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Junctin and triadin each activate skeletal ryanodine receptors but junctin alone mediates functional interactions with calsequestrin

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Summary

Normal Ca^{2+} signalling in skeletal muscle depends on the membrane associated proteins triadin and junctin and their ability to mediate functional interactions between the Ca^{2+} binding protein calsequestrin and the type 1 ryanodine receptor in the lumen of the sarcoplasmic reticulum. This important mechanism conserves intracellular Ca^{2+} stores, but is poorly understood. Triadin and junctin share similar structures and are lumped together in models of interactions between skeletal muscle calsequestrin and ryanodine receptors, however their individual roles have not been examined at a molecular level. We show here that purified skeletal ryanodine receptors are similarly activated by purified triadin or purified junctin added to their luminal side, although a lack of competition indicated that the proteins act at independent sites. Surprisingly, triadin and junctin differed markedly in their ability to transmit information between skeletal calsequestrin and ryanodine receptors. Purified calsequestrin inhibited junctin/triadin-associated, or junctin-associated, ryanodine receptors and the calsequestrin re-associated channel complexes were further inhibited when luminal Ca^{2+} fell from 1mM to $\leq 100\mu\text{M}$, as seen with native channels (containing endogenous calsequestrin/triadin/junctin). In contrast, skeletal calsequestrin had no effect on the triadin/ryanodine receptor complex and the channel activity of this complex increased when luminal Ca^{2+} fell, as seen with purified channels prior to triadin/calsequestrin re-association. Therefore in this cell free system, junctin alone mediates signals between luminal Ca^{2+} , skeletal calsequestrin and skeletal ryanodine receptors and may curtail resting Ca^{2+} leak from the sarcoplasmic reticulum. We suggest that triadin serves a different function which may dominate during excitation-contraction coupling.

Keywords

triadin; junctin; calsequestrin; sarcoplasmic reticulum; calcium stores; calsequestrin regulation of RyR1 channel activity

INTRODUCTION

Contraction in skeletal muscle depends on the release of Ca^{2+} from the intracellular store in the sarcoplasmic reticulum (SR), through ryanodine receptor type 1 (RyR1) calcium release

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channels. The efficacy of this Ca^{2+} release depends on the activity of the RyR1 channels and on the amount of Ca^{2+} stored within the SR. These factors are interdependent since the activity of RyR1 is regulated by the luminal Ca^{2+} binding protein, calsequestrin (CSQ1 or type 1 CSQ), in a manner that depends on the Ca^{2+} load within the store (Beard et al., 2002, Beard et al., 2005, Wei et al., 2006). CSQ1 inhibits RyR1 and prevents an increase in native RyR1 channel activity when luminal Ca^{2+} falls, but only when associated proteins remain with the channel complex (Beard et al., 2002, Wei et al., 2006). Purified RyR1 channels are activated by CSQ1 (Szegedi et al., 1999, Beard et al., 2002). Thus the normal interaction between CSQ1 and RyR1 is assumed to depend on proteins that are associated with the native channel. The most likely candidates for such proteins are the membrane embedded triadin and junctin which bind to both CSQ1 and RyR1 (Zhang et al., 1997, Wang et al., 1998, Glover et al., 2001).

Both triadin and junctin span the SR membrane and contain a short cytoplasmic N-terminal region and a longer C-terminus located within the SR. There are several isoforms of triadin and the 95kDa isoform associates with RyR1 in skeletal muscle (Vassilopoulos et al., 2005). Junctin is a non-catalytic splice variant of the aspartate- β -hydroxylase gene and is expressed in a range of tissues including skeletal muscle. There is indirect evidence that triadin and junctin may regulate different aspects of RyR1 activity. EC coupling and Ca^{2+} release are modified when triadin binding is abolished and junctin binding retained (Goonasekera et al., 2007). Knock down of triadin in skeletal myotubes also suggests a role for triadin in depolarisation induced Ca^{2+} release, while knockdown of junctin suggests that it has a separate role (Wang et al., 2008). Triadin overexpression stimulates cardiac EC coupling and arrhythmias in transgenic mice in some studies (Kirchhof et al., 2007, Terentyev et al., 2005). In contrast, junctin overexpression or its down-regulation in myocytes indicates that junctin suppresses RyR2 activity (Kirchhefer et al., 2003, Yuan et al., 2007).

Thus our hypothesis was that molecular interactions between RyR1, CSQ1 and triadin may differ from those between RyR1, CSQ1 and junctin. Our aim was to examine the actions of the individual proteins a) on the luminal domain of the RyR1 channel and b) in transmitting signal between CSQ1 and RyR1. These molecular interactions have not been previously examined. To achieve the aims, novel methods were developed to purify triadin and junctin from rabbit skeletal muscle and to reassemble the proteins with purified RyR1 and CSQ1 in artificial lipid bilayers.

METHODS

SR vesicle and junctional face membrane (JFM) preparation

Back and leg muscle from New Zealand White Rabbits was prepared as described in (Saito et al., 1984, Beard et al., 2002, Wei et al., 2006). JFM was prepared as described by (Costello et al., 1986, Beard et al., 2005, Wei et al., 2006) from B3 vesicles and stored at -20°C .

RyR1 purification was performed as described by (Lai et al., 1988). The purified RyR was concentrated, snap-frozen and stored at -70°C . The protein was run on SDS gels, and immunoblots probed with anti-CSQ, anti-triadin and anti-junctin antibodies to detect contamination by these proteins.

CSQ1 purification

CSQ1 was separated from JFM using native preparative gel electrophoresis and the Ornstein-Davis buffer system (Ornstein, 1964, Davis, 1964, Wei, 2008). Briefly, solubilized JFM was loaded onto a 7% cylindrical polyacrylamide gel and 2ml fractions containing CSQ were identified with mini SDS-PAGE and immunoblot. CSQ1 was concentrated, washed in a washing solution containing 20mM MOPS, 150mM NaCl and 200 μM EGTA, pH 7.4 and

stored at -20°C. SDS-PAGE and immunoblot are described in (Laemmli, 1970, Towbin et al., 1992).

Triadin and junctin purification

Triadin and junctin were isolated by SDS preparative gel electrophoresis (Wei, 2008), using a Laemmli denaturing buffer system (Laemmli, 1970). Briefly, to isolate triadin, solubilized JFM (as above) was loaded onto a 5% polyacrylamide gel, and fractions collected as for CSQ. To isolate junctin, we collected and concentrated the first 6 fractions from the triadin isolation and separated them using a secondary 12% preparative gel. Fractions containing triadin and junctin were identified as for CSQ (Fig. 1). Triadin and junctin were both co-precipitated from fractions by 400mM KCl (Carraro et al., 1991) and redissolved in washing solution (above) with 0.2% Triton X-100 to elute purified protein from SDS-KCl precipitates and induce protein refolding (by detergent exchange, replacing the denaturing buffer with a non-denaturing buffer to induce protein refolding). SDS-KCl precipitates were subsequently removed by centrifugation and protein supernatant diluted and washed at least 3 times in washing buffer (to removed triton X-100). Numerous proteins have been purified under denaturing conditions, refolded and shown to be functionally active (Gao et al., 2007, Goulhen et al., 2004, Song et al., 1998, Vohra et al., 2007, Mulvey et al., 2003).

Co-immunoprecipitation *In vitro* binding of anti CSQ/CSQ to purified triadin and junctin was performed using co-immunoprecipitation (Beard et al., 2008) using 50µg of CSQ isolated from muscle, and 25µg of junctin or triadin in the assay. Immunoprecipitated samples were eluted by boiling for 5mins in a buffer containing 62.5mM Tris-HCl pH 6.8, 10% (w/v) glycerol, 2.5% SDS, 5% mercaptoethanol and 0.02% bromophenol blue.

Single channel recording and analysis

Artificial planar bilayers separating two baths (*cis* and *trans*) were formed and purified RyR incorporated as previously described (Beard et al., 2002). Purified RyR1 (~10µg) was added to the *cis* solution. In general channels incorporated with their cytoplasmic surface of the SR and RyRs faced the *cis* solution and this was confirmed by characteristic increase in channel activity with ATP addition to the *cis* chamber or the characteristic small increase in activity when *cis* Ca²⁺ was lowered from an inhibiting concentration of 1 or 5 mM to a marginally activating concentration for RyR1 of 100nM immediately after incorporation. For channel incorporation, the solutions were: *cis*: 230mM CsMS, 20mM CsCl, 1 or 5mM CaCl₂, and 10mM TES (pH 7.4); and *trans*: 30/230mM CsMS, 20mM CsCl, 1mM CaCl₂, and 10mM TES (pH 7.4). After channel incorporation, *trans* [Cs⁺] was raised to 250mM with addition of 200mM CsMS. The *cis* solution was altered by the addition of 4.5mM BAPTA (free [Ca²⁺] = 100nM) and 2mM ATP. *Trans* [Ca²⁺] was maintained at 1mM under all conditions unless otherwise stated. Channels were identified as RyRs by their characteristic conductance and block by 20µM ruthenium red at the end of the experiment. Voltage is expressed as cytoplasm relative to lumen. Channel currents were filtered at 1kHz and sampled at 5KHz (Beard et al., 2008). Voltage was changed between +40mV and -40mV every 30s. Measurements were carried out at 23±2°C.

Single channel parameters were obtained using the Channel 2 program (developed by P.W. Gage and M. Smith, JCSMR, Canberra, Australia) as described previously (Beard et al., 2002, Beard et al., 2008, Wei et al., 2006). Channel activity was assessed from 60s to 180s of recording, 30s to 90s at -40mV and 30s to 90s at +40mV under each condition. Since all effects were independent of voltage, data from the two potentials was pooled and included in all averages. The average data shown in Fig. 6 were obtained from 120 to 180s of recording and is very similar to that in Figs 4 and 5 which was obtained from 60s of recording, indicating that the results in Figs. 4 and 5 were not biased by the shorter analysis period. In most

experiments, second and third channels opened in a bilayer after addition of triadin and junctin, when only one channel was opening before addition of the proteins. Therefore open probability was assessed from fractional mean current (I'_F), which is the average of all data points obtained during a recording period, divided by the maximum channel current (Beard, 2002 #9). I'_F is approximately equal to P_o measured using a threshold discrimination (Beard et al., 2008). In addition, brief segments of recordings in which only one channel opened, as shown in Figs 4 and 5, were analysed to obtain an estimate of open and closed times (Pouliquin et al., 2006). At least 50 openings or closings were measured under each condition in each bilayer. Running histograms of I'_F were obtained for two experiments (shown in Fig. 5), in which I'_F was measured over sequential 2s periods through the entire experiment. These histograms show the variability of I'_F as a function of time throughout the experiment.

Statistics

Average data are presented as mean $\pm$ SEM. The significance of differences were tested using a Student's *t*-test for paired or unpaired data, or the non-parametric Sign test, as appropriate. A P value of <0.05 was considered significant.

Materials

Monoclonal VD111D1₂ (anti-CSQ) was from Bio Scientific (Gynea, Australia). Polyclonal anti-junctin was a generous gift from Dr Steven Cala. Monoclonal anti-triadin antibody was purchased from Sigma (Castle Hill, Australia). All other chemicals were from Sigma-Aldrich.

RESULTS

Triadin and junctin purification

Triadin and junctin were purified using SDS preparative gel electrophoresis (Methods). The large difference between the molecular masses of the two proteins meant that they could be separated using consecutive electrophoresis steps with 5% and then 12% gels. Triadin was present at ~100kDa in fractions 28-37 of the 5% gel (Fig. 1A) and was precipitated using 400mM KCl and renatured through detergent exchange (Methods). Triadin resolved at its expected molecular mass (i.e. it was not degraded) and there was no detectable RyR, CSQ, or junctin in the triadin sample (Fig. 1B, lane 3&4). The purified triadin was functionally competent in binding CSQ (Fig. 1C). Fractions 1-6 from the 5% gel, containing proteins <70kDa (including junctin), were applied to a 12% preparative gel. Junctin was enriched in fractions 50-60 (at ~25kDa, Fig. 1D) and eluted and renatured as triadin (Fig. 1E). No RyR, CSQ, or triadin was detected in the junctin sample (Fig. 1F).

The effect of triadin and junctin on RyR1 activity

Purified RyR1 channels (Methods) were incorporated into lipid bilayers in the presence of 100nM *cis* Ca²⁺ and 1mM *trans* Ca²⁺. Both triadin and junctin increased RyR1 activity when added individually to the *trans* solution at a concentration of 5 μ g/ml (Fig. 2A,B&C). The concentrations of 5 μ g/ml were effective in regulating RyR1 and elicited a maximum response (see below). There was a similar increase in activity at +40mV and -40mV in each of the experiments in this section and below, so that measurements from both potentials are combined in all average data. I'_F (reflecting open probability) increased >2-fold from a control value of 0.10 $\pm$ 0.02 after junctin addition to the *trans* chamber (Fig. 2B). There was a similar 2-fold increase in the I'_F when triadin was added to the *trans* chamber (Fig. 2C). Therefore junctin and triadin are both potent luminal activators of RyR1. We found that the number of channels opening in the bilayer increased following addition of triadin or junctin and the channels tended to open more often to the full conductance level (as shown in Fig. 2A and Fig. 6A and C). It is likely that purified channels incorporated into bilayers as rafts of two or more channels as

others have observed (Yin et al., 2000, Yin et al., 2005, Dulhunty et al., 2005) and that, before adding triadin and/or junctin, opening of more than one channel is prevented (by a yet unidentified mechanism). Addition of these associated proteins removed this breaking factor and allowed the opening of more than one channel.

To determine whether the RyR1 response was saturated with 5 $\mu\text{g/ml}$ of triadin and whether addition of junctin could further influence RyR1 activity once the response to triadin was saturated, the concentration of triadin was increased to 10 $\mu\text{g/ml}$ and then a further 5 $\mu\text{g/ml}$ of junctin added. There was a ~ 1.9 -fold rise in I'_F with the first triadin addition (Fig. 2D). A further 5 $\mu\text{g/ml}$ of triadin (total *trans* [triadin]=10 $\mu\text{g/ml}$) did not further increase average activity, indicating that the triadin-sensitive sites on the channel were saturated. When 5 $\mu\text{g/ml}$ junctin was subsequently added to the *trans* solution there was however a further significant ~ 1.5 -fold increase in channel activity. The activation by junctin when the triadin effect was saturated, indicated that there were independent interaction sites for the two anchoring proteins. This conclusion was consistent with a previous observation that junctin binds to RyR1 when triadin binding is interrupted by mutation of the triadin binding residues on RyR1 (Goonasekera et al., 2007).

This interaction of our refolded triadin and junctin with CSQ here and in the following section was similar to that of the possibly less pure triadin and triadin obtained under non-denaturing conditions (Zhang et al., 1997, Shin et al., 2000, Gyorke et al., 2004). The similarity between the results indicates that the triadin and junctin used here were refolded to the extent that they were physiologically functional. In addition, we have shown that triadin and junctin isolated using essentially the same method as that used here, bind to CSQ1 in the presence of 100nM, 100 μM and 1mM Ca^{2+} (Beard et al., 2008).

Changes in RyR1 channel gating induced by triadin and junctin

By selecting brief sections of recording in which only one channel opened under each condition (Introduction and Fig. 4 below), we were able to obtain an indication of the effects of triadin and junctin on channel open and closed times (Pouliquin et al., 2006). It should be noted that these segments of recording were selected only for measurement of open and closed times and thus necessarily had a low I'_F that was not representative of the overall I'_F for that bilayer or for the average I'_F for the series of experiments. The results of this open and closed time analysis show that the increase in activity following addition of triadin or junctin was associated with a significant increase in mean channel open time (T_o) (Fig. 3A&B). The average 4.1 ± 0.8 -fold increase in T_o after triadin association was significantly greater than the average 2.3 ± 0.3 -fold increase after association of junctin with RyR1. The mean channel closed times were reduced by each of the proteins (Fig. 3C&D). The average ~ 2 -fold reduction with triadin (to 0.51 ± 0.05 of control) was significantly less than the ~ 5 -fold reduction with junctin (to 0.29 ± 0.05 of control). Thus association of triadin with RyR1 has a greater effect on T_o and a smaller effect on T_c than junctin association. These different effects of triadin and junctin on channel gating are consistent with the two proteins interacting with different sites on the luminal domain of RyR1.

Reassembly of the RyR1 luminal complex and restoration of CSQ1 regulation

Triadin, junctin and CSQ were sequentially added to the *trans* solution bathing purified RyRs at 1 mM Ca^{2+} to reassemble the luminal complex containing these four proteins. Addition of triadin and then junctin led to the expected stepwise increases in channel activity and increase in average fractional mean current from a control of 0.1 ± 0.02 to 0.18 ± 0.03 with addition of 5 $\mu\text{g/ml}$ triadin to the *trans* solution and then to 0.23 ± 0.04 after junctin addition (Fig. 4). RyR activity was then depressed when 16 $\mu\text{g/ml}$ CSQ was added (16 $\mu\text{g/ml}$ CSQ1 maximally inhibits RyR1 (Beard et al., 2002)). Average fractional mean current was reduced by ~ 2.5 -fold from

that prior to CSQ1 addition and was close to the initial control value. This inhibition by CSQ1 was not significantly different from the 2 to 3-fold reduction observed when CSQ1 is added to native RyR1 channels, which contain endogenous triadin and junctin, but have been stripped of endogenous CSQ1 (Beard et al., 2002, Beard et al., 2005). This result indicates that the two CSQ anchoring proteins are sufficient to transmit the inhibitory effect of CSQ1 to RyR1 channels and that other associated proteins in the native SR are not required for this action.

The increase in RyR1 channel activity with triadin and junctin was imposed by increases in mean open time (Fig. 4D) and decreases in mean closed time (Fig. 4E). Fractional mean current and mean open time were then reduced to near control levels when CSQ1 was added.

Luminal Ca^{2+} concentrations can fall during repetitive activity (Launikonis et al., 2006) to levels of $\sim 100\mu\text{M}$. The ability of CSQ1 to depress native RyR1 activity when the luminal Ca^{2+} is reduced from the usual resting value of 1mM was examined with the reassembled RyR1/triadin/junctin/CSQ1 complex. When the *trans* $[\text{Ca}^{2+}]$ is lowered to 100nM , the CSQ1 polymer, which forms when *trans* Ca^{2+} is 1mM (Park et al., 2004, Wei et al., 2006), remains associated with RyR1 for $\sim 3\text{min}$ before it depolymerizes and loses its ability to regulate the channel (Wei et al., 2006). Therefore channel activity was assessed (i) between 30s and 1min of decreasing *trans* $[\text{Ca}^{2+}]$, to determine the response of the RyR while CSQ was polymerized and regulating the RyR and (ii) after 3min to reveal the effect of CSQ depolymerization. In the initial period after lowering *trans* $[\text{Ca}^{2+}]$, average activity fell significantly (Fig. 4C), there was a trend towards lower mean open time (Fig. 4D) and a significant increase in mean closed time (Fig. 4E). The early drop in activity is seen when native channels are exposed to low *trans* Ca^{2+} and is in contrast to the activation seen when luminal $[\text{Ca}^{2+}]$ is lowered in the absence of CSQ (Wei et al., 2006). However after 3min exposure to low *trans* $[\text{Ca}^{2+}]$, there was a sudden increase in channel activity (Fig. 4C) which is seen in native RyRs and attributed to the depolymerization of CSQ1 and loss of its regulation of the RyR1 (Wei et al., 2006). Thus the responses of the reconstituted RyR1 complex to a fall in luminal Ca^{2+} are similar to the response of native RyRs, indicating that triadin and/or junctin are the only proteins required to transmit the stabilising luminal Ca^{2+} signal from CSQ1 to RyR1.

RyR1 regulation by the CSQ1/triadin or CSQ1/junctin complex

To answer the question of whether triadin or junctin or both proteins could relay signals from CSQ1 to the RyR1, CSQ1 was added to either junctin-regulated (junctin/RyR1) or triadin-regulated (triadin/RyR1) RyR1 channels in the presence of 1mM *trans* Ca^{2+} . Fig. 5 shows running histograms of I_F measured every 2s during the entire experiment, where either junctin was added then CSQ1 (Fig. 5A) or triadin added then CSQ1 (Fig. 2B). Data is presented in this way to show the variability in the steady state distribution of I_F under each condition and to show that even when there was some variability in I_F over time (as in the presence of CSQ in Fig. 5A), the values remained significantly lower than values under the preceding condition in the absence of CSQ1 and reflected the average changes in I_F for all channels in the experiment (Fig. 6). The control condition was that in the presence of 100nM *cis* Ca^{2+} before addition of triadin/or junctin. The very low activity in 1mM *cis* Ca^{2+} , and the increase with lowering *cis* Ca^{2+} to 100nM , is shown as it confirmed the orientation of the purified RyR1 channel in bilayer (see legend to Fig. 5). The changes in I_F with lowering luminal Ca^{2+} at the end of the experiment are discussed below with the experiments shown in Fig. 6.

Average data is given in Fig. 6 as well as representative short segments of channel activity. Junctin addition caused the usual increase in channel activity (Fig. 5A and Fig. 6A&E, middle trace) and increase in average fractional mean current (Fig. 5A and Fig. 6B). The subsequent addition of CSQ1 was followed after $\sim 2\text{min}$ by a fall in channel activity (Fig. 5A and Fig. 6A&E bottom trace), with a significant reduction in average fractional mean current (Fig. 5A and Fig. 6B). This was similar to the response of the reassembled triadin/junctin/RyR1 complex

to addition of CSQ1 (Fig. 4 above). Triadin also caused the usual ~ 2 fold increase in RyR1 activity (Fig. 5B and Fig. 6C&H middle trace) and average fractional mean current (Fig. 5B and Fig. 6D). Unexpectedly, CSQ1 added to these triadin-activated channels did not cause any significant change in activity (Fig. 6C&H bottom trace) or average fractional mean current (Fig. 6D). Therefore junctin alone, but not triadin, mediated CSQ1 inhibition of the RyR1.

Addition of junctin (Fig. 6F) or triadin (Fig. 6J) increased the mean open time of channels in this experiment, whilst the mean closed times fell significantly (Fig. 6G&I). Subsequently, addition of CSQ1 to the RyR1/junctin complex caused a significant decline in mean open time, although this parameter remained significantly longer (~ 1.5 fold) than the control open time before junctin addition. The mean closed time increased significantly. In contrast to the junctin-regulated RyR1, there was no significant change in either the mean open times or mean closed times when CSQ1 was added to triadin-regulated RyR1 channels (Fig. 6I&J).

In summary, junctin alone mediated the inhibition of native RyR1 by CSQ1. This observation suggests a hypothesis in which the inhibitory action of CSQ1 might be achieved by CSQ1 interrupting the junctin-induced activation of the RyR1.

Responses of the CSQ1/triadin/RyR1 and CSQ1/junctin/RyR1 complexes to a fall in luminal Ca^{2+} concentration

To further test the functional role of triadin and junctin, we examined the response of the individual CSQ1/triadin/RyR1 and CSQ1/junctin/RyR1 complexes to lowering luminal Ca^{2+} from 1mM to 100nM. Addition of either triadin or junctin to the *trans* solution produced the usual increases in fractional mean current of the purified RyR1 channels (Fig. 7A&B). Consistent with results in Fig. 5&6, addition of CSQ1 caused a significant reduction in the activity of the junctin/RyR1 complex (Fig. 7A), but change in the fractional mean current of the triadin/RyR1 complex (Fig. 7B). It is worth noting that there is a two-fold difference between the average control I_F (in the presence of 100nM *cis* Ca^{2+}) in the series of experiments shown in Figs. 5&6 and that shown in Fig. 7. A 2-fold difference between control values is commonly seen in RyR channel activity and this emphasises the importance of obtaining control and test measurements from each bilayer (Marengo et al., 1998).

When the *trans* Ca^{2+} concentration was then lowered from 1mM to 100nM, the CSQ1/junctin/RyR1 complex responded initially with a significant fall in activity, which was followed after >4min by a sudden significant increase (Fig. 7A). This response was the similar to that seen with the CSQ1/junctin/triadin/RyR1 (Fig. 4) and in native RyR1 channels (Wei et al., 2006). In marked contrast, the CSQ1/triadin/RyR1 complex responded to the reduction in *trans* Ca^{2+} concentration with a small and maintained increase in activity (Fig. 7B), typical of that seen in channels that are not regulated by CSQ1 (Wei et al., 2006). Similar changes in activity and response to lowering luminal Ca^{2+} were seen in other experiments in which luminal Ca^{2+} was lowered from 1mM to 100 μM after addition of luminal triadin or junctin and then CSQ1 in the presence of 1mM Ca^{2+} (Fig. 5 above). These results show clearly that the CSQ1-dependent response of RyR1 to a transient reduction in luminal Ca^{2+} concentration is mediated by junctin, not by triadin.

Discussion

This is the first report of functional interactions between the purified RyR1, triadin, junctin and CSQ1 and the first study from any tissue to examine these interactions at a physiological luminal Ca^{2+} concentration of 1mM. The novel results show that both triadin and junctin augment RyR1 channel activity and promote long channel openings. It was apparent that junctin is the only protein that supports the inhibition of RyR1 by CSQ1 and permits the CSQ1-

imposed suppression of the increase in channel activity that otherwise occurs during a transient reduction in luminal Ca^{2+} concentration in this isolated protein system.

Different functions of triadin and junctin in calcium signalling

The *in vitro* interaction between the native RyR1 and CSQ1 includes: 1) CSQ1 inhibition of RyR1 activity in the presence of 1mM luminal Ca^{2+} (Beard et al., 2002); 2) prevention of an increase in activity for 1-2min after a decline in luminal $[\text{Ca}^{2+}]$ to $\leq 100\mu\text{M}$ (Wei et al., 2006); and 3) a sudden increase in activity after 2-3min exposure to low luminal $[\text{Ca}^{2+}]$ due to CSQ depolymerization (Wei et al., 2006). Each of these interactions was restored when CSQ1 was reassociated with the triadin/junctin/RyR1 and with the junctin/RyR1 complex, indicating that CSQ1 regulates the RyR1 response to luminal Ca^{2+} through the CSQ1/junctin/RyR1 interaction alone. However we cannot exclude the possibility that CSQ1 also interacts with other luminal proteins *in vivo* and that the effects of these interactions may impinge on the effects mediated through the CSQ1/junctin/RyR1 complex. The triadin/RyR1 complex appeared insensitive to CSQ1 addition, although we have shown that CSQ1 binds to triadin under similar conditions (Beard et al., 2008). In addition, it is unlikely that the formation of triadin oligomers prevented CSQ1 binding to triadin in the bilayer situation as it has been clearly demonstrated that the presence of CSQ1 in fact prevents triadin oligomer formation (Ohkura et al., 1998, Groh et al., 1999).

The demonstration that junctin may be the major player in CSQ1 regulating RyR1 activity and store load provides a physical basis for conclusions from knockdown experiments in skeletal myotubes which indicated that junctin maintains the SR Ca^{2+} store size (Wang et al., 2008). Our results predict that RyR1 activity would increase when the inhibitory action of CSQ1 on the junctin/RyR1 complex was removed and that this would increase Ca^{2+} leak from the SR and decrease store size. Our results indicate that triadin is not involved in a simple signal transmission between CSQ1 and RyR1, although triadin may play a yet to be defined role in regulating the Ca^{2+} store, since triadin binding deficient RyR1 have altered caffeine-induced Ca^{2+} release (Goonasekera et al., 2007), while pan-triadin knockdown alters SR Ca^{2+} loading (Shen et al., 2007). Additionally prevention of triadin binding to RyR1 abolishes depolarisation-induced Ca^{2+} release in skeletal myotubes, even though junctin binding is preserved, and we speculated that triadin plays an essential role in skeletal EC coupling (Goonasekera et al., 2007). Both transient siRNA-mediated triadin knockdown and pan-triadin knockout are accompanied by a reduction in depolarisation-dependent Ca^{2+} release (Wang et al., 2008). This is consistent with the proposed role of triadin in EC coupling. However, triadin knockout mice appear to have normal muscle contractility, which could result from a multitude of different compensatory mechanisms (Shen et al., 2007) (see Discussion in (Goonasekera et al., 2007)). Overall, it is tempting to speculate that CSQ1 binding to triadin *in vivo* may allow CSQ1 to directly influence skeletal EC coupling.

The data in the present manuscript show that the degree of inhibition by CSQ1 was similar with the triadin/junctin/RyR1 and the junctin/RyR1 complexes, so we would predict that removing triadin would not remove this inhibition. Indeed there was no change in Ca^{2+} release with caffeine, 4-cmc or thapsigargin and no change in Ca^{2+} uptake after triadin knockdown (Wang et al., 2008), indicating that both Ca^{2+} leak and store load were normal. The conclusion that triadin and junctin have vastly different actions in Ca^{2+} homeostasis in skeletal muscle is supported by evidence that the two proteins in fact bind to different luminal sites on RyR1. This evidence is the additive effects of saturating concentrations of triadin and junctin (this study) and by the fact that disrupting the putative triadin binding sites on the skeletal RyR does not impair junctin binding (Goonasekera et al., 2007).

Triadin and junctin are activators of RyR1

Triadin and junctin each enhanced purified RyR1 activity when added to the *trans* solution. Curiously, a triadin over-expression model indicated that triadin inhibits skeletal RyR1 channels (Rezgui et al., 2005, Fodor et al., 2008). This inhibition could be explained if the over-expressed proteins self-aggregated, disrupting their binding to the RyR and removing their activating influences. The strong activation observed in the bilayer experiments suggests a model in which CSQ1 inhibits channel activity by removing or overriding the activating effect of junctin on RyR1. It is interesting to note that one KEKE motif in residues 200-232 of skeletal triadin is thought to be involved in binding to both CSQ1 and RyR1 (Kobayashi et al., 2000). More complex interactions are suggested with junctin because multiple regions appear to be involved in its binding to the RyR and CSQ (Kobayashi et al., 2000). Given the different binding sites for triadin and junctin on CSQ1 and RyR1, it is hardly surprising that triadin and junctin transmit different signals between CSQ1 and RyR1.

There is some evidence that the effects of triadin and junctin on cardiac RyR2 are the same as those on skeletal RyR1 channels. Cardiac triadin and junctin increase cardiac RyR2 activity (Gyorke et al., 2004) and acute triadin over-expression resulted in enhanced RyR2 channel activity in one study (Terentyev et al., 2005). However, other data suggests that both proteins inhibit cardiac RyR2 channels (Kirchhefer et al., 2006, Kirchhefer et al., 2001). Changes in expression of other proteins (Kirchhefer et al., 2003, Hong et al., 2002) as well as in the cardiac t-tubule/SR junction (Franzini-Armstrong et al., 2005) 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).

Triadin/junctin/CSQ interactions in skeletal muscle and the heart

In contrast to the possibly similar actions of triadin and junctin on isolated RyR activity in the heart and skeletal systems, there appear to be differences between the triadin/junctin/CSQ interactions in the two muscle types. Cardiac CSQ2 does not polymerise in the presence of physiological ionic strength and luminal $[\text{Ca}^{2+}]$ (while CSQ1 is polymerised (Wei et al., 2006, Qin et al., 2008, Park et al., 2004). Furthermore, native RyR2 channels (containing triadin and junctin) are more active in the presence CSQ2 than in its absence, when the luminal Ca^{2+} is 1mM (Wei, 2008, Qin et al., 2008) (as opposed to CSQ1 which inhibits RyR1 (Beard et al., 2002)). Curiously purified RyR2 channels are inhibited when CSQ2 is added after reconstitution with triadin or junctin (Gyorke et al., 2004). The reason for this difference is not clear. The functional effects of CSQ2 are not clearly revealed by knockout and mutation studies because Ca^{2+} regulation is also affected by changes in expression of other proteins, changes in Ca^{2+} stored in the SR and changes in the structure of the SR (Paolini et al., 2007, Knollmann et al., 2006).

The question of whether CSQ2 regulates RyR2 by binding to triadin or junctin is unresolved. Two studies conclude that CSQ2 binding to triadin is essential for CSQ2 regulation of RyR2 in the cardiac system (Terentyev et al., 2007, Qin et al., 2008), in apparent contrast to our finding in the skeletal system. However junctin may well have mediated the effects of CSQ2 in both these studies. Triadin and junctin were both present in the native RyR2 channels used in one study (Qin et al., 2008), while the peptide used to interrupt CSQ2 binding to triadin in the second study (Terentyev et al., 2008) may have interrupted CSQ2 binding to junctin, given the similarity of the CSQ binding sequences on the two proteins (Kobayashi et al., 2000). Thus the separate roles of triadin and junctin are yet to be established in the cardiac situation.

The ability of CSQ1 to inhibit RyR1 is an exclusive property of the skeletal isoforms of the two proteins since CSQ2 not only activates native (triadin/junctin associated) RyR2 but also native RyR1, while CSQ1 activates native RyR2 (Wei, 2008, Wei et al., 2009). The action of CSQ1 on the juvenile splice variants of RyR1 (Futatsugi et al., 1995) has not been investigated, nor has the association of triadin and junctin with the splice variants. Further evidence for the isoform specificity of CSQ1 inhibition of adult RyR1 may be provided by the observation that CSQ1 activated RyR1 and RyR3 channels in vesicles isolated from C2C12 myotubes (Wang et al., 2006).

Do triadin and junctin insert into a lipid bilayer?

It is worth noting that both triadin and junctin span the SR membrane with a short N-terminal cytoplasmic tail and a long highly acidic C-terminal tail located in the lumen of the SR. A question that arises is whether triadin and junctin added to the *trans* chamber insert into the bilayer in their normal orientation and whether this insertion is necessary for functional changes to reflect the *in vivo* interactions. It is highly likely that both proteins do insert into the bilayer, given the numerous isolated membrane proteins that reconstitute into bilayers. These include a plethora of ion channels, such as the RyR (this study and many others including (Gyorke et al., 2004)). In addition, many small peptides, with lengths similar to or less than triadin or junctin and containing membrane spanning sequences, insert into bilayers and are functionally active (Premkumar et al., 2005, Premkumar et al., 2004). Indeed, triadin and junctin have well-defined hydrophobic domains (Kobayashi et al., 1999, Jones et al., 1995), which would facilitate their incorporation into the bilayer. It is likely that triadin and junctin would insert with the correct orientation since their short electrically neutral N-terminal domains would pass through the bilayer (and face the cytoplasmic solution) in preference to the much longer acidic C-terminal tail. If triadin does insert correctly, the question remains of why we do not see a reflection of the reported cytoplasmic inhibition by the N-terminal domain of triadin (Ohkura et al., 1998, Groh et al., 1999).

Both triadin and a peptide corresponding to its N-terminal domain inhibit RyR1 when added to the **cytoplasmic** solution (Ohkura et al., 1998, Groh et al., 1999). Curiously, Ohkura et al. (Ohkura et al., 1998) did not see an effect of triadin added to the luminal solution, in contrast to the results reported here and those of (Gyorke et al., 2004). Questions that arise from these studies are the location and functional significance of the cytoplasmic binding site for triadin, considering the fact that the only triadin binding site identified to date is on the luminal domain of RyR1 (Goonasekera et al., 2007, Lee et al., 2004). Mutations in this binding site abolish triadin binding and EC coupling and alter caffeine-induced Ca^{2+} release (Goonasekera et al., 2007). It remains possible that a cytoplasmic binding site for the N-terminus of triadin is also important for RyR1 function and that binding to this site is stabilised by the binding to luminal domain of RyR1. However this remains to be tested.

Physiological implications

The results suggest a model in which a beneficial low calcium leak from the SR and low RyR1 activity at rest is maintained by the luminal interaction between CSQ1, junctin and RyR1. This interaction also insures that the transient reduction in luminal Ca^{2+} during strong activity (Launikonis et al., 2006) is not exacerbated by the increase in RyR1 activity which is seen when luminal Ca^{2+} is lowered in the absence of the CSQ1 polymer (Wei et al., 2006). Since the affinity of both triadin and junctin for CSQ increases as $[\text{Ca}^{2+}]$ falls (Zhang et al., 1997, Shin et al., 2000), it is possible that the initial reduction in channel activity after luminal $[\text{Ca}^{2+}]$ is lowered is due to a stronger interaction between junctin and CSQ, which produces a stronger inhibitory effect on the RyR.

The role of triadin and CSQ1 binding to RyR1 is a mystery yet to be solved. The observation that elimination of triadin binding to RyR1 abolished depolarisation-dependent Ca^{2+} release (Goonasekera et al., 2007) was surprising. The effect was attributed to a long-range interaction between the triadin binding site on the luminal domain of RyR1 and a cytoplasmic or pore site that regulates channel opening in response to the depolarisation evoked signal from the DHPR. Our results now suggest that regulation of EC coupling may be the primary role of triadin in skeletal muscle and raise the possibility that CSQ1 (and the amount of Ca^{2+} in the SR store) may influence EC coupling through this long range interaction. In this context, it is interesting to note that early experiments detected changes in CSQ1 following voltage activation, which preceded Ca^{2+} release from the SR (Ikemoto et al., 1991). It will be important in the future to explore the role of the luminal CSQ1/triadin/RyR1 complex in skeletal EC coupling.

In summary, the RyR, junctin, triadin and CSQ1 form a tightly interacting machine that *in vivo* ensures optimal EC coupling and SR Ca^{2+} release. The activation achieved by junctin binding to RyR1 is negated by the antagonistic effect of CSQ1 on junctin activation. Junctin and triadin have vastly different roles in skeletal muscle function. Junctin facilitates interactions between CSQ1 and RyR1 that are important in maintaining and conserving Ca^{2+} concentrations within the stores. Triadin is not associated with this function and appears to have an independent role in maintaining EC coupling.

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Figure 1. Purification of rabbit skeletal triadin and junctin using SDS preparative gel electrophoresis. Gels shown are 10% SDS polyacrylamide gels. (A) – (C) – triadin purification
 (A) A silver-stained gel of the parent JFM and selected fractions from the 5% preparative gel. Fractions 30-35 (asterisks) were enriched with triadin (~100kDa, broken line). (B) Purified triadin was analysed by SDS-PAGE silver staining (lane 1-2) and immuno-blot (lane 3-4), using the anti-triadin antibody. (C) The function of purified triadin and junctin was assessed from its ability to bind to CSQ using CSQ co-immunoprecipitation; the silver-stained gel (top) and immuno-blot (bottom; with anti-triadin on the left and anti-junctin on the right) shows purified triadin (left) and junctin (right) before (lanes 1&3) and after (lanes 2&4) co-immunoprecipitation by adding to A/G sepharose bound anti-CSQ/CSQ matrix. Co-immunoprecipitation was done in the presence of 1mM Ca^{2+} . The asterisk (*) shows the presence of protein A/G and the anti-CSQ antibody (which resolve around the same molecular weight of 45-50 kDa), which was eluted from the sepharose along with the precipitated proteins.

Figure 2. Addition of junctin and/or triadin to the *trans* chamber activates purified RyR1
(A) - records of 3s of purified RyR1 channel activity at -40mV with 1mM *trans* Ca²⁺ and 100nM *cis* Ca²⁺. Channel opening is downward from zero current (c, continuous line) to maximum open conductance (o, broken lines). The top recording shows control activity. The lower panel shows activity after addition of 5µg/ml junctin. **(B)** & **(C)** - average I'_F , data at +40mV and -40mV was combined since there was no voltage-dependent effect. Average data is shown for junctin (**B**, $n=22$), triadin (**C**, $n=40$). **(D)** Average relative I'_F ($n=18$) of purified RyR1 at the start of the experiment (i); after adding 5µg/ml triadin to the *trans* solution (ii); after adding a further 5µg/ml triadin (iii); then 5µg/ml junctin (iv). Averages are combined data from +40mV and -40mV recordings. Single asterisks (*) indicate average values significantly different from the control and double asterisk (**) denotes the data value significantly different from data obtained under the previous condition.

Figure 3. Triadin and junctin induce longer channel openings and briefer channel closures in purified RyR1
 (A) - (D), average data (combined data for +40mV and -40mV) for mean open time (T_o , A&B), or mean closed time (T_c C&D) before (RyR1) and after the addition of 5 μ g/ml triadin (A&C) and 5 μ g/ml junctin (B&D) to purified RyR1. Asterisks (*) indicate values that are significant different from the control ($n = 22-40$).

Figure 4. CSQ1 modification of RyR1 through the re-assembled CSQ1/triadin/junctin/RyR1 complex

(A & B), 3s of channel activity at +40mV with 1mM *trans* Ca^{2+} and 100nM *cis* Ca^{2+} . Channel opening is upward from zero current (continuous line, c) to maximum conductance for one channel (broken line, o or o_1) or for two channels opening simultaneously (o_2). Changes in RyR1 activity indicative of assembly of the RyR/triadin/junctin/CSQ complex are shown. The upper record shows control purified RyR1 activity, the 2nd record was obtained after adding 5µg/ml triadin, the 3rd record after 5µg/ml junctin and the final record after adding 16µg/ml CSQ. The records in (A) show representative data used to measure P_F , while segments of records like those in (B) were used to obtain mean open times and mean closed times. (C) – (E) Average data (30s at +40mV plus 30s at -40mV) for P_F (C), mean open time (T_o , D) and mean closed time (T_c , E). Bins 1 to 4 show average data with 1mM *trans* Ca^{2+} . The 1st bin shows control data (RyR). The 2nd bin shows data after addition of either 5µg/ml triadin (R/

T) or 5 μ g/ml junctin (R/J). The data was combined since either protein increased RyR activity by a similar amount. The 3rd bin is data with 5 μ g/ml triadin plus 5 μ g/ml junctin (R/J/T) and the 4th bin after adding 16 μ g/ml CSQ (R/T/J/C). Bins 5 and 6 show data after lowering *trans* Ca²⁺ to 100nM. The 5th bin, is average data obtained within 30s of lowering Ca²⁺ and the 6th bin, data obtained >3min after lowering Ca²⁺. A single asterisk (*) indicates a significant difference from control and a double asterisk (**) denotes a significant difference from the previous condition ($n = 6 - 18$).

Figure 5. Running I_F analysis of entire records of representative experiments in which purified RyR1 channels were incorporated into lipid bilayers, followed by addition of junctin (A) or triadin (B) to the *trans* (luminal) chamber and then addition of CSQ1

I_F was measured for sequential 2s periods throughout the experiment. Blank regions indicate periods of compound addition with stirring where noise prevented analysis. Grey bars show activity at -40 mV, black bars show activity at +40mV. Arrows above the histograms indicate the time of changes in Ca^{2+} concentration or addition of proteins as indicated. The purified channels were incorporated with 1mM *cis* Ca^{2+} and 1mM *trans* Ca^{2+} . *Cis* Ca^{2+} was lowered to 100nM which resulted in a small increase in activity in both channels in (A) and (B), due to relief of the high $[\text{Ca}^{2+}]$ inhibition. This increase in activity confirmed the orientation of the channel in the bilayer. After recording control activity for 3-5 mins, either junctin (A) or triadin (B) was added to the *trans* chamber, causing a 2-3 fold increase in activity. Subsequent addition of CSQ produced a delayed decrease in activity in the junctin/RyR1 complex (A), but no change in the triadin/RyR1 complex (B). Reducing luminal Ca^{2+} to 100 μM caused a transient decline in activity in the CSQ/junctin/RyR1 complex, consistent with the low $[\text{Ca}^{2+}]$ causing a depolymerisation and dissociation of CSQ (Wei et al., 2006). In marked contrast, there was no change in the activity of the CSQ1/triadin/RyR1 complex. The broken horizontal lines show average I_F for the period covered by the lines. Standard errors are not shown to avoid clutter but are sequentially in (A): ± 0.0041 ; ± 0.0039 ; ± 0.0116 ; ± 0.0067 ; ± 0.0052 ; ± 0.0074 . Standard errors in (B) are: ± 0.0059 ; ± 0.0047 ; ± 0.0036 ; ± 0.0045 .

Figure 6. CSQ1 suppresses activity of junctin-regulated RyR1, but not triadin-regulated, RyR1 (A, C, E, H) - records of 3s of channel activity at +40mV (A&B) or -40mV (E&H) with 1mM *trans* Ca^{2+} and 100nM *cis* Ca^{2+} . Single channel opening is upward (A&B) or downward (C&D) respectively, from zero current (continuous line, c) to maximum open conductance for one channel opening (broken line, o or o_1) or for two channels opening simultaneously (o_2). The top trace in each panel shows control purified RyR1 activity (labelled RyR in B and D), the middle trace shows activity after adding 5 μ g/ml junctin (A&E) or 5 μ g/ml triadin (E&H), and the lower trace shows effects of addition of 16 μ g/ml CSQ1. The recordings in A&C are representative of those used to calculate I'_F , while those in E&H are typical of stretches of activity with one channel opening, used to calculate mean open and mean closed times. (B&D) – average data (30s at +40mV plus 30s at -40mV) for I'_F . (E-G & I-J), average data (30s at +40mV plus 30s at -40mV) for mean open time (T_o , E&I) and mean closed time (T_c , G&J). Data is shown for control channel activity (RyR, 1st bin), after junctin (R/J) or triadin (R/T) addition (2nd bin) and after CSQ1 addition to junctin/RyR1 (R/J/C) or triadin (R/T/C) (3rd bin). A single asterisk (*) indicates a significant difference from control and a double asterisk (**) denotes a significant difference from the previous condition ($n=16$).

Figure 7. Responses of the CSQ1/junctin/RyR1 and the CSQ1/triadin/RyR1 complexes to a reduction in luminal Ca^{2+} concentration
 (A) and (B) show average data (from 60s – 90s at +40mV plus 60s – 90s at -40mV) for I'_F during experiments which examined the effects of lowering luminal Ca^{2+} on the CSQ1/junctin/RyR1 (A) and the CSQ1/triadin/RyR1 (B). The 1st bin shows control data for purified RyR1. The 2nd bin shows data after addition of either 5 μ g/ml junctin (R/J in A) or 5 μ g/ml triadin (R/T in B). The 3rd bin shows data obtained after adding 16 μ g/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 100nM. The 4th bin, is average data obtained within 30s of lowering Ca^{2+} and the 5th bin, data obtained >4min after lowering Ca^{2+} . The asterisks indicate a significant difference from the previous condition ($n=6$ in A or 8 in B).