing the ryanodine receptor, triadin, junctin and calsequestrin. **b** Terminal cisternae vesicles added to the *cis* side of the artificial lipid bilayer, diffuse to the bilayer (step *1*) and then incorporate into the bilayer (step *2*) in such a way that their cytoplasmic side faces the *cis* solution and their luminal side faces the *trans* solution

as the inhibitory action of CSQ1 is retained over the entire range of Ca^{2+} concentrations ($\leq$1 nM to $\geq$3 mM) for periods of 1–3 min after a change in Ca^{2+} concentration from the normal resting level of ~1 mM. A transient fall in luminal Ca^{2+} can occur within this time frame after strong Ca^{2+} release in intact muscle fibres (Launikonis et al. 2006).

(5) RyR1 activity increases when luminal Ca^{2+} concentration declines if CSQ1 is not bound in the normal way to the RyR1 complex. In contrast, RyR1 activity falls after a sudden decrease in luminal Ca^{2+} concentration while CSQ1 remains bound to the RyR1 complex for 1–3 min after the drop in Ca^{2+} concentration. This suggests that CSQ1 acts to conserve Ca^{2+} in the lumen of the SR when intraluminal Ca^{2+} falls briefly as it can during normal activity (Launikonis et al. 2006).

This model incorporates published data about molecular interactions between the isolated skeletal RyR1, CSQ1, the 95 kDa triadin and junctin. Molecular interactions between RyR2, CSQ2, the 32 kDa isoform of triadin and junctin have been studied in less detail. The model, and the work which supports it, suggest that CSQ1 plays a dynamic role in regulating Ca^{2+} release and in maintaining store load in skeletal muscle.

The role of CSQ2 in the heart may be quite different from that of CSQ1 in skeletal muscle

Despite their general similarities, there are major differences in the detailed properties of CSQ1 and CSQ2 that have significant functional implications. The Ca^{2+} binding capacity of CSQ1 is greater than that of CSQ2 (Park et al. 2004; Wei et al. 2009b) and the isolated proteins undergo different structural changes over a [Ca^{2+}] range of zero to 1 mM (Slupsky et al. 1987; Wei et al. 2009b). These differences raise the possibility that CSQ regulates RyRs in different ways in the heart and skeletal muscle, and indeed that there may be considerable differences between CSQ1 regulation of RyR1 in fast-twitch skeletal muscle and CSQ2 regulation of RyR1 in slow-twitch skeletal muscle.

Our recent studies provide the first detailed comparison of CSQ1 binding to RyR1 and CSQ2 binding to RyR2 under physiological conditions and support the hypothesis that there are significant isoform-specific differences (Wei et al. 2009b). These differences are apparent in bilayer experiments where endogenous CSQ is first removed from the RyR (by exposure to either high ionic strength or to $Ca^{2+} \geq$ 5 mM) and then exogenous CSQ added back to the luminal solution bathing lipid bilayers (Beard et al. 2002). Under these conditions, CSQ1 inhibits RyR1 when added back at a physiological luminal [Ca^{2+}] of 1 mM (Beard et al. 2002), while CSQ2 activates RyR2 under the same conditions (Wei et al. 2009b) (Fig. 3). Two factors should be kept in mind when comparing these data with results obtained by other groups (Gyorke et al. 2004; Szegedi et al. 1999). First, our experiments were performed with native RyR channels where triadin and junctin remain bound to the RyR, so that exogenous CSQ added back to the bilayer solution can bind to endogenous triadin and junctin and most likely influences RyR activity through this binding (Beard et al. 2002). Second, the luminal Ca^{2+} concentration in our experiments was 1 mM. This second point is significant because the CSQ1 monomer in fact activates RyR1 when luminal Ca^{2+} is $\leq$100 μM, while supercompacted CSQ1 does not bind to triadin and junctin and does not inhibit RyR1 activity when luminal Ca^{2+} is 3–5 mM, as outlined in the model in "Regulation of RyR1 channels by CSQ1: the role of CSQ1 as a Ca^{2+} sensor for RyR1". When the Ca^{2+} concentration is increased to 3–5 mM, CSQ becomes supercompacted and dissociates from triadin and junctin over a period of 2–3 min (Beard et al. 2005). On the other hand, when luminal Ca^{2+} is lowered to $\leq$100 μM, CSQ1 depolymerises and all but the terminal monomer dissociate from triadin and junctin, again over a few minutes (Wei et al. 2006). Thus, to re-iterate, the inhibitory action of CSQ1 on RyR1 is maintained in a steady-state only with ~1 mM Ca^{2+}, and is maintained only during brief transitions to higher or lower Ca^{2+} concentrations.

Native cardiac RyR2 channels are activated by CSQ2 in the presence of 1 mM Ca^{2+} as well as at lower luminal Ca^{2+} concentrations (Wei et al. 2008; Qin et al. 2008). Curiously, and in contrast to these results, CSQ2 has been reported to inhibit RyR2 channels when the luminal Ca^{2+} concentration is less than 20 μM (Gyorke et al. 2004; Terentyev et al. 2008). However, these experiments were performed under different conditions, using purified RyR2 channels which were inserted into the bilayer and then the triadin/junctin/RyR complex reconstituted by adding recombinant triadin and junctin to the luminal bilayer solution before CSQ2 addition. Overall, our preliminary data and that of (Qin et al. 2008) indicate that the structure and function of CSQ1 and CSQ2 are indeed different. CSQ1 acts to reduce the gain of RyR1 channel activity, thereby curtailing Ca^{2+} release during a single action potential, while CSQ2 increases the gain of RyR2 channels to ensure maximal Ca^{2+} release in response to a single action potential (Wei et al. 2009b).

The different responses of native RyR1 to CSQ1 and native RyR2 to CSQ2 are likely to be related in part to the different Ca^{2+}-dependence of the structures of the CSQ isoforms. In the presence of 1 mM Ca^{2+}, CSQ1 is a polymer, while CSQ2 is a monomer (Wei et al. 2009b). This difference is apparent in cross-linking experiments and also in the amounts of CSQ bound to the junctional face membrane

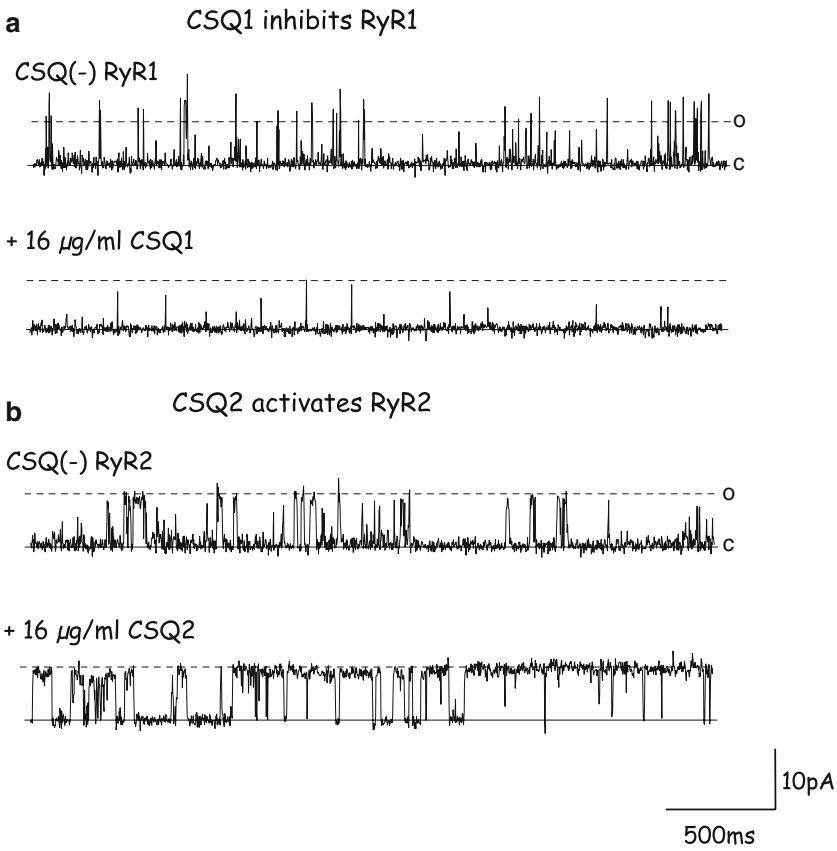

Fig. 3 CSQ1 inhibits RyR1, while CSQ2 activates RyR2 in the presence of 1 mM luminal Ca^{2+}. **a** Record of 3 s of single skeletal RyR1 channel activity before (control) and after addition of CSQ1. Channel opening is upward from zero current (*c*) to maximum open conductance (*o*) at +40 mV. **b** Identical to **a** except that data are recorded from RyR2 before and after addition of CSQ2. Reproduced from (Wei et al. 2009b)

(JFM) (~30% of CSQ2 is bound to cardiac JFM compared with ~70% of CSQ1) and in the ratio of CSQ2 to RyR2 which is only 50% of the CSQ1 to RyR1 ratio (Wei et al. 2009b). Our studies with the skeletal isoforms of the proteins suggest that CSQ1 *polymers inhibit* RyR1, while CSQ1 *monomers activate* RyR1 (Sect. 2, Wei et al. 2006). If this also applies to the CSQ2 interactions with RyR2, we would predict the CSQ2 monomer which exists in the presence of 1 mM Ca^{2+} might activate RyR2, and that CSQ1 might inhibit RyR2. However, the situation is more complex because CSQ1 also activates native RyR2 channels under the same conditions (Wei et al. 2009b). Thus, the response also clearly depends on other differences between CSQ1 and CSQ2 as well as differences between triadin/junctin/RyR2 and the triadin/junctin/RyR1 complexes. These isoform specific differences remain to be investigated in greater detail.

Disruption to Ca^{2+} signaling caused by CSQ mutations or knockout

An alternative approach to elucidating the roles of the luminal Ca^{2+} transduction proteins is to study the effects of mutating or deleting the proteins on whole cell muscle fiber or myotube function. In contrast to molecular interactions which have been studied most extensively in isolated systems, the majority of this transgenic work has focused on cardiac myocytes. This is likely to be a consequence of the discovery that CPVT and sudden cardiac death result from mutations in CSQ2 or failure of CSQ2 expression. In many cases the results of mutations or deletions are not easily reconcilable with results obtained with the isolated proteins. This is hardly surprising since on the one hand isolated proteins cannot be studied in an environment that fully replicates the conditions within the intact cells, while on the other hand mutation or deletion of one protein in a cell can impact on the expression of other proteins and on factors such as store load and the geometry of the SR (discussed below and in "Exploring the role of triadin and junctin in intact cells"). Thus results from each type of study must be interpreted in the context of these constraints and of results from other experimental approaches.

Contraction is preserved in the absence of CSQ1 in mice through major adaptive changes in muscle structure and protein composition (Paolini et al. 2007). The kinetics of the contraction, however, is not normal, with significant changes observed in fast twitch extensor digitorum longus muscle fibers. These changes include a prolonged rise and decay in tension, a decline in the amount of Ca^{2+} released from the SR and decline in the amplitude of the Ca^{2+} transient. Ultrastructural changes in the SR include a massive

proliferation of SR junctional domains although the volume of the individual terminal cisternae is in fact decreased. The numbers of Ca^{2+}-release channels increased and there was enhanced [^{3}H]ryanodine binding. The density of mitochondria also increased. The authors concluded that CSQ1 is essential for the normal development of the SR and its calcium release domains and for the storage and release of appropriate amounts of Ca^{2+} from the SR. The reduced amplitude of the Ca^{2+} transient could be explained by reduced EC coupling (if CSQ1 activated RyR1 rather than the inhibition seen in single channel studies, or if CSQ2 remained and activated RyR1 as outlined above), but could also be explained by reduced Ca^{2+} loading in the SR. These results (Paolini et al. 2007) do indicate a profound effect of CSQ1 on calcium handling in mature muscle fibres, in marked contrast to results obtained in the C2C12 myotubes which indicate that CSQ1 is essential for effective stored Ca^{2+} release (Wang et al. 2006). In the same study, rabbit CSQ1 was added to the luminal side of RyR channels isolated from the C2C12 myotubes inserted into artificial bilayers and it enhanced the activity of RyR channels (Wang et al. 2006). The apparent difference between the results obtained with C2C12 myotubes and those with adult skeletal muscle RyR1 (Beard et al. 2002; Wei et al. 2006; 2009a, b) might be explained by the RyR isoform expression, given the possible RyR and CSQ isoform dependence of CSQ action (Wei et al. 2009b) (see "The role of CSQ2 in the heart may be quite different from that of CSQ1 in skeletal muscle"). C2C12 cells express both the RyR1 and RyR3 isoforms, and likely also express the juvenile isoform of RyR1 whose properties differ in several respects from those adult isoform (Kimura et al. 2005, 2007, 2009). There have been no molecular studies of interactions between CSQ and either RyR3 or the juvenile isoform of RyR1 and CSQ1 might well activate these channels in the same way as it activates RyR2.

Single residue mutations in CSQ2 or lack of CSQ2 expression lead to either the CPVT phenotype or an altered capacity for luminal Ca^{2+} to regulate RyR2 in mice (Dirksen et al. 2007; Houle et al. 2004; Knollmann et al. 2006; Qin et al. 2008; Terentyev et al. 2008). As with CSQ1, the knockout and mutation studies show that CSQ2 is essential for the normal structure and function of cardiac myocytes. Contraction is again preserved in the absence of CSQ2 through major adaptive changes in structure and protein composition (Knollmann et al. 2006). CSQ2 knockout (Knollmann et al. 2006) and the CPVT mutations, D307H (Dirksen et al. 2007) and R33Q (Terentyev et al. 2008), result in an increased diastolic Ca^{2+} leak, spontaneous Ca^{2+} release and Ca^{2+} oscillations under basal conditions and with catecholamine stimulation (which presumably underlies CPVT). At the same time, there is a reduction in the amplitude of caffeine-induced or I_{Ca}-induced Ca^{2+} transients with the D307H mutation (Dirksen et al. 2007; Viatchenko-Karpinski et al. 2004) and contraction with CSQ2 knockout (see records in Fig. 4 of Knollmann et al. 2006). These changes could be caused by any, or a combination, of changes in several factors including CSQ2, SR volume (Knollmann et al. 2006), expression of RyR2 and calreticulin (which increase in CSQ2 knockout and the D307H mutation Song et al. 2007), triadin and/or junctin expression (which is reduced with CSQ2 knockout and the R33Q mutation Knollmann et al. 2006; Rizzi et al. 2008) or binding of mutant CSQ2 to triadin and junctin (which is reduced in the D307H or truncation mutations Houle et al. 2004; Terentyev et al. 2008). Finally, the Ca^{2+} binding capacity of CSQ2 and its ability to dimerize or polymerize with low ionic strength or high Ca^{2+} is disrupted to a greater or lesser

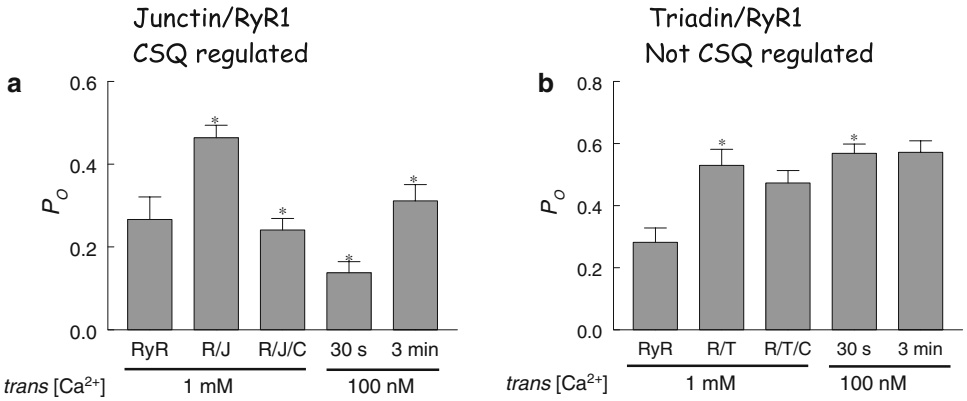

Fig. 4 Junctin and triadin each activate purified RyR1, but junctin alone dictates the normal response to CSQ1 and to a fall in luminal Ca^{2+}. **a** the CSQ1/junctin/RyR1 complex. **b** the CSQ1/triadin/RyR1 complex. **a**, **b** show average data for open probability. The first bin shows control data for purified RyR1. The second bin shows data after addition of either 5 μg/ml junctin (R/J in **a**) or 5 μg/ml triadin (R/T in **b**). The third bin shows data obtained after adding 16 μg/ml CSQ1 (R/J/C in **a**, or R/T/C in **b**). Bins 4 and 5 show data after lowering *trans* Ca^{2+} to 100 nM. The fourth bin, is average data obtained within 30 s of lowering Ca^{2+} and the fifth bin, data obtained >3 min after lowering Ca^{2+}. The *asterisks* indicate a significant difference from the previous condition. Reproduced from (Wei et al. 2009a)

extent in the D307H, R33Q and L167H mutations (Houle et al. 2004; Kim et al. 2007; Valle et al. 2008). Since Ca^{2+} regulation is a complex function of the amount of Ca^{2+} in the SR, the open probability of RyR2 channels, the amounts of SR and number of RyR channels, these whole cell studies are valuable in assessing the multitude of systems that CSQ impacts on, but do not reveal the molecular nature of interactions between CSQ2 and RyR2.

Several of the results obtained with the CSQ2 knock out and CPVT mutations in CSQ2 are consistent with the observed activation of RyR2 channels by CSQ2 (Qin et al. 2008; Wei et al. 2009b). These include a depression of caffeine-activated Ca^{2+} release and contraction (Dirksen et al. 2007; Houle et al. 2004; Knollmann et al. 2006) although this depression could also be explained by reduced amounts of Ca^{2+} in the SR (Terentyev et al. 2008). Also consistent with the concept that wild-type CSQ2 activates RyR2, is the increased amplitude of spontaneous Ca^{2+} release events that occurs with CSQ2 overexpression (Terentyev et al. 2008; Viatchenko-Karpinski et al. 2004) and the decreased amplitude with the deletion mutant and D307H that prevents CSQ2 binding to triadin and regulating RyR2 (Terentyev et al. 2008; Viatchenko-Karpinski et al. 2004).

It is clear that CSQ plays a major role in Ca^{2+} homeostasis in skeletal and cardiac muscle fibers and in the regulation of RyR channels in both tissues. Although we have come a long way in defining molecular mechanisms of this regulation, there are clearly many questions that remain to be answered.

Triadin and junctin

Triadin and junctin are thought to be closely involved in communications between CSQ1 and RyR1 channels because the normal inhibitory interaction between CSQ1 and RyR1 ("Regulation of RyR1 channels by CSQ1: the role of CSQ1 as a Ca^{2+} sensor for RyR1", "The role of CSQ2 in the heart may be quite different from that of CSQ1 in skeletal muscle") is seen only with native RyR1 channels that are isolated from skeletal muscle with triadin, junctin and other associated proteins intact (Beard et al. 2002) or in purified RyR1 channels that have been reconstituted with junctin (Wei et al. 2009a). In marked contrast to its inhibitory action on native RyR1 channels, CSQ1 enhances the activity of purified RyR1 channels (lacking associated proteins), presumably by binding directly to the channel protein (Beard et al. 2002; Szegedi et al. 1999). This indicates that the native inhibitory interaction requires the presence of additional proteins that are associated with the native channel. The most likely candidates are the membrane-spanning triadin and junctin which each bind to both CSQ1 and RyR1 (Glover et al. 2001; Wang et al. 1998; Zhang et al. 1997).

Both triadin and junctin contain a short cytoplasmic N-terminal tail, a hydrophobic helix that spans the SR membrane and a longer C-terminal tail located within the lumen of the SR. There are several isoforms of triadin which are splice variants of the one triadin gene. The 95 kDa triadin isoform is associated with RyR1 in skeletal muscle (Vassilopoulos et al. 2005), while the 32 kDa isoform is associated with RyR2 in the heart (Guo et al. 1996). Junctin is a non-catalytic splice variant of the aspartate-β-hydroxylase gene which is expressed in a wide range of tissues including skeletal muscle and the heart. Because of the structural similarity between the triadin and junctin and the ability of both to interact with the RyR and with CSQ, their functions have previously been thought to be redundant and they have been lumped together and designated "anchoring proteins". However, our recent evidence indicates that triadin and junctin have independent and functionally significant actions in regulating RyR1 activity. EC coupling and Ca^{2+} release are modified when triadin binding is abolished although junctin binding remains intact (Goonasekera et al. 2007). The binding sites for triadin and junctin on the luminal loops of RyR1 also appear to differ. Triadin binds to the luminal residues D4907, E4908, and D4878 of RyR1 and its binding is abolished by mutation of these residues; on the other hand, RyR1 binding to junctin is unaffected by the mutations (Goonasekera et al. 2007). Cardiac triadin (32 kDa isoform) and junctin activate RyR2 channels in lipid bilayers when added to the luminal solution (Gyorke et al. 2004). We have shown that skeletal triadin (95 kDa isoform) enhances [^{3}H]ryanodine binding to recombinant RyR1 channels (Goonasekera et al. 2007) and we have since found that highly purified skeletal triadin or junctin, added independently to the luminal solution bathing purified RyR1 channels, each increases the open probability of the channel (Wei et al. 2009a) (Fig. 4). A completely unexpected finding was that, when CSQ1 was added to the reconstituted triadin/RyR1 or junctin/RyR1 complexes, only the junctin/RyR1 complex (not the triadin/RyR complex) responded with a decline in activity and that this low activity was maintained briefly when the luminal Ca^{2+} concentration was lowered to 100 nM (Fig. 4, Wei et al. 2009a). This exciting observation showed that junctin alone is responsible for normal CSQ1 regulation of RyR1 and confirmed the hypothesis that triadin and junctin have independent roles on regulation of RyR1. The binding site on RyR1 for junctin and the binding sites on RyR2 for both triadin and junctin remain to be identified. In addition, it is not known whether triadin and junctin are the only proteins that are required for the functional interactions between CSQ1 and RyR1 or whether other associated proteins may also contribute.

The conclusions from our experiments have been supported by the effects of knockdown of triadin and/or junctin in skeletal myotubes. Triadin knockdown compromises depolarization-dependent Ca^{2+} release, again suggesting a role for triadin in EC coupling, while knockdown of junctin suggests a separate role for this protein in maintaining Ca^{2+} stores (Wang et al. 2008). Similarly, triadin knockdown reduces the amplitude of depolarization-dependent Ca^{2+} transients (Shen et al. 2007). Overexpression studies in the heart also suggest separate roles for the two proteins. Triadin overexpression stimulates cardiac EC coupling and arrhythmias in mice (Kirchhof et al. 2007; Terentyev et al. 2005). In contrast, junctin overexpression or its down-regulation in cardiomyocytes indicates that it suppresses RyR2 activity (Kirchhefer et al. 2003; Yuan et al. 2007).

A role of triadin in EC coupling begs the question of how a protein that binds to the luminal domain of the RyR can influence a process that depends interactions with the extreme cytoplasmic surface of the protein (see Fig. 1). One not unreasonable answer is that there are long-range interactions within the RyR which allow cross-talk between luminal and extreme cytoplasmic residues (Goonasekera et al. 2007). However, another possibility is that triadin can also interact with the cytoplasmic domain of the RyR1, with RyR1 residues that are spatially closer to the DHPR binding site. In this context it is interesting to note that triadin was originally thought to be located in the cytoplasm and to bind to both the DHPR and RyR1 and thus to be intimately involved in EC coupling (Brandt et al. 1992; Caswell et al. 1991; Fan et al. 1995). Although it was later shown that the bulk of triadin is located in the lumen of the SR, not in the cytoplasm (Guo et al. 1996; Knudson et al. 1993a, b), there are two reports indicating that the cytoplasmic N-terminal tail of triadin can bind to the cytoplasmic side of the RyR and in so-doing, inhibit the channel (Groh et al. 1999; Ohkura et al. 1998). The possible role of this cytoplasmic interaction in skeletal EC coupling remains to be established as does the binding site on the cytoplasmic domain of RyR1. It is worth noting that triadin binding to the cytoplasmic binding site on the RyR is likely to be weak, since no binding is observed when the luminal binding site is disrupted (Goonasekera et al. 2007).

Exploring the role of triadin and junctin in intact cells

As mentioned previously ("Triadin and junctin"), experiments with isolated RyR, triadin and junctin show that the two anchoring proteins activate RyR1 and RyR2 channels in lipid bilayers and may thus activate the channels in the heart and in skeletal muscle (Goonasekera et al. 2007; Gyorke et al. 2004). Some whole cell studies support this conclusion. In one such study, acute triadin over-expression resulted in enhanced RyR2 channel activity, suggesting that triadin activates RyR2 channels (Terentyev et al. 2005). Similarly, both acute skeletal triadin and junctin knockdown in myocytes, as well as in muscle fibers from triadin knockout mice, demonstrate reduced depolarization induced Ca^{2+} release which is consistent with a stimulatory action of the proteins on the RyR and Ca^{2+} release (Shen et al. 2007; Wang et al. 2008). However, other junctin and triadin over-expression data in cardiac myocytes suggest that both proteins inhibit cardiac RyR2 channels (Kirchhefer et al. 2001, 2006). Similarly, triadin over-expression has been reported to inhibit RyR1 channels (Rezgui et al. 2005). The inhibition by triadin over-expression could be explained either by a direct inhibitory action of the proteins on the RyR, but could equally well be explained if the over-expressed proteins self-aggregated and thus removed an excitatory influence. Self aggregation is a characteristic property of triadin (Knudson et al. 1993a). Changes in expression of other proteins (Hong et al. 2002; Kirchhefer et al. 2003) as well as in the ultrastructure of cardiac t-tubule/SR junctin (Franzini-Armstrong et al. 2005) with changes in triadin or junctin expression must also impact on Ca^{2+} release. An inhibitory effect of junctin on cardiac RyR2 channels is also indicated by enhanced Ca^{2+} release in cardiac myocytes from junctin knock-down mice (Yuan et al. 2007) and with acute down-regulation (Fan et al. 2007), although both knockdown and overexpression can also induce changes in expression levels of other proteins (Wang et al. 2008). In addition, it is worth noting that changes in expression of triadin and junctin might impact on the interactions between CSQ and the RyR, so that functional changes may be due to alterations in CSQ regulation, rather than the effects of triadin or junctin per se.

Conclusions

For almost half a decade, CSQ was thought of simply as a passive Ca^{2+} binding protein that was anchored to the RyR by triadin and junctin to provide a pool of Ca^{2+} ions that are readily available for release through the RyR channel. When the geometry of the SR is considered, this becomes a rather weak argument, since the terminal cisternae extend longitudinally only ~100 nm from the junctional face so that it is ~15 nm by 100 nm in cross section. The terminal cisternae can extend for greater distances in its transverse dimension, but the junctions extend in the same dimension. Since CSQ is targeted to the terminal cisternae and thought not extend into the longitudinal SR, most CSQ would be located within 100 nm of the RyR channels even when not tethered to the RyR, compared to some located within ~20–50 nm when anchored. Given that the time required for an ion to diffuse a distance of 1 μm is ~1 ms, and that

diffusion time changes with the square of the distance, the time required for Ca^{2+} to diffuse 100 nm would be of the order of 10 μs. The difference between a diffusion distance of 100 nm and 30 nm may reduce to time to ~1 μs. However, these times are insignificant given that Ca^{2+} release proceeds over a period of several milliseconds in skeletal muscle fibers (see, e.g. Launikonis et al. 2006) and for longer periods in the heart. These considerations have long suggested that that there must be a more compelling reason for CSQ being anchored to the RyR, but this reason has remained elusive until the recent studies outlined above.

We now have a picture of CSQ, triadin and junctin as playing an active role in relaying information on the Ca^{2+} load in the SR to the RyR, so that resting and voltage-activated Ca^{2+} release can be modified in response to changes in store load. Transmission of this information requires a physical coupling between the proteins. Therefore, the purpose of anchoring CSQ to the RyR is to facilitate this communication between CSQ and the RyR. Although many of the details about this system remain to be deciphered, it is clear that the proper function of this luminal Ca^{2+} transduction machine is vitally important for normal skeletal and cardiac muscle function.

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