

Regulation of Ryanodine Receptors by Calsequestrin: Effect of High Luminal Ca^{2+} and Phosphorylation

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ABSTRACT Calsequestrin, the major calcium sequestering protein in the sarcoplasmic reticulum of muscle, forms a quaternary complex with the ryanodine receptor calcium release channel and the intrinsic membrane proteins triadin and junctin. We have investigated the possibility that calsequestrin is a luminal calcium concentration sensor for the ryanodine receptor. We measured the luminal calcium concentration at which calsequestrin dissociates from the ryanodine receptor and the effect of calsequestrin on the response of the ryanodine receptor to changes in luminal calcium. We provide electrophysiological and biochemical evidence that: 1), luminal calcium concentration of ≥ 4 mM dissociates calsequestrin from junctional face membrane, whereas in the range of 1–3 mM calsequestrin remains attached; 2), the association with calsequestrin inhibits ryanodine receptor activity, but amplifies its response to changes in luminal calcium concentration; and 3), under physiological calcium conditions (1 mM), phosphorylation of calsequestrin does not alter its ability to inhibit native ryanodine receptor activity when the anchoring proteins triadin and junctin are present. These data suggest that the quaternary complex is intact in vivo, and provides further evidence that calsequestrin is involved in the sarcoplasmic reticulum calcium signaling pathway and has a role as a luminal calcium sensor for the ryanodine receptor.

INTRODUCTION

In skeletal muscle, Ca^{2+} release through the sarcoplasmic reticulum (SR) Ca^{2+} release channel, the ryanodine receptor (RyR), triggers muscle contraction. The RyR is known to associate with other proteins, such as the FK506-binding protein, DHPR, triadin, junctin, and calsequestrin (CSQ), forming a large macromolecular complex in the junctional SR membrane (Meissner, 2004). CSQ, the major Ca^{2+} binding protein within the SR lumen is located in the direct vicinity of the RyR1 (Franzini-Armstrong et al., 1987), binds triadin and junctin (Guo and Campbell, 1995; Guo et al., 1994; Jones et al., 1995), and presumably these proteins form a quaternary complex that controls Ca^{2+} release (Wang et al., 1998; Yano and Zarain-Herzberg, 1994). Both triadin and junctin span the SR membrane and are the only proteins known to associate with both the RyR and CSQ (Brandt et al., 1990; Groh et al., 1999; Guo and Campbell, 1995; Kim and Caswell, 1990; Kobayashi et al., 2000; Zhang et al., 1997), thus providing the physical link between the RyR and CSQ. Although CSQ was thought to have the main role of maintaining Ca^{2+} homeostasis and providing Ca^{2+} for release by the RyR, recent reports have demonstrated its regulatory ability on both native RyRs, presumably by binding to coproteins triadin and junctin on the luminal side of the channel complex (Beard et al., 2002; Györke et al.,

2004; Wang et al., 2001), and purified RyR channel activity, by binding directly to the luminal portion of the RyR (Szegedi et al., 1999). Therefore, CSQ is ideally placed to act as a sensor for luminal $[\text{Ca}^{2+}]$ in the transduction pathway linking RyR activity with store load.

The release of Ca^{2+} from the SR is strongly dependent on the SR Ca^{2+} load (Györke et al., 2002; Lamb et al., 2001). This may be in part due to the electrochemical driving force on Ca^{2+} ions, although this is unlikely to be a major factor because the free $[\text{Ca}^{2+}]$ is strongly buffered at ~ 1 mM by CSQ (Fryer and Stephenson, 1996). A more important feature is the ability of luminal $[\text{Ca}^{2+}]$ to regulate single RyR channel activity (Fill et al., 1990; Herrmann-Frank and Lehmann-Horn, 1996; Ma et al., 1988; Sitsapesan and Williams, 1995; Szegedi et al., 1999; Tripathy and Meissner, 1996), via Ca^{2+} sensors associated with luminal domains of the RyR (Ching et al., 2000), one of which is likely to be CSQ (Györke and Györke, 1998). However, CSQ can only function in this capacity if it is physically coupled to the RyR at the normal free luminal $[\text{Ca}^{2+}]$ of ~ 1 mM. CSQ could act as a Ca^{2+} sensor because many of its properties are Ca^{2+} dependent. Ca^{2+} is important in stabilizing both the polymer structure of CSQ and the quaternary complex (Wang et al., 1998), whereas CSQ binding to junctin and triadin is inhibited in the presence of > 10 mM Ca^{2+} (Zhang et al., 1997).

Our recent data suggest that CSQ is associated with the RyR when the free luminal $[\text{Ca}^{2+}]$ is 1 mM. Raising luminal Ca^{2+} from 1 to 13 mM increases RyR activity in two phases (Beard et al., 2002). Firstly, an initial fast phase, which is most likely due to direct Ca^{2+} activation of the RyR (at cytoplasmic and/or luminal sites). Secondly, a slower phase is likely to be

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due to CSQ dissociation and hence the removal of CSQs inhibitory effect. These results are in contrast to other studies that suggest that CSQ may be dissociated from triadin and junctin with 1 mM luminal $[\text{Ca}^{2+}]$ (Shin et al., 2000). To determine more precisely the luminal $[\text{Ca}^{2+}]$ at which CSQ dissociates and whether or not CSQ is associated with the RyR/triadin/junctin (RyR/T/J) complex in working muscle, the effect of varying luminal $[\text{Ca}^{2+}]$ between 1 and 5 mM has been examined and is reported here. In addition, we explore the possibility that CSQ communicates changes in luminal $[\text{Ca}^{2+}]$ to the RyR. We have also investigated the effects of CSQ phosphorylation on its inhibition of native RyRs (where both triadin and junctin are present) and the interaction with these anchoring proteins, at 1 mM Ca^{2+} . The results show that: a), CSQ remains associated with the junctional face membrane in the presence of 1 and 2 mM Ca^{2+} , but that CSQ is removed from native RyRs when luminal Ca^{2+} is increased to 4 mM; b), the presence of CSQ amplifies the response of native RyRs to changes in luminal $[\text{Ca}^{2+}]$; c), phosphorylation of CSQ does not influence CSQs ability to inhibit native RyRs at a physiological $[\text{Ca}^{2+}]$; and d), phosphorylation of CSQ does not alter the physical coupling between CSQ and triadin and junctin in an in vivo environment.

MATERIALS AND METHODS

Materials

The polyclonal anti-CSQ antibody was obtained from Swant Chemicals (Bellinzona, Switzerland), whereas both the monoclonal VD111D1₂ (anti-CSQ) antibody and anti-phosphothreonine antibody was obtained from Bio Scientific (Gymea, Australia). Other chemicals were from Sigma-Aldrich (Castle Hill, Australia).

Methods

SR vesicle preparation

Back and leg muscles were removed from New Zealand white rabbits and SR vesicles prepared using methods of Saito et al. (1984), with minor changes (Ahern et al., 1994).

Junctional face membrane

Junctional face membrane was isolated from either heavy SR (Kim et al., 1983), or SR vesicles as previously described (Costello et al., 1986), with minor changes (Beard, 2003).

Calsequestrin purification from rabbit skeletal muscle

CSQ was purified from RyR-enriched SR, using the methodology reported previously (Costello et al., 1986), except that centrifugation after solubilization was performed at $48,000 \times g$ for 1 h (Professor Cecilia Hidalgo, Instituto de Ciencias Biomedicas, Universidad de Chile, Santiago, Chile, personal communication).

To determine the effect of $[\text{Ca}^{2+}]$ on CSQ dissociation from junctional face membrane, the concentration of junctional face membrane was adjusted to 3.3 mg/ml by the addition of 230 mM Cs methanesulfonate (CsMS),

20 mM CsCl, 1 mM CaCl_2 , 10 mM TES (which corresponds to the solution used in single channel recordings; see below), with 0.5% triton X-100, 0.4 mM benzamidine, and 1 $\mu\text{g}/\text{ml}$ leupeptin. After incubation on ice for 30 min and centrifugation at $100,000 \times g$ for 15 min, the solubilized junctional face membrane pellet was suspended at 5 mg/ml, in 230 mM CsMS, 20 mM CsCl, 1 mM CaCl_2 , 10 mM TES, 0.5% triton X-100, 0.4 mM benzamidine, and 1 $\mu\text{g}/\text{ml}$ leupeptin. The final $[\text{Ca}^{2+}]$ was adjusted to 1, 2, 3, 4, 5, or 10 mM, by the addition of appropriate aliquots of a 1 M CaCl_2 stock (in 10 mM TES), pH 7.4, and after incubation on ice for ~ 1 h, the suspension centrifuged as above. The resulting pellets and supernatants were analyzed by electrophoresis and immunoblot.

Expression of rabbit skeletal recombinant CSQ

Rabbit skeletal calsequestrin was subcloned into a pGEX5x1 vector (*Bam*HI at the 5' end and *Xho*I at the 3' end), containing an N-terminal GST tag. Calsequestrin was expressed as GST fusion proteins in *Escherichia coli* strain BL21DE3 colonies and purified by glutathione Sepharose 4B chromatography. A single colony was grown at 37°C to an optical density at 600 nm of ~ 0.5 , and expression was induced by 0.5 mM isopropyl *B*-D-thiogalactoside. Bacteria were pelleted and resuspended, the cell membrane disrupted by lysozyme and French press, centrifuged, and supernatant incubated with glutathione Sepharose 4B beads. After incubation, calsequestrin was cleaved from the GST-glutathione Sepharose 4B complex by incubation with Factor Xa for 4 h at 25°C. CSQ was dialyzed against either 20 mM MOPS, 150 mM KCl, and 1 mM CaCl_2 (pH 7.4) or against *cis* solutions (see Single Channel Recording and Analysis below).

Electrophoresis and immunoblot

SDS-PAGE was performed using the Laemmli buffer system (Laemmli, 1970), with 10% polyacrylamide gels, whereas immunoblot was as per Towbin et al. (1992).

³¹P-NMR spectroscopy

CSQ phosphorylation status was determined using ³¹P-NMR spectroscopy. All spectra were acquired on a Varian-Inova 500 spectrometer (Palo Alto, CA), using a spectral width of 15,000 Hz, a pulse width of $\sim 15 \mu\text{s}$, a spectral frequency of 202,421 MHz, and an acquisition time of 0.33 s. Samples were kept at a constant temperature of 5°C. CSQ samples for NMR spectroscopy were prepared at a concentration of ~ 0.17 mM in an H_2O solution containing 10% $\text{D}_2\text{O}/90\% \text{H}_2\text{O}$. Phosphoric acid was used as an internal standard (0 ppm).

Acid phosphatase and casein kinase II treatment

Dephosphorylation (by acid phosphatase treatment) and phosphorylation (by casein kinase II) of recombinant CSQ (coupled to a GST-glutathione Sepharose matrix) were undertaken as previously described (Cala and Jones, 1991). To remove enzymes, samples were washed with 10 vol of 20 mM MOPS, 1 mM Ca^{2+} , and 150 mM KCl.

GST fusion protein affinity chromatography

To determine whether triadin and junctin can bind to both phosphorylated and dephosphorylated CSQ, GST fusion protein affinity chromatography was undertaken as described by Shin et al. (2000), with the following changes. Solubilized junctional face membrane (in 0.1% triton X-100) was added to both phosphorylated and dephosphorylated glutathione Sepharose 4B bound CSQ-GST, in 20 mM MOPS, 150 mM NaCl, and 1 mM CaCl_2 .

Single channel recording and analysis

Artificial planar bilayers separating two baths (*cis* and *trans*) were formed as previously described (Beard et al., 2002; Laver et al., 1995). SR vesicles (50 μg) were added to the *cis* solution so that the cytoplasmic surface of the SR and RyRs faced the *cis* solution after incorporation into the lipid bilayer. For SR vesicle incorporation, the solution compositions were as follows: *cis*, 230 mM CsMS, 20 mM CsCl, 1 mM CaCl_2 , and 10 mM TES (pH 7.4); and *trans*, 30 mM CsMS, 20 mM CsCl, 1 mM CaCl_2 , and 10 mM TES (pH 7.4). After incorporation of a channel, *trans* $[\text{Cs}^+]$ was raised from 50 to 250 mM with the addition of 200 mM CsMS and the *cis* solution was altered by the addition of 4.5 mM BAPTA (free $[\text{Ca}^{2+}] = 100 \text{ nM}$) and 2 mM ATP. Single channel parameters were obtained using the Channel 2 program (developed by P. W. Gage and M. Smith, John Curtin School of Medical Research, Canberra, Australia). Channel activity was assessed from a 30-s record, either from fractional mean current (I'_F , which is the average of all data points obtained during a recording period divided by the maximum single channel current), relative mean current (I'_F/I'_c , which is the fractional mean current under test conditions, divided by the fractional mean current under control conditions), or open probability (P_o). All electrical potentials are expressed here using standard physiological convention (i.e., cytoplasmic side relative to the luminal side at virtual ground). Unless otherwise stated, single channel recordings were obtained using a bilayer potential difference of $-/+40 \text{ mV}$. Measurements were carried out at $23 \pm 2^\circ\text{C}$. Channel activity is also expressed as relative P_o to include data in which activity varied from ~ 0.01 to ~ 0.6 and would more accurately reflect the population response in the intact fiber ($N = 4\text{--}14$).

Statistics

Average data are presented as mean \pm SE. The significance of differences between control and test values was tested using a Student's *t*-test for paired data or a sign test (Minium et al., 1993), as appropriate. A *p*-value of <0.05 was considered to be significant.

RESULTS

Effects of varying luminal Ca^{2+} concentration between 1 and 5 mM

Upon increasing *trans* Ca^{2+} from 1 to 5 mM, there was an almost immediate and significant increase in the fractional mean current (I'_F) of the channel, from $0.086 (\pm 0.01)$ to $0.186 (\pm 0.04)$ in six experiments (Fig. 1, *B* and *F*). A secondary rise in channel activity was then observed to always occur within 3 min of exposure to 5 mM *trans* Ca^{2+} , with I'_F increasing to $0.291 (\pm 0.03)$ (Fig. 1 *C*). The average dissociation time due to 5 mM Ca^{2+} ($110 \pm 17 \text{ s}$) was consistent with the time course of high Cs^+ dissociation of CSQ ($89 \pm 15 \text{ s}$) (Beard et al., 2002). Perfusion of the *trans* chamber to lower Ca^{2+} from 5 to 1 mM Ca^{2+} reduced channel activity somewhat, but not to control levels, despite observing channel activity for up to 12 min. Subsequent addition of a final concentration of $16 \mu\text{g/ml}$ *trans* CSQ reduced channel activity to a level that was not significantly different from control (Fig. 1 *E*). Furthermore, $0.5 \mu\text{g/ml}$ *trans* polyclonal anti-CSQ antibody (which inhibits RyR activity only when CSQ is present) (Beard et al., 2002), was added to one channel after CSQ addition and further reduced channel activity (data not shown). These results are consistent with firstly, an initial direct activation of the RyR by luminal Ca^{2+} when it was increased from 1 to 5 mM, and a further activation when the 5 mM *trans* Ca^{2+} dissociated CSQ from the RyR/T/J complex. Secondly, lowering $[\text{Ca}^{2+}]$ from 5 to 1 mM reversed the direct activation of the RyR, and addition of $16 \mu\text{g/ml}$ *trans* CSQ

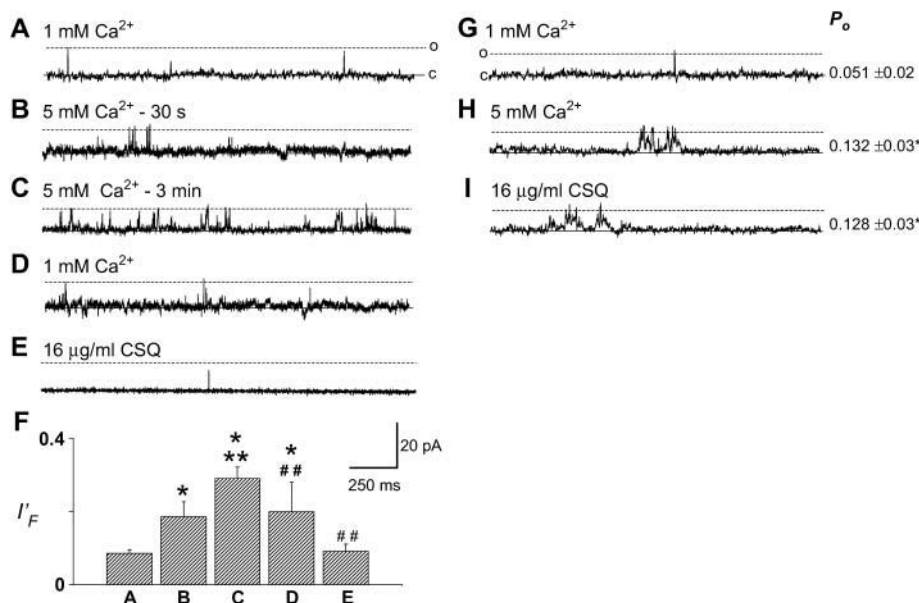


FIGURE 1 Increasing *trans* Ca^{2+} from 1 to 5 mM resulted in a biphasic RyR activation. (A–E) Records of 2 s of single channel activity. Single channel opening is upward from zero current (solid line) to maximum open conductance (dashed line). (A) Control, with 1 mM *trans* Ca^{2+} ; (B) 30 s after increasing *trans* Ca^{2+} to 5 mM; (C) 3 min after increasing *trans* Ca^{2+} to 5 mM; (D) after perfusing the *trans* chamber with 1 mM Ca^{2+} ; (E) addition of 16 $\mu\text{g/ml}$ CSQ; (F) average data ($n = 5\text{--}6$) for fractional mean current (I'_F) for conditions shown in panels A–E. (G) Control, with 1 mM *trans* Ca^{2+} ; (H) after perfusing the *trans* chamber with 5 mM Ca^{2+} , after CSQ dissociation; (I) 5 min after addition of 16 $\mu\text{g/ml}$ CSQ. Average P_o ($n = 5$) is given to the right of panels G–I. Asterisks (*) indicate average values significantly ($p < 0.05$, *t*-test) different from control, and double asterisk (**) and double cross-hatch (##) indicate average values significantly different ($p < 0.05$, *t*-test and sign test, respectively) from the previous condition. $V_m - E_{\text{Cs}^+}$ was $+40 \text{ mV}$ for all records presented in Figs. 1, 2, 4, and 5, except where indicated.

resulted in a further decline of activity, presumably by allowing reassociation of CSQ with the RyR/T/J complex (possible once *trans* Ca^{2+} had been restored to 1 mM). Although data are only shown at +40 mV, similar effects were obtained at -40 mV in all experiments.

In a second set of experiments CSQ was dissociated from the RyR/T/J complex, after incorporation, by either increasing $[\text{Cs}^+]$ to 500 mM or increasing *trans* Ca^{2+} to 5 mM. A late irreversible increase in activity was seen with CSQ dissociation using both Cs^+ and Ca^{2+} dissociation. There was also an immediate increase in activity with 5 mM Ca^{2+} (data not shown). The *trans* chamber was then perfused with solution containing 5 mM Ca^{2+} to remove dissociated CSQ from the chamber (Fig. 1 *H*). Subsequent addition of 16 $\mu\text{g}/\text{ml}$ exogenous CSQ did not significantly reduce channel activity, despite incubation for 7 min (Fig. 1 *I*). These data illustrate that 5 mM Ca^{2+} can both dissociate and prevent reassociation of CSQ to the RyR/T/J complex. CSQ slowly dissociated from channels exposed to 4 mM *trans* Ca^{2+} . If channels were maintained in 4 mM *trans* Ca^{2+} , (Fig. 2, *left panels*), the immediate Ca^{2+} activation was followed in five out of six channels, by a late activation observed after

~9 min (where I_F rose from 0.147 ± 0.05 to 0.285 ± 0.04) (Fig. 2, *C and F, left panels*). Unlike 5 mM Ca^{2+} dissociation (Fig. 1), no secondary change in channel activity was observed within 3 min (Fig. 2 *F, left panel*). Replacement of control *trans* Ca^{2+} (1 mM) could only partially remove the activation induced by prolonged exposure to 4 mM Ca^{2+} , and in one out of one channel, 16 $\mu\text{g}/\text{ml}$ exogenous CSQ returned channel activity to levels similar to control (Fig. 2, *D and E, left panels*).

Exposing RyRs to 2 or 3 mM *trans* Ca^{2+} resulted in a fast phase of RyR activation, but not the late phase within the time frame of the experiment (Fig. 2, *middle and right panels*). I_F increased almost immediately from control levels ($I_F = 0.1323 \pm 0.02$ to 0.3017 ± 0.03 ; Fig. 2 *F, middle panel*) with no further increase during incubation in 3 mM Ca^{2+} for up to 18 min (Fig. 2, *A–C, middle panel*). Similarly, upon exposure to 2 mM *trans* Ca^{2+} , channel activity rose immediately from control levels ($I_F = 0.083 \pm 0.01$ to 0.153 ± 0.02 ; Fig. 2 *F, right panel*). No further rise in activity was observed, despite incubation in 2 mM Ca^{2+} in one channel for up to 15 min. Reperfusion of the *trans* chamber with 1 mM Ca^{2+} restored activity to control levels in both experiments ($I_F = 0.081 \pm$

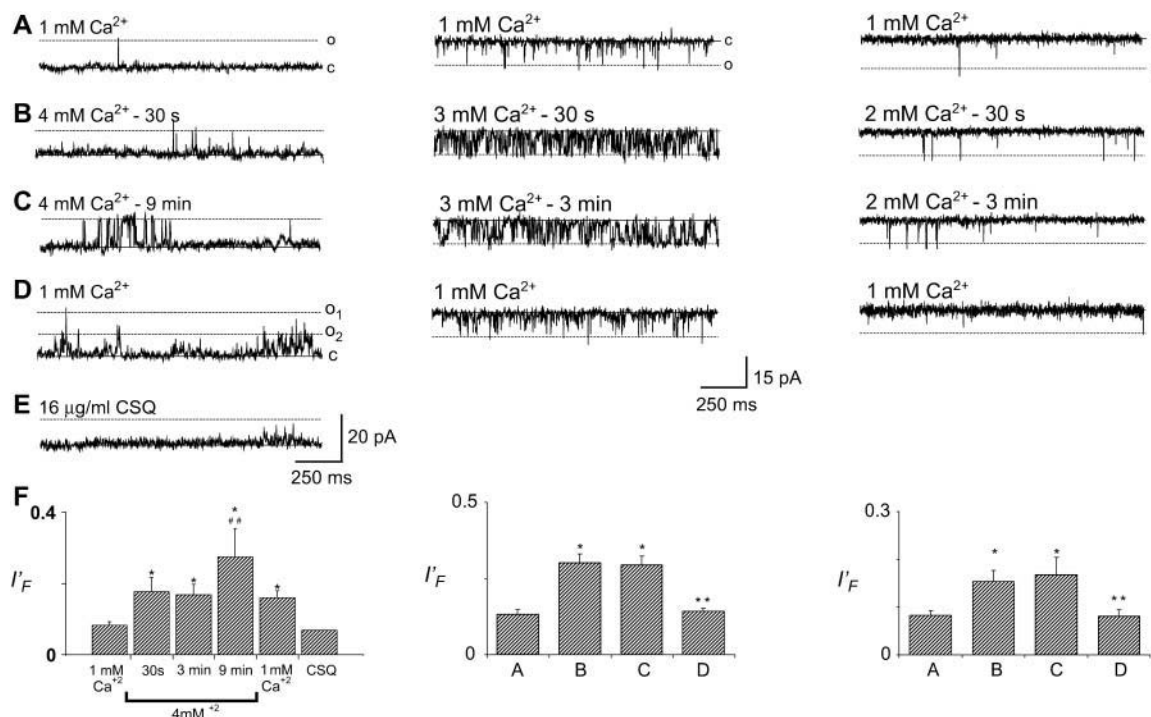


FIGURE 2 Extended exposure to *trans* Ca^{2+} of 4 mM, and not 2 or 3 mM, resulted in a biphasic RyR activation. (A–E) Records of 2 s of single channel activity. Single channel opening is upward from zero current (solid line) to maximum open conductance (dashed line) for the left panels, and downwards from zero current (solid line) to maximum open conductance (dashed line) for the middle and right panels. (A) Control, with 1 mM *trans* Ca^{2+} ; (B) 30 s after increasing *trans* Ca^{2+} to 4 mM (left panel), 3 mM (middle panel), and 2 mM (right panel); (C) 3 min (middle and right panels) and 9 min (left panel) after increasing *trans* Ca^{2+} to 4, 3, or 2 mM; (D) after perfusing *trans* chamber with 1 mM Ca^{2+} ; (E) addition of 16 $\mu\text{g}/\text{ml}$ CSQ; (F) average and individual data ($n = 4-8$ for A–D and $n = 1$ for E) for fractional mean current (I_F), at $[\text{Ca}^{2+}]$ s listed under the graph (left panel) or for conditions shown in panels A–D (middle, right panels). Asterisks (*) indicate average values significantly ($p < 0.05$, *t*-test) different from control, and double asterisk (**) and double cross-hatch (##) indicate average values significantly different ($p < 0.05$, *t*-test and sign test, respectively) from the previous condition. $V_m - E_{\text{Cs}^+}$ was +40 mV for left panel and -40 mV for middle and right panels.

0.01 and 0.140 ± 0.02 for channels previously exposed to 2 and 3 mM Ca^{2+} , respectively; Fig. 2, *D* and *F*). This shows that the activity increase upon raising $[\text{Ca}^{2+}]$ to 2 or 3 mM was fully reversible (Fig. 2 *D*, middle and right panels), and suggested that 2 or 3 mM Ca^{2+} did not dissociate a significant proportion of CSQ from the RyR/T/J complex within 15–18 min.

The luminal Ca^{2+} dependence of CSQ dissociation from junctional face membrane was followed using SDS-PAGE. Comparison of native SR vesicles (used in bilayer experiments) and solubilized junctional face membrane (used in this section) have shown that the relative amounts of RyR, CSQ, triadin, and junctin are the same in both preparations (Beard, 2003). Thus solubilization per se does not dissociate the quaternary complex. Exposure of junctional face membrane to 1 or 2 mM Ca^{2+} did not detach CSQ (Fig. 3, *A* and *B*, lanes 2–5), shown by Coomassie stain (Fig. 3 *A*), and immunoblot with monoclonal VIIID1₂ anti-CSQ antibody (Fig. 3 *B*). There was a significant band at 55 kDa (the apparent

molecular weight of CSQ) in the original junctional face membrane (Fig. 3, *A* and *B*, lane 1) and in the pellet, but not in the supernatant, from the solubilized fractions after exposure to either 1 or 2 mM Ca^{2+} $[\text{Ca}^{2+}]$ (Fig. 3, *A* and *B*, lanes 2–5). In contrast, 4, 5, and 10 mM Ca^{2+} resulted in significant dissociation of CSQ from the junctional face membrane, indicated by increasing amounts of CSQ in the supernatant (Fig. 3, *A* and *B*, lanes 9, 11, and 13), and reduced (Fig. 3, *A* and *B*, lane 8) or undetectable amounts in the membrane pellet (Fig. 3, *A* and *B*, lanes 10 and 12). CSQ dissociated by 3 mM Ca^{2+} was undetectable (Fig. 3, *A* and *B*, lane 7), with the profile of the JFM and membrane pellet essentially identical (Fig. 3, *A* and *B*, lanes 1 and 6). It is unlikely that a small dissociation that might occur with 3 mM Ca^{2+} would alter the regulatory effect on RyR channels in lipid bilayer experiments (Fig. 2). This dissociation data confirmed our interpretation of the single channel data, i.e., that CSQ was attached to RyRs in the lipid bilayers under control conditions (1 mM Ca^{2+}), that 2 and 3 mM Ca^{2+} did not induce a CSQ dissociation-dependent RyR activation, and that ≥ 4 mM Ca^{2+} can dissociate significant amounts of CSQ from the RyR/T/J complex. From both biochemical and lipid bilayer data, it appears that 4 mM Ca^{2+} is the minimal Ca^{2+} concentration required for CSQ dissociation and that dissociation critically depends on a $[\text{Ca}^{2+}]$ in the vicinity of 4 mM.

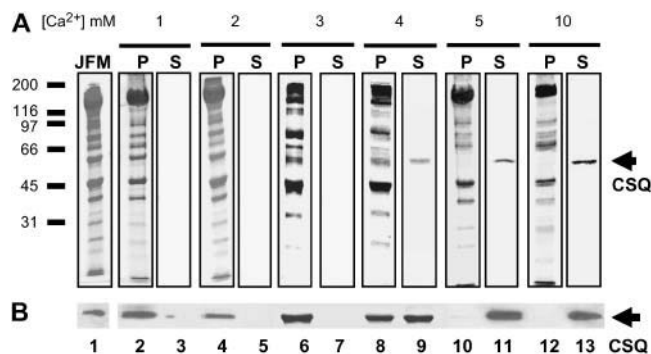


FIGURE 3 Only $[\text{Ca}^{2+}] \geq 4$ mM can dissociate CSQ from the solubilized junctional face membrane. (*A*) Ten percent SDS polyacrylamide gel showing the original junctional face membrane pellet (*JFM*), insoluble junctional face membrane pellet (*P*), and solubilized supernatant (*S*), after the junctional face membrane was exposed to 1, 2, 3, 4, 5, or 10 mM Ca^{2+} . CSQ was absent in the solubilized sample (*S*, lanes 3 and 5) after exposure to 1 or 2 mM Ca^{2+} , with the gel profile of the insoluble pellet (*P*, lanes 2 and 4) being identical to the original junctional face membrane sample (*JFM*, lane 1). Only trace amounts of CSQ were found in the solubilized sample (*S*, lane 7) after incubation with 3 mM Ca^{2+} , leaving the profiles of the original junctional face membrane and the insoluble pellet (compare lanes 1 and 6) virtually identical. In contrast, increasing amounts of CSQ were dissociated from the original junctional face membrane sample by exposure to 4, 5, and 10 mM Ca^{2+} (*S*, lanes 9, 11, and 13) with significantly reduced (*P*, lane 8) or undetectable levels of CSQ observed in the insoluble pellet (*P*, lanes 10 and 12). (*B*) Immunoblot of protein products shown in panel *A*, immunoprobed with VIIID1₂ monoclonal anti-CSQ antibody. Approximate position of the molecular weight markers are indicated to the left of lane 1 in panel *A*, with CSQ indicated by the arrows. As $[\text{Ca}^{2+}]$ was kept at 1 mM throughout the CSQ purification procedure, no additional Ca^{2+} was added to the 1-mM sample. The following amount of protein was added to the appropriate lanes; 50 μg original junctional face membrane, 40 μg pellet, and 10 μl solubilized supernatant (which equates to 2.7 μg in lane 9, 3.3 μg in lane 11, and 4.7 μg in lane 13). As a protein concentration cannot be determined for the supernatants obtained after 1, 2, and 3 mM Ca^{2+} extraction, an equivolume (10 μl) of all supernatant fractions was loaded for both the Coomassie stained gel and the immunoblot.

Changes in RyR activity caused by varying luminal Ca^{2+} between 1 and 5 mM

Native RyRs were incorporated into bilayers in the presence of 1 mM *trans* Ca^{2+} , $[\text{Ca}^{2+}]$ was then increased to 1.5, 2, 2.5, 3, and 5 mM in a stepwise fashion (Fig. 4), and channel activity was followed at both positive and negative potentials.

For CSQ-attached (CSQ(+)) RyRs, currents were analyzed within 1 min of exposure to the increased $[\text{Ca}^{2+}]$ to avoid any effects of CSQ dissociation. However, in agreement with results presented above (Fig. 2), channels exposed to 1.5–3 mM Ca^{2+} did not show a secondary increase in activity under these conditions for up to 18 min, again indicating that 1–3 mM luminal Ca^{2+} did not dissociate CSQ during this period. For experiments examining increases in luminal Ca^{2+} to 1.5–3 mM, CSQ-depleted (CSQ(–)) channels were obtained increasing *trans* ionic strength before *trans* $[\text{Ca}^{2+}]$ was raised. The increase in ionic strength effectively dissociates CSQ from the RyR (Beard et al., 2002). The *trans* Cs^+ was increased from 250 to 500 mM and maintained at that concentration until an increase in channel activity was observed (89 ± 15 s), indicating CSQ dissociation (Beard et al., 2002). Once activity increased and remained stable for 1 min, *trans* $[\text{Cs}^+]$ was restored to 250 mM by perfusion. It should be noted that returning to 250 mM Cs^+ (control conditions) did not restore control RyR activity, as expected if CSQ had been dissociated from the RyR/T/J complex. For experiments with 5 mM Ca^{2+} , the high *trans* Ca^{2+} itself was used to dissociate CSQ, and activity after the

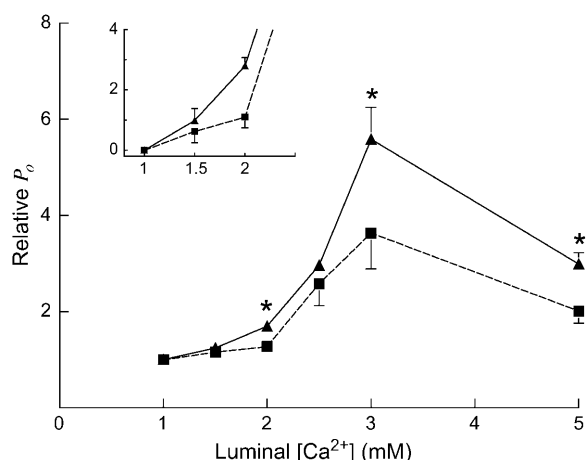


FIGURE 4 RyR responses to changes in luminal $[\text{Ca}^{2+}]$ between 1 and 5 mM, in the presence and absence of CSQ. Experimental conditions: *cis* (mM) 250 Cs^+ , 2 ATP (activating), and 1 nM Ca^{2+} (subactivating); *trans* (mM) 250 Cs^+ , 1–5 Ca^{2+} . *Trans* $[\text{Ca}^{2+}]$ was altered by aliquot additions of 200 mM stock Ca^{2+} ($[\text{Ca}^{2+}]$ determined by a Ca^{2+} electrode). CSQ was dissociated from RyRs before increasing $[\text{Ca}^{2+}]$ by exposure to 500 mM Cs^+ , or to high $[\text{Ca}^{2+}]$ (in the case of the data obtained at 5 mM Ca^{2+}). Each data point is the mean relative open probability (relative P_0), which is test P_0 (2–5 mM Ca^{2+}) relative to control P_0 (1 mM), for RyRs in the absence (CSQ(–); \blacksquare) and presence (CSQ(+); \blacktriangle) of CSQ. The bars are means \pm SE for $n = 4$ –14. Absolute mean P_0 values for control activities are listed in the Results section. The inset chart shows relative P_0 changes at luminal Ca^{2+} from 1 to 2.0 mM Ca^{2+} in more detail. Asterisks (*) indicate average values significantly different from control ($p > 0.05$, t -test).

secondary increase (analyzed 3 min after exposure to 5 mM Ca^{2+}), was used as the measure of the response of channel activity in the absence of CSQ. Channel activity is reported as relative P_0 , i.e., P_0 under test conditions (2–5 mM) relative to P_0 under control (1 mM Ca^{2+}) conditions. Removal of CSQ made RyRs less responsive to increasing *trans* $[\text{Ca}^{2+}]$ at each $[\text{Ca}^{2+}]$ tested (Fig. 4), with significant differences in relative P_0 seen at 2, 3, and 5 mM *trans* Ca^{2+} . It should be noted that although absolute channel P_0 under control conditions was higher for CSQ(–) RyRs than for CSQ(+) RyRs (0.14 ± 0.02 and 0.09 ± 0.01 , respectively), due to the removal of CSQs inhibiting effect, CSQ(+) channels were more responsive to changes in $[\text{Ca}^{2+}]$ from 1 mM than CSQ(–) RyRs (Fig. 4). The apparently smaller increase in channel activity at 5 mM Ca^{2+} compared with 3 mM Ca^{2+} in both CSQ(+) and CSQ(–) channels was not explored further, but is indicative of a small decline in activation seen at higher Ca^{2+} concentrations and is perhaps due to a separate inhibitory mechanism (Ching et al., 2000; Tripathy and Meissner, 1996). Together, the data clearly demonstrate that CSQ amplified the activating effect of luminal $[\text{Ca}^{2+}]$ between 1 and 5 mM.

Determination of CSQs phosphorylation status

The phosphorylation status of the isolated CSQ was determined by both ^{31}P -NMR analysis and immunoblot

with anti-phosphothreonine. The ^{31}P -NMR analysis indicated that CSQ from rabbit skeletal muscle was phosphorylated (Fig. 5 A, *top trace*). In the spectra, the horizontal axis corresponds to a chemical shift (ppm), and reflects the environment experienced by the phosphorous atoms. The original CSQ sample (*top trace*) displays a resonance peak at ~ 0.2 ppm, suggesting that CSQ is at least in part phosphorylated. CSQ, therefore, is probably phosphorylated *in vivo* as it is unlikely that CSQ underwent autophosphorylation during the isolation procedure as ATP was absent from all isolation media.

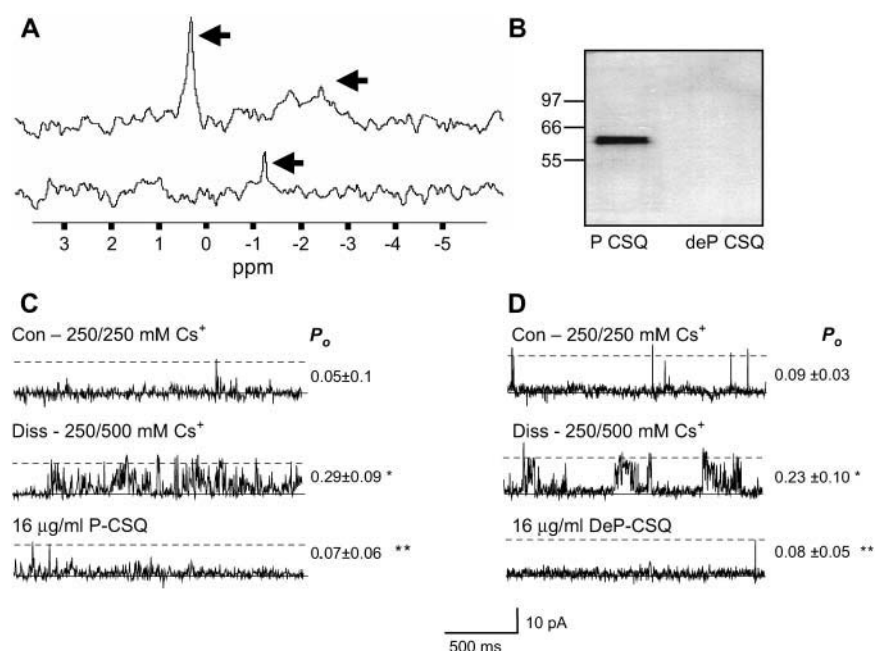
The appearance of a broad resonance peak, at between -1.5 and -2.8 ppm (*arrow, top trace*) in the phosphorylated sample, suggests that the phosphorous atoms may reside in an aggregated form of CSQ. Indeed, protein precipitation was observed after NMR analysis, suggesting that some CSQ aggregated during the 24-h testing period. This NMR data show clearly that CSQ was phosphorylated but does not allow determination of the number of phosphorylated residues.

After dephosphorylation by acid phosphatase treatment and subsequent dialysis to remove free phosphorous, the peaks observed in the phosphorylated sample of CSQ at ~ 0.2 and between -1.5 and -2.8 ppm (Fig. 5 A, *arrows, top trace*) disappeared. The appearance of a small peak near -1.2 ppm (*arrow, bottom trace*), may be explained by residual (free) phosphorous atoms in the sample, most probably due to incomplete dialysis of the CSQ sample after dephosphorylation. Nevertheless, the dephosphorylated CSQ sample contained at least 10 times less phosphorous than the phosphorylated CSQ sample (indicated by the area under each spectrum).

As Thr^{353} is phosphorylated in rabbit skeletal CSQ, (Cala and Jones, 1991) both the original and acid phosphatase-treated CSQ samples were probed with a polyclonal anti-phosphothreonine antibody. Only the native CSQ and not the dephosphorylated CSQ could be detected by this antibody (Fig. 5 B). This observation confirms the NMR data showing that isolated CSQ is in a phosphorylated form, and can be significantly dephosphorylated by acid phosphatase treatment.

Native RyR regulation by phosphorylated and dephosphorylated CSQ

The actions of both phosphorylated and dephosphorylated CSQ on native skeletal RyRs were examined under physiological Ca^{2+} conditions (1 mM *trans* Ca^{2+}). RyRs were exposed to 500 mM *trans* Cs^+ to dissociate endogenous CSQ, and this procedure resulted in a rise in P_0 at positive potentials (Fig. 5, C and D, *middle panel*). After subsequent perfusion of the *trans* chamber with 250 mM Cs^+ , 16 $\mu\text{g/ml}$ of phosphorylated CSQ was added (Fig. 5 C, *bottom panel*). As seen previously (Figs. 1 and 2), phosphorylated CSQ significantly reduced channel P_0 (Fig. 5 C).



Similarly, reassociation of 16 $\mu\text{g/ml}$ of dephosphorylated CSQ also reduced channel activity to control (predissociation) levels (Fig. 5 D). Similar inhibitory effects of phosphorylated and dephosphorylated CSQ were seen at -40 mV. To ascertain whether the addition of exogenous CSQ buffered $[\text{Ca}^{2+}]_{\text{free}}$ (and lower it significantly from 1 mM), the $[\text{Ca}^{2+}]_{\text{free}}$ was measured in the absence and presence of CSQ using a Ca^{2+} electrode. No significant difference in $[\text{Ca}^{2+}]_{\text{free}}$ was detected.

The phosphorylation status of CSQ did not influence its ability to inhibit native RyRs at 1 mM *trans* Ca^{2+} . Phosphorylated CSQ decreased I_F relative to postdissociation activity to 0.51 ± 0.10 ($N = 16$), whereas dephosphorylated CSQ decreased relative I_F to 0.43 ± 0.15 ($N = 8$). Apparently, altering the phosphorylation status of exogenous CSQ did not alter its functional interaction with the native RyR/T/J complex, or the degree of inhibition it imposed.

CSQ interactions with triadin and junctin

Determination of whether phosphorylation of CSQ altered its binding to triadin and junctin was carried out using GST fusion protein affinity chromatography. GST-tagged CSQ was bound to glutathione Sepharose 4B matrix, and was experimentally phosphorylated or dephosphorylated (see Methods) *in situ*. Only the phosphorylated CSQ sample was detectably phosphorylated, as determined by anti-phosphothreonine immunoblot (see Methods; data not shown).

Solubilized junctional face membrane (in 0.1% triton X-100) was applied to either phosphorylated or dephosphorylated GST-tagged CSQ under near physiological conditions (1 mM CaCl_2 , 150 mM NaCl), and after a 2-h incubation, unbound proteins were washed and separated from the protein-bound CSQ-GST-glutathione Sepharose complex. This complex was subsequently collected by centrifugation. Junctional face membrane proteins that interacted with GST-tagged CSQ were separated by SDS-PAGE and immunoprobated with anti-triadin and anti-junctin after Western blot (Fig. 6). The results indicated that CSQ will still form a complex with triadin and junctin regardless of its phosphorylation status, as both triadin and junctin interacted with both phosphorylated and dephosphorylated CSQ (Fig. 6).

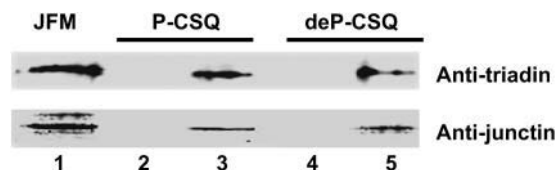


FIGURE 6 Interactions of triadin and junctin with CSQ are independent of phosphorylation status. Western blot, after 10% SDS-PAGE, of the GST-CSQ coupled to glutathione Sepharose 4B before (lanes 2 and 4) and after (lanes 3 and 5) exposure to solubilized junctional face membrane (JFM; lane 1). Blot was immunoprobated with anti-triadin and anti-junctin. Both phosphorylated (P-CSQ; lanes 2 and 3) and dephosphorylated (deP-CSQ; lanes 4 and 5) CSQ could bind triadin and junctin (lanes 3 and 5) under close to physiological conditions (1 mM Ca^{2+} , 150 mM NaCl).

DISCUSSION

Novel observations on the Ca^{2+} dependence of the association between CSQ and the RyR, and the effect of CSQ on the luminal Ca^{2+} dependence of RyR activity, are described. Using two different techniques, we show that CSQ is associated with the RyR when the luminal $[\text{Ca}^{2+}]$ is maintained at the physiological level of ~ 1 mM. Furthermore, CSQ remains associated with the RyR/T/J complex when luminal Ca^{2+} is increased from 1 to 3 mM, but dissociates at 4 and 5 mM (and cannot reassociate at 5 mM Ca^{2+}). In addition, this is the first report of the effects of high luminal $[\text{Ca}^{2+}]$ on skeletal RyR activity under conditions in which CSQ would have remained associated with the RyR/T/J complex after RyRs were incorporated into bilayers. We show that under these conditions, channel activity increases as luminal Ca^{2+} is increased and that the response of the RyR to small increases in luminal $[\text{Ca}^{2+}]$ from 1 to 5 mM is amplified by CSQ. Finally, we show that CSQ in native SR is phosphorylated and that both a physical and functional coupling of CSQ with native RyR channels and with triadin and junctin is independent of CSQ phosphorylation.

Is CSQ associated with the RyR/T/J complex under physiological conditions?

If CSQ plays an active role in communicating luminal $[\text{Ca}^{2+}]$ to the RyR *in vivo*, it must be physically coupled either directly or indirectly to the RyR under normal physiological conditions. Using both biochemical (Fig. 3) and electrophysiological techniques (Figs. 1 and 2), we show that CSQ is associated with the junctional face membrane when the luminal $[\text{Ca}^{2+}]$ is ~ 1 mM, i.e., the free $[\text{Ca}^{2+}]$ that is considered to exist in the lumen of the SR *in vivo* (Fryer and Stephenson, 1996). In addition, electron micrographs of terminal cisternae, and electron tomography of frozen hydrated triad junctions from skeletal muscle (fixed under physiological conditions), show CSQ located close to the RyR, suggesting that under physiological conditions, CSQ is associated with the RyR (Franzini-Armstrong, 1973; Wagenknecht et al., 2002). This is not unexpected because CSQ association with triadin and junctin in skeletal muscle requires Ca^{2+} (Guo and Campbell, 1995; Wang et al., 1998). However, other conflicting evidence suggests that binding of solubilized skeletal SR to a GST-CSQ fusion protein is gradually reduced as Ca^{2+} increased from 0 to 5 mM, with maximal binding occurring at 0 mM Ca^{2+} , leading to the conclusion that CSQ may not associate with the skeletal RyR/T/J complex in the presence of physiological (1 mM) Ca^{2+} (Shin et al., 2000). It should be noted that data presented by Shin et al. (2000) show significant CSQ association with the solubilized SR at 1 mM Ca^{2+} . In the light of our data (Figs. 1–3 and 6) and those of Guo and Campbell (1995) and Costello et al. (1986), skeletal CSQ

association with triadin and junctin under physiological $[\text{Ca}^{2+}]$ seems to be likely.

How does high Ca^{2+} dissociate CSQ from the RyR/T/J complex?

It is likely that the ability of CSQ to associate with and dissociate from triadin and junctin at different $[\text{Ca}^{2+}]$, depends on the structure of the CSQ protein, which is strongly Ca^{2+} dependent. It is not clear how high $[\text{Ca}^{2+}]$ dissociates CSQ from the RyR/T/J complex. At low $[\text{Ca}^{2+}]$, CSQ assumes a mostly random coil structure, with α -helical content increasing as Ca^{2+} binds (Ikemoto et al., 1972, 1974; Ostwald et al., 1974). To form dimers and polymers, both skeletal and cardiac CSQ require Ca^{2+} , presumably above 10 μM . High $[\text{Ca}^{2+}]$ (≥ 5 mM) dissociates CSQ from the junctional face membrane (this study) and has been successfully used to selectively elute recombinant CSQ from phenyl-Sepharose affinity matrix (Cala and Jones, 1983). Recent studies with the cardiac RyR show that exposure of the reconstituted quaternary complex to 5 mM luminal Ca^{2+} resulted in channel activation due to CSQ dissociation (Györke et al., 2004). Additionally, increasing $[\text{Ca}^{2+}]$ to ≥ 5 mM decreases the stokes radius and increases the apparent compaction of CSQ (Cozens and Reithmeier, 1984), and results in CSQ aggregation (N. A. Beard and A. F. Dulhunty, unpublished data; Park et al., 2003). As the $[\text{Ca}^{2+}]$ rises, hydrophobic side chains are buried within the polymer, reducing the ability of CSQ to bind to other proteins (Mitchell et al., 1988). This is suggestive of a somewhat “supercompacted” CSQ within the polymer (Beard et al., 2004). Supercompaction of CSQ upon exposure to increasing luminal $[\text{Ca}^{2+}]$ may disrupt the interactions with triadin and junctin (and indeed the RyR), allowing selective dissociation of CSQ.

Role for CSQ in regulating RyRs at 1–5 mM luminal Ca^{2+}

Ikemoto et al. (1972) and Ostwald et al. (1974) reported changes in CSQ conformation occurring within the range of $[\text{Ca}^{2+}]$ tested here. Alterations in protein conformation with luminal $[\text{Ca}^{2+}]$ changes between 0.1 and 3 mM have been postulated to increase SR Ca^{2+} release rate constants (Donoso et al., 1995). In this study, the channel response to varying $[\text{Ca}^{2+}]$ between 1 and 5 mM was amplified in the presence of CSQ. The change in activity observed upon exposure to 2 mM Ca^{2+} cannot be attributed to an effect of CSQ dissociation, as exposure of junctional face membrane to 2 mM Ca^{2+} does not dissociate CSQ (Fig. 3). It is possible that regulation of RyRs by luminal Ca^{2+} is partly due to Ca^{2+} -dependent changes in CSQ conformation that do not result in CSQ dissociation (He et al., 1993; Ikemoto et al., 1989).

In cardiac muscle, increasing luminal $[\text{Ca}^{2+}]$ induced an increase in activity in native, but not purified RyRs (Györke

et al., 2004). Only restoration of the quaternary complex, by adding exogenous triadin, junctin, and CSQ (but not by adding triadin and junctin alone), could restore RyR responsiveness to luminal Ca^{2+} (Györke et al., 2004). Although in the absence of CSQ, increasing luminal $[\text{Ca}^{2+}]$ resulted in RyR activation, we show that in skeletal muscle, RyR responsiveness to increased luminal $[\text{Ca}^{2+}]$ was substantially augmented by the presence of CSQ. Both studies (Fig. 4; Györke et al., 2004) provide evidence for the role of CSQ (as part of an intact quaternary complex) as a luminal Ca^{2+} sensor.

CSQ activation versus CSQ inhibition of RyRs

The reported effects of CSQ on RyRs vary between laboratories and preparations, as seen with the response of the RyR to phosphorylated and dephosphorylated CSQ. With physiological luminal $[\text{Ca}^{2+}]$, the ability of CSQ to inhibit native skeletal RyRs was independent of phosphorylation (Fig. 5). In contrast, Szegedi et al. (1999) showed that CSQ activates purified skeletal RyRs only when it is dephosphorylated and we have confirmed these findings in our laboratory (N. A. Beard, unpublished data). Thus, the differences in RyR regulation observed in these two studies can be explained by the different preparations used; the 3-[(3-Cholamidopropyl)Dimethyl-Ammonio]-1-Propanesulfonate solubilized and purified RyR (with presumably no significant amounts of accessory proteins triadin and junctin present) in Szegedi's study (and our observations), and the native RyR, with triadin and junctin present (in this study). Although we cannot discount that another unknown accessory protein found within the SR lumen may be responsible for anchoring CSQ to the RyR in the native preparation, the only two proteins known to bind both CSQ and the RyR in the lumen are triadin and junctin.

In addition, the reported differences in CSQ regulation of RyRs support the hypothesis that CSQ modulates RyR activity via two mechanisms; firstly, by inducing RyR inhibition through interactions with triadin and junctin in a phosphorylation-independent manner (this study), and secondly, by binding directly to the RyR (Herzog et al., 2000) and activating the channel in a phosphorylation-dependent manner, as shown by Szegedi et al. (1999). Whether or not both mechanisms of CSQ regulation operate in native RyRs in vivo is unknown. If so, the reported CSQ activation induced by a direct RyR-CSQ interaction is overshadowed by the triadin/junctin mediated CSQ inhibition, seen as an overall inhibition of the channel.

Phosphorylation as an in vivo modulator of CSQ regulation of RyRs?

Regulation of RyRs by cyclic phosphorylation/dephosphorylation of CSQ in vivo depends on whether CSQ is phosphorylated before luminal segregation, or after targeting

to the lumen. This, in turn, depends on the location of specific kinases responsible for CSQ phosphorylation, and whether or not ATP could be transported into the lumen of the SR. It is not known which kinase specifically phosphorylates CSQ in vivo, or whether such kinases are present in the SR lumen (or indeed whether CSQ is phosphorylated inside the lumen). Casein kinase II phosphorylates Thr³⁵³ in skeletal CSQ (Cala and Jones, 1991). To date, the presence of casein kinase II within the SR lumen has been inferred, but not proven (Shoshan-Barmatz et al., 1996). Thr³⁵³ or another phosphorylatable residue may also be phosphorylated by other kinases, which might be present in the lumen. CSQ has also been identified as a potentially good substrate for casein kinase I and ϵ protein kinase C, but current evidence suggests that these kinases reside only in the cytoplasm and not within the SR lumen (Rodriguez et al., 1999; Salvatori et al., 1994). CSQ is phosphorylated when isolated from muscle homogenates (Fig. 5 B) and this observation shows that CSQ in the SR lumen is phosphorylated. Cala and Jones (1991) found that rabbit skeletal CSQ was not isolated in its phosphorylated form; Campbell and Shamoo (1980) show that CSQ can be phosphorylated in skeletal SR muscle preparations, whereas Varsanyi and Heilmeyer (1980) report that CSQ is capable of autophosphorylation and the isolated calsequestrin from skeletal muscle can be obtained in fully or partially phosphorylated form (Varsanyi and Heilmeyer, 1979). Recently, O'Brian et al. (2002) reported that presumably phosphorylation and glycosylation processes are involved in both common and distinct cellular compartmentation of the calsequestrin isoforms.

Physiological implications

Taken together, these results show that luminal Ca^{2+} has two actions on RyR channels. Firstly, Ca^{2+} can bind to activation sites, found on the RyR or an associated protein (when luminal Ca^{2+} is raised from 1 to ≥ 4 mM). Secondly, the increase in luminal $[\text{Ca}^{2+}]$ can consistently dissociate CSQ, inducing a further significant rise in RyR activity, and 5 mM luminal Ca^{2+} can prevent reassociation of CSQ with the RyR/T/J complex. It is not likely that the dissociation of CSQ from the junctional face membrane would be caused by normal physiological changes in luminal $[\text{Ca}^{2+}]$, and therefore CSQ dissociation-induced changes in RyR activity may not be of physiological importance. However, levels of luminal Ca^{2+} of ~ 10 mM are obtained experimentally when loading the SR to above-normal levels (Lamb et al., 2001), and such loading leads to enhanced Ca^{2+} release. The contribution of CSQ dissociation from the junctional face membrane in the SR to this increase in activity remains to be investigated.

Although not investigated here, CSQ might influence RyR function in response to changes in total luminal $[\text{Ca}^{2+}]$ in a similar manner to calmodulin (associated with cytoplasmic

$[\text{Ca}^{2+}]$) (Meissner 1986; Plank et al., 1983; Rodney et al., 2000; Tripathy et al., 1995; Xu and Meissner, 2004). Like calmodulin, the effect of CSQ on RyR function may depend on the Ca^{2+} binding status of CSQ (and CSQ conformation), which is likely to vary with changes in total luminal $[\text{Ca}^{2+}]$ (i.e., store loading). Changes in the amount of Ca^{2+} bound to CSQ could occur with only minor changes in the free $[\text{Ca}^{2+}]$ and might alter CSQ conformation without dissociation, thus causing subtle changes in RyR activity or its response to cytoplasmic ligands such as Ca^{2+} . Indeed, CSQ has been implicated as a major luminal Ca^{2+} sensor, whose presence is required (along with triadin and junctin), to enhance RyR responsiveness to changes in luminal Ca^{2+} concentration (this article; Györke et al., 2004).

In conclusion, we find that CSQ is associated with the RyR/T/J complex when free luminal Ca^{2+} is at a physiological concentration of ~ 1 mM, and we provide novel data showing that CSQ dissociates from the skeletal RyR complex when the free $[\text{Ca}^{2+}]$ increases to between 3 and 5 mM. The results show that CSQ sensitizes the RyR to changes in free $[\text{Ca}^{2+}]$ between 1 and 5 mM. Finally, we show that although CSQ is most likely phosphorylated in vivo, its ability to inhibit RyR activity does not depend on its phosphorylation status. These data provide mounting evidence that an intact quaternary complex between CSQ, triadin, junctin, and the RyR forms a signaling pathway that communicates the luminal $[\text{Ca}^{2+}]$ to the RyR channel.

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