Calsequestrin Is an Inhibitor of Skeletal Muscle Ryanodine Receptor Calcium Release Channels

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ABSTRACT We provide novel evidence that the sarcoplasmic reticulum calcium binding protein, calsequestrin, inhibits native ryanodine receptor calcium release channel activity. Calsequestrin dissociation from junctional face membrane was achieved by increasing luminal (*trans*) ionic strength from 250 to 500 mM with CsCl or by exposing the luminal side of ryanodine receptors to high [Ca^{2+}] (13 mM) and dissociation was confirmed with sodium dodecyl sulfate-polyacrylamide gel electrophoresis and Western blotting. Calsequestrin dissociation caused a 10-fold increase in the duration of ryanodine receptor channel opening in lipid bilayers. Adding calsequestrin back to the luminal side of the channel after dissociation reversed this increased activity. In addition, an anticalsequestrin antibody added to the luminal solution reduced ryanodine receptor activity before, but not after, calsequestrin dissociation. A population of ryanodine receptors (~35%) may have initially lacked calsequestrin, because their activity was high and was unaffected by increasing ionic strength or by anticalsequestrin antibody: their activity fell when purified calsequestrin was added and they then responded to antibody. In contrast to native ryanodine receptors, purified channels, depleted of triadin and calsequestrin, were not inhibited by calsequestrin. We suggest that calsequestrin reduces ryanodine receptor activity by binding to a coprotein, possibly to the luminal domain of triadin.

INTRODUCTION

Contraction in skeletal and cardiac muscle is initiated by a cascade of events collectively known as excitation-contraction coupling, in which depolarization of the sarcolemma triggers Ca^{2+} release through ryanodine receptor (RyR) Ca^{2+} release channels in the sarcoplasmic reticulum (SR). Releasable Ca^{2+} is loosely bound to, and stored on, a glycoprotein calsequestrin (CSQ), which is located within the SR lumen. CSQ is highly acidic with a moderate affinity ($K_D = 4 \times 10^{-5}$ M) and high capacity (40–50 mol Ca^{2+}/mol CSQ) for Ca^{2+} (MacLennan and Wong, 1971).

CSQ monomers form a linear polymer that is tethered through accessory proteins to the RyR (Costello et al., 1986) and can be visualized in thin section electron micrographs, where it appears to be anchored to the junctional face membrane (JFM) (Franzini-Armstrong et al., 1987). Anchoring to the JFM and the RyR occurs via triadin and junctin (Jones et al., 1995; Zhang et al., 1997; Mitchell et al., 1988). Like calmodulin, CSQ monomers undergo a conformational change upon Ca^{2+} binding, resulting in compaction and folding with an associated increase in α-helical content (Ikemoto et al., 1972). The Ca^{2+}-induced folding of monomeric CSQ is followed by aggregation into a polymer (He et al., 1993; Wang et al., 1998). Amino acids 354 to 367, a region near the N-terminus of CSQ, is rich in aspartate residues and is thought to form the binding site for triadin (Zhang et al., 1997; Wang et al., 1998; Shin et al., 2001). Another region near the N-terminus of CSQ is thought to form a binding site for junctin (Wang et al., 1998; Zhang et al., 1997).

Both triadin and junctin span the SR membrane, both bind to CSQ and the RyR, and both share structural similarities and significant sequence homology (Mitchell et al., 1988; Guo et al., 1996; Knudson et al., 1993; Jones et al., 1995; Zhang et al., 1997). The binding site on triadin for CSQ has recently been localized to a KEKE motif at amino acids 210 to 224 (multiple alternate lysine and glutamic acid residues), whereas junctin is thought to have several sites involved in CSQ binding (Kobayashi et al., 2001). A binding site for CSQ on the RyR has not yet been identified.

Several reports show that CSQ activates purified RyRs (Szegedi et al., 1999; Herzog et al., 2000). The effect of CSQ on native RyRs is more controversial because the calcium binding protein has been variously reported to activate (Kawasaki and Kasai, 1994; Ohkura et al., 1995) or inhibit RyR channel activity (Beard et al., 1999, 2000; Wang et al., 2001). Because the conformation of CSQ changes over the physiological range of luminal [Ca^{2+}] (Ikemoto et al., 1989), it has been suggested that CSQ plays a role in regulating RyR activity in response to changes in luminal [Ca^{2+}] (Donoso et al., 1995; Yamaguchi et al., 1995).

Here we examine the effects of CSQ dissociation and reassociation on single channel activity under carefully controlled conditions. The results show that 1) CSQ dissociation enhances native RyR activity, while CSQ binding suppresses channel opening, 2) CHAPS solubilized RyRs (without triadin and presumably junctin) showed a marginal increase in activity when exposed to CSQ, and 3) antibody

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binding to CSQ further inhibits native RyR channel activity. Preliminary results have been presented (Beard et al., 1999, 2000).

MATERIALS AND METHODS

Materials

The polyclonal anti-CSQ antibody was obtained from Swant Chemicals, (Bellinzona, Switzerland). Other chemicals were from Sigma-Aldrich (Castle Hill, Australia).

SR vesicles

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). Briefly, the muscle was differentially centrifuged to yield a crude microsomal fraction, which was fractionated by loading onto a discontinuous sucrose gradient. Heavy SR vesicles were collected from the 35 to 45% (wt/vol) interface, centrifuged, resuspended in buffer, then snap frozen, and stored in liquid N_2 or at -70°C.

RyR purification

RyRs were purified from SR vesicles using methods of Lai et al. (1988). SR vesicles were incubated at room temperature for 2 h in a solubilization medium containing: 25 mM Pipes, 1 M NaCl, 0.5% CHAPS, 0.25% phosphatidylcholine (PC), with protease inhibitors (0.8 mM benzamadine, 0.1 mM phenylmethylsulfonyl fluoride (PMSF), 1 μg/ml leuopeptin, and 0.6 μg/ml pepstatin A). After centrifugation for 15 min at 100,000 $\times$ *g*, the supernatant was applied to a sucrose gradient of between 5 to 25% and ultracentrifuged at 80,000 $\times$ *g* for 16 h. Fractions enriched with RyRs were collected and RyRs visualized after sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting. Fractions enriched with RyR were concentrated by centrifugation at 5000 $\times$ *g* for 30 min in a centricon-30 centrifugal concentrator and then dialysed overnight against: 500 mM NaCl, 10 mM NaPipes, 1 mM dithiothreitol, 100 μM EGTA, 200 μM $CaCl_2$, and 1.5 μM PMSF (pH 7.4) at 4°C. Aliquots ($<$200 μl) were stored frozen at -70°C.

Calsequestrin purification

JFM was isolated as described Kim et al. (1983). Briefly, 200 g of chopped muscle was homogenized in a Waring Blender in 800 ml of a solution containing 2.5 mM NaOH and 20 mM MES (pH 6.8), centrifuged at 10,000 $\times$ *g* for 3 min, the supernatant filtered, centrifuged twice at 17 000 $\times$ *g* for 30 min, and the heavy SR pellet collected. CSQ was isolated as described by Ikemoto et al. (1972, 1989). SR was suspended in a solution containing 0.3 M sucrose, 20 mM MES (pH 6.8) plus protease inhibitors (0.5 mM PMSF, 1 μg/ml leupeptin, 1 μg/ml pepstatin A, 1 mM benzamadine), solubilized in 0.5% Triton X-100 (at 0°C), and centrifuged at 350,000 $\times$ *g* for 15 min. The pellet was homogenized in 500 mM NaCl, 20 mM MES (pH 6.8) plus protease inhibitors (above) to dissociate CSQ from JFM. JFM was removed by centrifugation at 350,000 $\times$ *g* for 15 min. The supernatant was dialyzed twice overnight at 4°C against milliQ water to remove residual Triton X-100, concentrated by centrifugation at 5000 $\times$ *g* for 30 min in a centricon-30 centrifugal concentrator, resuspended at $\sim$1 mg/ml in 10 mM TES, 100 mM KCl, and 1 mM $CaCl_2$, and stored in 20-μl aliquots at -70°C.

To demonstrate selective dissociation of CSQ from solubilized SR using high [Ca^{2+}] on SDS-PAGE and Western blots, the following alterations to the above procedure were made. After centrifugation of the Triton X-100 solubilized SR at 110,000 $\times$ *g* for 15 min (Beckman bench top ultracentrifuge using a TLA-100.3 rotor), the pellet was resuspended at 1 mg/ml in a solution containing 300 mM sucrose, 13 mM $CaCl_2$, 20 mM MOPS-Tris (pH 6.8) plus protease inhibitors (above). The resultant suspension was homogenized in a dounce glass homogenizer and then centrifuged at 110,000 $\times$ *g* for 15 min. The supernatant, containing calsequestrin, was collected and after dialysis to remove residual Triton X-100, the final pellet was suspended at an approximate concentration of 0.5 mg/ml in 20 mM MOPS-Tris, 100 mM KCl, and 1 mM $CaCl_2$, in small aliquots ($<$100 μl) at -70°C

Electrophoresis and Western blotting

SDS-PAGE gels (6.5, 12, 5–17, or 3–12%) (Laemmli, 1970) were stained with Commassie Brilliant Blue or silver stain, as appropriate. Proteins were transferred onto Immobillon-P polyvinylidene difluoride (PVDF) transfer membranes (Towbin et al., 1992). Either an alkaline phosphatase or horseradish peroxidase development system was used for immunodetection.

Single channel recording

Lipid bilayers were formed from phosphatidylethanolamine (PE), phosphatidylserine (PS), and PC (5:3:2 wt/wt) or PE and PC (8:2 wt/wt), across a 200- to 250-μm aperture in the wall of a delrin cup, separating *trans* (luminal) and *cis* (cytoplasmic) solutions (Ahern et al., 1994), containing *cis*: 230 mM Cs methanesulfonate (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. SR vesicles were added to the *cis* solution. Three-hundred millimolar mannitol was added to the *cis* chamber in some experiments to aid incorporation. After incorporation 1) *cis* [Ca^{2+}] was lowered to $\sim$100 nM with 4.5 mM 1,2-bis(2-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-terra-acetic acid (BAPTA) to prevent further incorporation. Free [Ca^{2+}] was calculated using the Bound and Determined program (Brooks and Storey, 1992), 2) 2 mM Na-ATP was added to the *cis* solution, and 3) 200 mM CsMS added to the *trans* solution. During initial experiments 3 mM $MgCl_2$ (1 mM free Mg^{2+}) was included in the *cis* solution to mimic the effects of free Mg^{2+} in muscle. However, Mg^{2+}, a RyR inhibitor was omitted from later experiments, to record measurable channel activity. Solutions were changed by perfusion using back-to-back syringes. Potential was controlled and current recorded via an Axopatch 200A amplifier. The potential of the *trans* solution was held at ground and the *cis* potential varied. However potential is expressed conventionally as $V_{cis} - V_{trans}$. Currents were filtered at 1 kHz and digitized at 5 kHz. 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). Overall channel activity was measured by either mean current (I', average of all data points obtained during a recording period), relative mean current (I'_t/I'_c in which I'_t is the mean current under test conditions, and I'_c is the mean current under control conditions) or fractional mean current (I'_F, i.e., I' divided by the maximal current I_{max}). A half-amplitude detection method was used to determine channel open probability (P_o), opening frequency (F_o), mean open (T_o), and mean intraburst closed times (T_c, a burst is defined as a group of sequential openings terminated by a closed interval longer than 1 s). Channel activity was analyzed over 30 s of continuous recording at $\pm$40 mV with 250/250 mM Cs^+ (*cis*/*trans*) or at +57 mV and -23 mV with 250/500 mM Cs^+. In either case the membrane potential, V_m, was 40 mV positive or negative to the current reversal potential, E_{Cs}, of the RyR (i.e., $V_m - E_{Cs} = \pm 40$ mV).

Statistics

Average data are presented as mean $\pm$ SE. The significance of differences between control and test values were tested using a Student's *t*-test for

paired or independent data or a sign test (Minium et al., 1993), as appropriate.

RESULTS

Calsequestrin purification

JFM contained a 55-kDa protein corresponding to CSQ (Fig. 1 *A*, lane 1). Exposure of JFM to a high salt solution allowed isolation of this protein (Fig. 1 *A*, lane 2), which was detected by the polyclonal anti-CSQ antibody (lane 3). Approximately 5 mg of CSQ was purified from 200 g of muscle. A faint band at ~30 kDa on SDS-PAGE (not apparent in Fig. 1 *A*), present after the first dialysis, was removed by the second dialysis. The protein may be the 30-kDa luminal SR protein thought to regulate RyRs (Yamaguchi et al., 1997). However, the presence of the protein in some experiments ($n = 5$) neither affected RyR activity nor altered the channel's response to CSQ.

RyR purification

SDS-PAGE of native SR showed a strong band at ~400 kDa (presumably the RyR, Fig. 1 *B*, lane 1) and at ~110 kDa (presumably Ca-ATPase). CHAPS-solubilized SR showed a strong band at ~400 kDa and a weak band at ~110 kDa (Fig. 1 *B*, lane 2), indicating that the solubilized fraction contained mostly RyR with some contaminant Ca-ATPase. Immunoprobing the gels with anti-CSQ or antitriadin antibodies showed that CSQ (Fig. 1 *B*, lane 3) and triadin (lane 5) were present in native SR but were removed in the solubilized fraction (lanes 4 and 6, respectively). A faint band at 26 kD, which corresponds to junctin, can be seen in the native SR (lane 1), which was not present in the solubilized fraction (lane 2). When the solubilized fraction was incorporated into bilayers, channel activity of 510 pS was observed and responded to cytoplasmic Ca^{2+}, ATP and ruthenium red in a similar way to native RyRs (data not shown).

Response of RyR channels to increased ionic strength

Channel activity is shown and analyzed at a potential 40 mV positive to the current reversal potential (Materials and Methods). Similar results were obtained at 40 mV negative to the reversal potential under all conditions. The CSQ purification method (above) was modified to dissociate CSQ from SR in bilayers. RyR openings were infrequent and brief in the presence of (*cis*) 250 mM Cs^+, 100 nM Ca^{2+}, 2 mM ATP, and 3 mM Mg^{2+} and (*trans*) 250 mM Cs^+ and 1 mM Ca^{2+} (luminal control conditions) (Fig. 2 *A*), but increased within 2 to 5 min after increasing *trans* ionic strength to 500 mM (with 250 mM CsCl) (Fig. 2 *B*). The *trans* ionic strength was then returned to 250 mM to determine whether the increased activity was due to increased ionic strength per se or to an irreversible process, such as CSQ dissociation. Channel activity remained high after

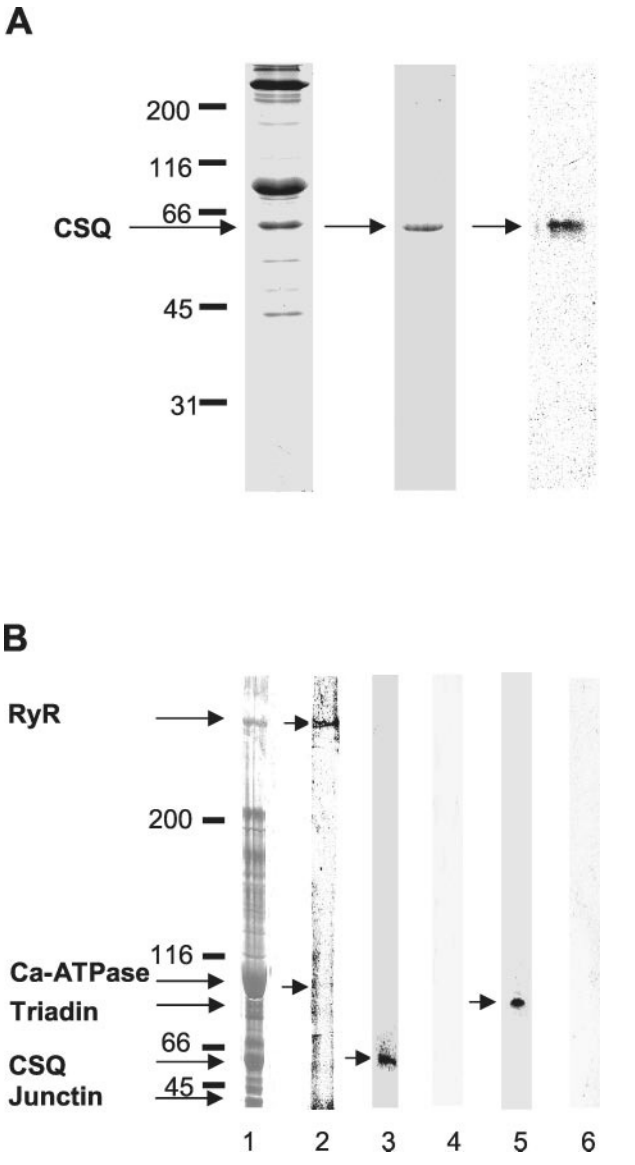

FIGURE 1 (*A*) SDS-PAGE (12%) showing a 55-kDa band in JFM (lane 1) and in supernatant after exposure of JFM to 500 mM NaCl (lane 2). The band was identified as CSQ on Western blots using anti-CSQ antibody (lane 3). The position of molecular weight markers is shown next to lane 1. Proteins were transferred onto Immobilon-P PVDF membranes before probing with anti-CSQ. (*B*) Silver stain of SDS-PAGE (5–17%) showing a ~110- and ~400-kDa protein in native SR (lane 1) after purification of RyRs (lane 2). The resolution of the silver stain in lane 2 has been enhanced to illustrate the removal of all SR proteins (besides RyR and Ca-ATPase) after the purification procedure. CSQ (lanes 3 and 4) and triadin (lanes 5 and 6) were detected in native SR membranes (lanes 3 and 5) but not in purified RyR fractions (lanes 4 and 6) after Western blotting with anti-CSQ or antitriadin antibodies. The protein located at ~110 kDa is presumed to be Ca-ATPase and the 400-kDa protein to be the RyR. The position of molecular weight markers is shown next to lane 1. There was no band at 26 kDa (lane 2) that would correspond to junctin.

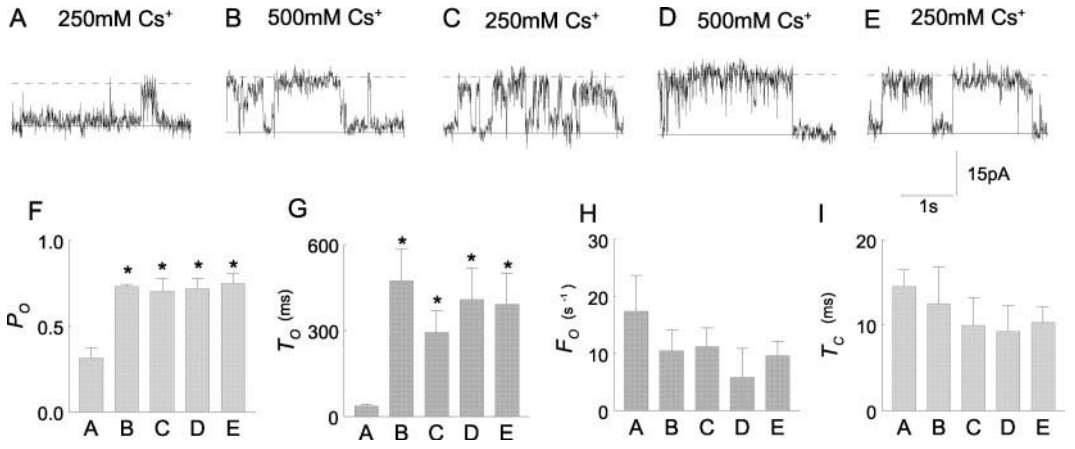

FIGURE 2 Increasing *trans* ionic strength to 500 mM causes an irreversible increase in single RyR channel activity. $V_m - E_{Cs}$ was +40 mV under all conditions. Single channel opening is upward from the zero current level (*continuous line*) to the maximal open conductance (*broken line*). The solutions contained: *cis* 100 nM free Ca^{2+}(1 mM $CaCl_2$ plus 4.5 mM BAPTA), 2 mM ATP, 3 mM $MgCl_2$, and 250 mM Cs^+; *trans* 250 or 500 mM Cs^+ and 1 mM $CaCl_2$. (*A*) Control activity with 250 mM *trans* Cs^+; (*B*) after increasing *trans* Cs^+ to 500 mM with CsCl; (*C*) perfusing the *trans* chamber with 250 mM Cs^+; (*D* and *E*) repeat of *B* and *C*. (*A–E*) *Trans* [Cs^+] is shown above each record. (*F–I*) Average data ($n = 5$) for P_o (*F*), T_o (*G*), F_o (*H*), and T_c (*I*) under conditions shown in *A* through *E*. Asterisks (*) indicate average values significantly different from control.

perfusion (Fig. 2 *C*) and repeated exposure to 500 mM *trans* Cs^+ (Fig. 2 *D*) and reperfusion with 250 mM Cs^+ (Fig. 2 *E*).

Activity in one of six channels did not change upon exposure to high *trans* ionic strength. In the other five channels, initial exposure to 500 mM Cs^+ increased P_o and T_o with significant increases in both average parameter values for the six channels (Fig. 2, *F* and *G*). Although F_o fell in four of the six channels and T_c fell in four channels, average F_o and T_c did not change significantly (Fig. 2, *H* and *I*). Single channel conductance at positive bilayer potentials did not change when increasing *trans* [Cs^+] from 250 to 500 mM, I_{max} was 11.3 ± 0.7 pA ($n = 10$) before and 12.1 ± 0.9 pA after exposure to high ionic strength. P_o and T_o remained significantly higher than control when *trans* ionic strength was subsequently varied between 250 and 500 mM. An irreversible increase in channel activity caused by the initial increase in *trans* ionic strength was consistent with CSQ dissociation. Presumably, once dissociated, CSQ did not reassociate with RyRs 1) because it was immediately diluted in the *trans* solution, and 2) it was further diluted with perfusion of the *trans* bath.

Reassociation of calsequestrin

If the increased RyR activity after exposure to high ionic strength was due to CSQ dissociation, addition of excess CSQ should then reduce channel activity. Activity fell in each of five bilayers (containing one or two channels) when 20 μg of purified CSQ was added to *trans* solutions containing 250 mM Cs^+ after they had been activated with 500 mM Cs^+ (Fig. 3, *A–D*). It seems extremely unlikely that this reduction in channel activity is a result of CSQ chelating Ca^{2+}, thereby lowering $[Ca^{2+}]_{free}$ to levels such that a direct Ca^{2+}-induced RyR inhibition was observed. CSQ (stored in 1 mM Ca^{2+}), which can bind 900 nmol Ca^{2+}/mg (Campbell, 1986), would produce an insignificant reduction in $[Ca^{2+}]_{free}$ (1 mM to over 0.999 mM). RyR activity that increased after initial exposure to high ionic strength then decreased significantly upon CSQ addition (Fig. 3 *D*). I'_F (relative mean current, see Materials and Methods and Fig. 3 legend) increased threefold upon exposure to 500 mM Cs^+ and was restored to control levels upon CSQ addition. The values of I'_F were 0.11 ± 0.02 under control conditions, 0.40 ± 0.10 with 500 mM CsCl, and 0.15 ± 0.04 with 20 μg CSQ. This result is consistent with the initial increase in activity being due to CSQ dissociation. Activity in another four bilayers did not increase with the initial increase in *trans* [Cs^+], but CSQ (20 μg) still inhibited channels in all four experiments. I'_F was 0.37 ± 0.03 under control conditions, 0.33 ± 0.02 with 500 mM CsCl, and 0.05 ± 0.02 with 20 μg CSQ. These channels, and the unresponsive channel in the previous section, may have initially lacked associated CSQ.

Effect of anticalsequestrin antibody on RyR activity

A polyclonal antibody to CSQ (0.5 μg/ml *trans*) inhibited five of eight RyRs (Fig. 4, *A* and *B*) with significant reductions in P_o and F_o and an increase in T_c (Fig. 4, *C*, *E*, and *F*). CSQ may not have been associated with the three channels that were not inhibited by either 5 or 50 μg/ml antibody. In control experiments, the anti-CSQ storage buffer (0.62 mM NaN_3) alone did not alter RyR activity when added to the *trans* chamber. I'_F was 0.23 ± 0.05 under

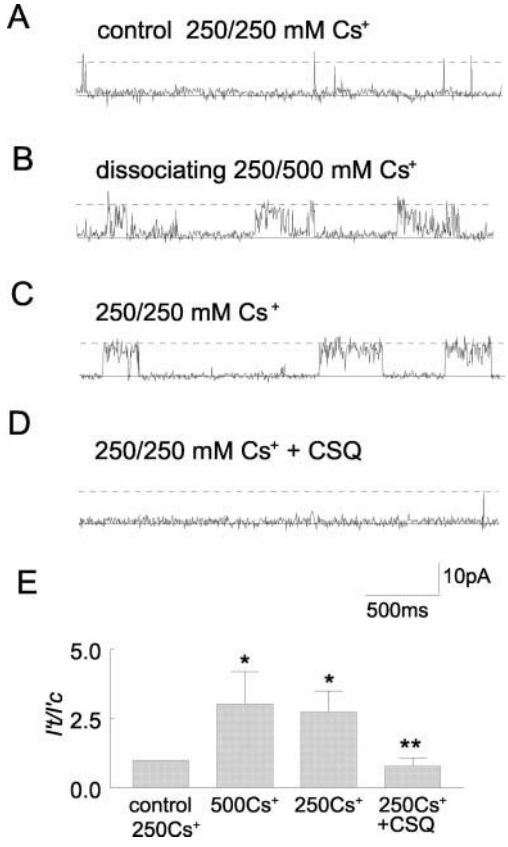

FIGURE 3 Excess CSQ reverses the increase in RyR activity seen with high ionic strength. $V_m - E_{Cs}$ was +40 mV under all conditions, and channel opening is upward from the zero current (*continuous line*) to the maximal open conductance (*broken line*). Solutions contained *cis* 100 nM Ca^{2+}, 2 mM ATP, and 250 mM Cs^+; *trans*, 250 or 500 mM Cs^+ and 1 mM $CaCl_2$. (*A*) Control channel activity; (*B*) after increasing *trans* Cs^+ to 500 mM; (*C*) after *trans* perfusion with 250 mM Cs^+; and (*D*) addition of 20 μg of *trans* CSQ. In *A* through *D*, [Cs^+]s *cis/trans* are shown for each record. (*E*) Average data ($n = 5$) for the relative mean current (I'_t/I'_c) under conditions in A through D (I'_t/I'_c is defined in Materials and Methods). *, Significant increase in I'_t/I'_c with 500 mM Cs^+ and perfusion; **, significant fall after adding CSQ.

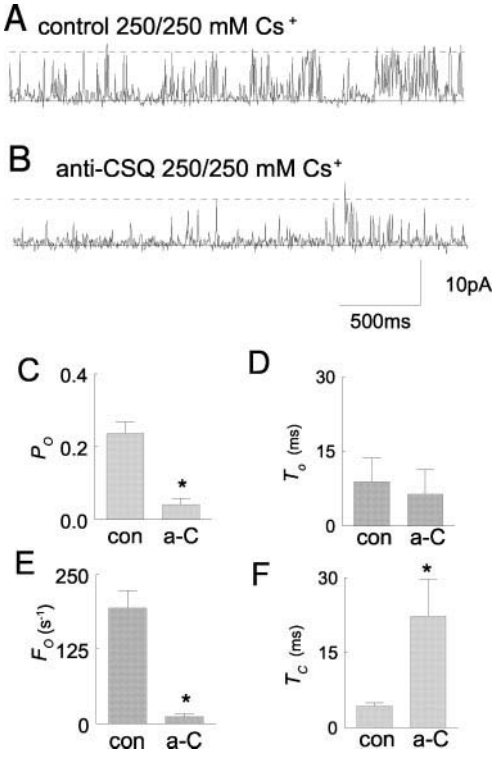

FIGURE 4 Anti-CSQ antibody reduces single RyR channel activity. $V_m - E_{Cs}$ was at +40 mV, single channel opening is upward from the zero current (*continuous line*) to the maximal open conductance (*broken line*). The solutions contained: *cis* 100 nM Ca^{2+}, 2 mM ATP, and 250 mM Cs^+; *trans*, 250 mM Cs^+ and 1 mM $CaCl_2$. (*A*) Single channel activity under control conditions; (*B*) after adding 0.5 μg/ml anti-CSQ to the *trans* solution. In *A* and *B*, [Cs^+]s *cis/trans* are shown above each record; (*C–F*) average data ($n = 5$) for P_o (*C*), T_o (*D*), F_o (*E*), and T_c (*F*) under control conditions (*con*) and after adding anti-CSQ antibody (*a-C*). *, Average values significantly different from control.

control conditions and 0.27 ± 0.04 with NaN_3 ($n = 4$). Note that Mg^{2+} was not present in the *cis* chamber during these experiments, so that the P_o and F_o values obtained here were higher than those obtained from channels in the presence of Mg^{2+} (Fig. 2, *F* and *H*).

The antibody did not alter activity in 10 of 10 RyRs when added to the *trans* solution after treatment with 500 mM *trans* Cs^+ (Fig. 5). I'_F in one channel, which possibly lacked CSQ, was 0.24 under control conditions, 0.24 in 500 mM Cs^+, and 0.23 with antibody. Conversely, RyR activity fell in each of three experiments in which RyRs were exposed to antibody (0.5 μg/ml) after they had been exposed to 500 mM *trans* Cs^+ and then to 20 μg of purified CSQ. I'_F fell from 0.05 ± 0.02 to 0.016 ± 0.007. Together these results 1) suggest that the antibody inhibited RyRs by binding to CSQ and 2) confirm that the high ionic strength solution dissociated CSQ from the RyR complex.

Some RyR channels may not have associated calsequestrin under control conditions

If RyRs that failed to respond either to an increase in *trans* ionic strength or to anti-CSQ antibody, lacked associated CSQ under initial control conditions, they should have had higher control activity than responsive channels, because activity increased when CSQ was removed (above results). A plot of I'_F for 33 responsive and 18 unresponsive channels (Fig. 6) shows mostly greater activity in unresponsive than in responsive channels. Unresponsive RyRs had a significantly ($p < 10^{-13}$) higher average I'_F (0.38 ± 0.03) than responsive RyRs (0.14 ± 0.01), indicating two populations of RyRs.

We further tested the hypothesis that channels with low activity contained associated CSQ, by exposing channels to CSQ (20 μg *trans*). Low activity channels did not show any

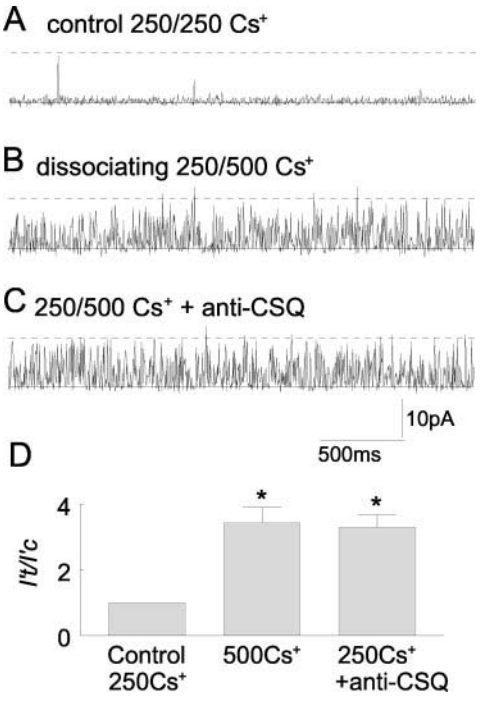

FIGURE 5 Anti-CSQ antibody does not alter activity in CSQ-depleted single RyRs. $V_m - E_{Cs}$ was +40 mV, single channel opening is upward from zero current (*continuous line*) to the maximal open conductance (*broken line*). The solutions contained: *cis* 100 nM Ca^{2+}, 2 mM ATP, 3 mM $MgCl_2$ (in 2 of 10 experiments) and 250 mM Cs^+; *trans*, 250 or 500 mM Cs^+ and 1 mM $CaCl_2$. (*A*) Control channel activity; (*B*) after increasing *trans* Cs^+ to 500 mM; (*C*) after adding 0.5 μg/ml anti-CSQ to the *trans* chamber. (*D*) Average relative mean current, I'_t/I'_c, ($n = 10$) under conditions in A through C. *, There is a significant increase in I'_t/I'_c above after increasing *trans* ionic strength to 500 mM.

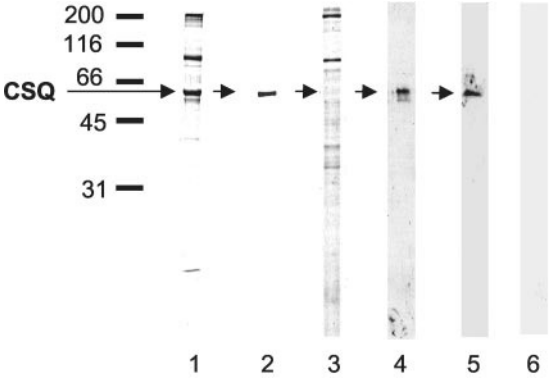

FIGURE 7 Increasing [Ca^{2+}] from 1 to 13 mM removed CSQ from the JFM. SDS-PAGE (5–17%) showing a 55-kDa band in JFM (lane 1) and in a purified CSQ fraction (lane 2) but virtually absent from JFM after exposure to 13 mM Ca^{2+} (lane 3). This band was confirmed as CSQ in heavy SR and purified CSQ fractions after Western blotting with anti-CSQ (lanes 4 and 5). No CSQ was detectable in the JFM pellet after exposure to 13 mM Ca^{2+} (lane 6). Proteins were transferred onto Immobilon-P PVDF membrane before probing with antibody. Note the very faint band at ~55 kDa in JFM after exposure to 13 mM Ca^{2+} (lane 3, *arrowhead*), so that a small fraction of CSQ remained associated with the JFM after exposure to 13 mM Ca^{2+}. Western blotting with anti-CSQ did not reveal the presence of CSQ in this band.

further reduction in channel activity with CSQ addition (four of four channels), whereas high activity channels showed a reduction in activity (six of six channels). This was consistent with added CSQ associating only with those channels that lacked CSQ (i.e., high activity channels) and the hypothesis that high activity channels lacked CSQ.

High luminal Ca^{2+} concentrations also cause an irreversible increase in RyR activity

Like high ionic strength, luminal Ca^{2+} concentrations greater than 10 mM have been reported to prevent the interaction between CSQ and triadin and CSQ and junctin (Zhang et al., 1997). We also found that there were no detectable levels of CSQ after JFM had been exposed to 13 mM Ca^{2+} (Fig. 7, lanes 3 and 6), suggesting that CSQ had

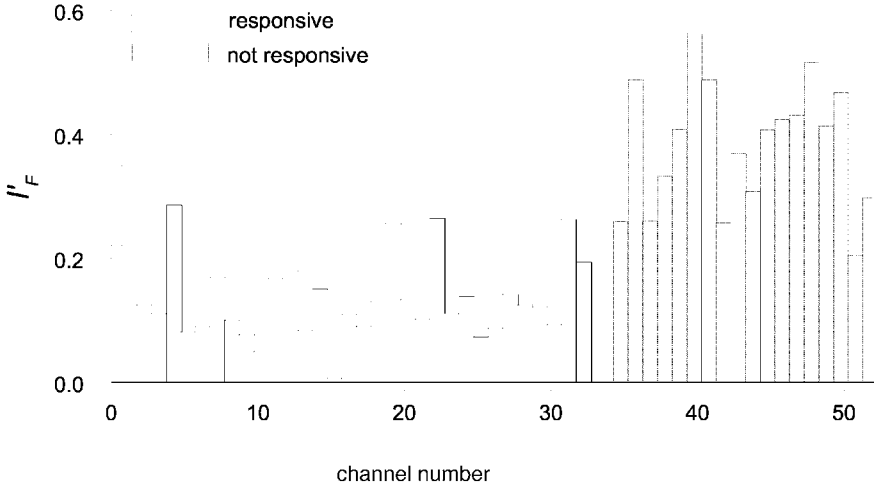

FIGURE 6 Control activity of single RyR channels that respond to procedures that influence CSQ is lower than control activity of responsive channels. Control mean current as a fraction of maximal current (I'_F) is shown. Channels whose activity increased with 500 mM *trans* Cs^+ (CSQ dissociation) or decreased with *trans* anti-CSQ antibody are numbered 1 to 33 (*empty bars*); channels that showed no consistent change in I'_F after either exposure to 500 mM *trans* Cs^+ or 0.5 μg/ml *trans* anti-CSQ are numbered 34 to 51 (*shaded bars*). The Student's *t*-test indicates that there are two populations of RyR channel (see Results).

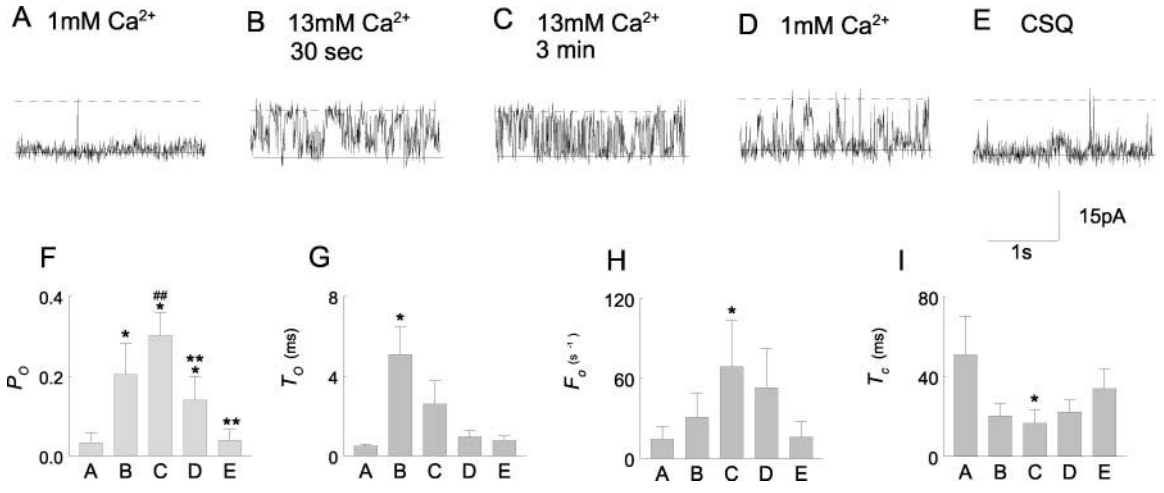

FIGURE 8 Increasing *trans* Ca^{2+} from 1 to 13 mM causes an increase in single RyR channel activity, which can only be fully reversed by adding excess CSQ to the solution. $V_m - E_{Cs}$ was +40 mV under all conditions. Single channel openings are upward from zero current (*continuous line*) to maximal open conductance (*broken line*). Solutions contained: *cis*, 100 nM Ca^{2+}, 2 mM ATP and 250 mM Cs^+; *trans*, 1 or 13 mM Ca^{2+} and 250 mM Cs^+. (*A*) Control channel activity with 1 mM *trans* Ca^{2+}; (*B*) 30 s after increasing *trans* Ca^{2+} to 13 mM; (*C*) 3 min after increasing *trans* Ca^{2+} to 13 mM; (*D*) after perfusing *trans* chamber with 1 mM Ca^{2+}; (*E*) addition of *trans* 20 μg/ml CSQ. *F* through *I* are average single channel parameter values ($n = 6$) for p_o (*F*), T_o (*G*), F_o (*H*), and T_c (*I*) under conditions shown in *A* through *E*. The * (*t*-test) indicates average values significantly different from control, ** (*t*-test) and cross-hatch (##) (sign test) indicate values that are significantly different from the preceding condition.

dissociated from the JFM. It is unlikely that a significant fraction of the CSQ was lost due to proteolytic degradation because proteolytic inhibitors were present throughout the isolation procedure (Materials and Methods).

The effect of high luminal [Ca^{2+}] on the RyR-CSQ complex and RyR activity was examined by exposing single RyRs to 13 mM *trans* Ca^{2+}. The time course of the response of RyRs showed two distinct phases: an immediate increase in activity followed by a slower increase after longer exposure to high [Ca^{2+}] (Fig. 8). The increase in activity was characterized by significant increases in average p_o, T_o, and F_o and a decline in T_c. When *trans* Ca^{2+} was returned to 1 mM, the channel activity fell significantly but did not return to its original level. However, addition of 20 μg/ml of purified CSQ to the *trans* chamber did cause a further reduction in activity to control levels. Because we have shown that addition of exogenous CSQ does not alter the activity of channels that are already associated with CSQ (above results), the CSQ-induced reduction in activity seen here provides additional evidence that high luminal Ca^{2+} had dissociated CSQ from the RyR complex.

To investigate the role of CSQ in the lumen on Ca^{2+}-dependent activation of RyRs, we measured the effect of high luminal [Ca^{2+}] on RyRs after CSQ had been removed by exposure to an ionic strength of 500 mM. Exposure to high luminal ionic strength produced a 1.8-fold increase in RyR activity, and increasing luminal [Ca^{2+}] to 13 mM then increased channel activity further. However, in contrast to the RyR-CSQ complex, the time course of activation of CSQ-depleted RyRs after an increase in luminal Ca^{2+} showed only a single, rapid phase with no slow phase. When *trans* [Ca^{2+}] was raised to 13 mM, RyRs activity increased within 20 s and did not increase further in activity over the next 5 min (Fig. 9). Returning [Ca^{2+}] to 1 mM by either perfusion of the *trans* chamber or the addition of 12 mM BAPTA, returned RyR activity to that seen before the increase in luminal [Ca^{2+}], but not to the low levels seen before dissociation of CSQ. Thus the different effects of luminal Ca^{2+} on RyR with and without CSQ indicates that 1) luminal Ca^{2+} activates RyRs by both CSQ dependent and independent mechanisms and 2) that CSQ dissociation underlies the slower component of RyR activation. The relatively slow secondary increase in RyR activity with high [Ca^{2+}] was similar to the slow increase in activity observed during ionic strength, suggesting that, in both cases, CSQ dissociation from the RyR complex takes 2 to 3 min.

Effects of calsequestrin on purified RyRs

The decrease in activity seen when CSQ is added to the luminal (*trans*) solution could have been due to CSQ binding to the RyR directly or to CSQ affecting the RyR after binding to triadin and junctin. To test these possibilities we used RyR purification, to remove triadin, CSQ, and presumably junctin (Fig. 1). Adding either 20 or 50 μg/ml of CSQ to purified RyRs either increased activity ($n = 3$, $I'_t/I'_c = 3.34 \pm 1.65$) or caused no significant change ($n = 2$, $I'_t/I'_c = 0.96 \pm 0.012$) in activity. The combined data for all five channels indicates an insignificant increase in activity ($I'_t/I'_c = 2.38 \pm 0.80$). Thus purified RyRs were not inhibited by CSQ, if anything they tended to be activated. The lack of

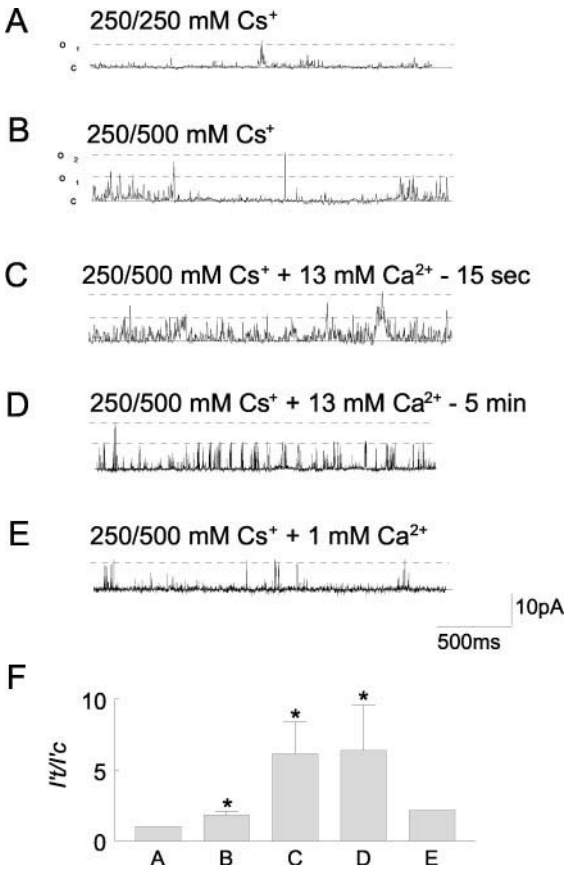

FIGURE 9 Addition of 13 mM Ca^{+} to the *trans* bath induces an immediate RyR activation in the absence of CSQ. $V_m - E_{Cs+}$ was +40 mV under all conditions. Channel openings are upward from the zero current level (*continuous line*) to the maximal open conductance (*broken line*) of each of two channels (O_1 and O_2). The solutions contained: *cis* 100 nM Ca^{2+}, 2 mM ATP and 250 mM Cs^{+}; *trans* 250 or 500 mM Cs^{+} and 1 or 13 mM $CaCl_2$. (*A*) Control activity with 250 mM *trans* Cs^{+}; (*B*) after increasing *trans* Cs^{+} to 500 mM; (*C*) [Ca^{2+}] increase from 1 mM to 13 mM; (*D*) reducing [Ca^{2+}] back to 1 mM; (*E*) relative mean current (I_t'/I_c') $n = 4$ for *A* through *C*, $n = 3$ for *D* and $n = 1$ for *E*, under conditions shown in *A* through *E*. Asterisks (*) indicate average values significantly different from control.

inhibition with purified RyRs is consistent CSQ requiring anchoring protein(s) to mediate its inhibitory effect.

DISCUSSION

Our results show that association of CSQ with the native RyR complex in lipid bilayers decreases channel activity. These results were obtained in the presence of ATP and Ca^{2+} concentrations which are present in resting muscle. We show that high luminal ionic strength (500 mM *trans*) or high luminal [Ca^{2+}] (13 mM), both of which dissociate CSQ from the JFM, cause an increase in RyR activity. In addition, our results suggest that the actions of CSQ on RyR activity depend on the presence of at least one coprotein (probably junctin and/or triadin) in the RyR complex. This is the first study to examine changes in channel activity during dissociation and reassociation of the Ca^{2+} binding protein.

Dissociation CSQ from RyRs

Several lines of evidence are presented that support the hypothesis that CSQ dissociation from the RyR complex can lead to an increase in channel activity. 1) Sixty-five percent of RyRs, initially observed with low activity, exhibited an increase in activity when exposed to high luminal (*trans*) ionic strength (Fig. 2). Lowering the ionic strength did not reverse the increase, but it was reversed by adding CSQ to the *trans* bath (Fig. 3). 2) These low activity RyRs were also activated when *trans* (luminal) [Ca^{2+}] was increased to concentrations known to dissociate CSQ from triadin and junctin (Figs. 7 and 8) (Zhang et al., 1997). Once again, adding CSQ reversed the effects. 3) Thirty-five percent of RyRs that were initially observed with high activity were unaffected by high ionic strength, but their activity could be lowered by adding CSQ. 4) An antibody that binds specifically to CSQ (Fig. 1) only affected RyR activity under conditions where it was expected that CSQ was associated with the RyR. For example, anti-CSQ antibody did not affect RyR activity after high ionic strength treatment (Fig. 5), but it did reduce activity if added after addition of CSQ. It is not clear how anti-CSQ caused the large decrease in RyR activity. Wang et al. (2001) have also reported the anti-CSQ antibody can reduce RyR activity. Regardless of the mechanism of action, anti-CSQ presumably exerted its effect by binding to CSQ and provides an additional method for confirming the presence of CSQ in the RyR complex.

Calsequestrin-depleted RyRs

Our results suggest that a discrete population of ~35% of RyRs did not initially have CSQ attached, because they did not respond to either high ionic strength or the anti-CSQ antibody. The absence of CSQ was not due to a lack of the necessary accessory protein (likely to be junctin/triadin), because channel activity fell after excess CSQ addition, showing that CSQ could bind and regulate channels. Our results alone do not show whether some RyRs lacked CSQ in vivo (suggested by Yamaguchi et al., 1997) or whether CSQ was dissociated during preparation of SR vesicles. It is possible that increasing luminal ionic strength from 50 to 250 mM before control recordings may have caused some dissociation, because 250 mM is higher than the ionic strength in vivo (~150 mM). If RyRs are partially depleted of CSQ in vivo, this would provide an explanation for the observation by Wang et al. (2000) that cardiac myocytes that over express CSQ have impaired Ca^{2+} release and a reduction of spontaneous Ca^{2+} sparks (i.e., reduced RyR

activity). Although protein over expression may induce nonspecific changes, it is interesting to speculate that some RyRs in cardiac myocytes that originally lacked CSQ, may be inhibited when CSQ was over expressed, because we observe CSQ-induced inhibition only in RyRs that lack CSQ.

The absence of CSQ in some cases provides additional insight into the marked heterogeneity commonly observed in RyR activity (Laver et al., 1995; Copello et al., 1997). The variation in activity within the CSQ-depleted and CSQ-replete RyR groups was less than the variability of the total RyR population, indicating that differences in CSQ status is an important factor in RyR variation. The variability observed within the two RyR groups may stem from RyRs that are differently coupled to other accessory proteins, known to affect RyR function, such as dihydropyridine receptor (Melzer et al., 1995), calsequestrin (Kawasaki and Kasai, 1994), FK506-binding (Timerman et al., 1993; Ahern et al., 1994), and calmodulin (Tripathy et al., 1995).

Different effects of CSQ on activity of native and purified RyRs

CSQ decreased the activity of native RyRs incorporated into bilayers from SR vesicles. However, with CHAPS solublized RyRs CSQ produced either no effect ($n = 2$) or caused a threefold increase in activity ($n = 3$). Because of the large difference between the two types of response, the combined mean ($n = 5$) showed a twofold activation that was not significant. None-the-less, these findings are not inconsistent with the CSQ-induced activation of purified RyRs reported by Szegedi et al. (1999). The different effects of CSQ seen in our experience may reflect differences in the phosphorylation state of each CSQ molecule that binds to the RyR (see below).

Different effects of CSQ on activity of native and purified RyRs suggest that the CSQ effect is somehow dependent on the composition of the RyR complex. For example, CHAPS may have removed a necessary coprotein from the RyR complex. Because triadin and junctin bind to both the RyR and CSQ (Zhang et al., 1997) and these proteins are absent in our purified RyRs (Fig. 1 *B*), it is likely that one or both triadin and junctin mediate the CSQ effect. Indeed, it has been shown that triadin modifies the effect of CSQ on RyR activity (Ohkura et al., 1998). The in vivo action of junctin/triadin on RyRs is not known, although the activity of purified RyRs is decreased by triadin (Ohkura et al., 1998; Groh et al., 1999) and peptides corresponding to the cytoplasmic domain of triadin inhibit RyR activity (Caswell et al., 1999; Groh et al., 1999). This indicates that the functional interaction between the proteins occurs on the cytoplasmic side of the membrane. In the light of these findings, we suggest that CSQ binding to the luminal domain of triadin somehow influences the interaction between its cytoplasmic domain and the RyR, enabling triadin to further inhibit RyR activity.

There have been conflicting reports of the effect of CSQ on single native RyR channels and ^{3}H-ryanodine binding. CSQ decreases RyR activity here (for preliminary reports, see Beard et al., 1999, 2000; Wang et al., 2001). In contrast, other studies show that CSQ increases both RyR activity and ^{3}H-ryanodine binding (Kawasaki and Kasai, 1994; Ohkura et al., 1995, 1998; Szegedi et al., 1999). It is unlikely that the conflicting reports of CSQ action on RyR are due simply to the incorporation of the RyR complex into an artificial membrane because this study and the studies of Kawasaki and Kasai (1994) and Ohkura et al. (1995) all used the lipid bilayer technique. A more likely explanation is that an essential coprotein remains associated with RyRs after the relatively mild dissociating procedures used here and by Wang et al. (2001) but was not present in other studies. Exposure to 500 mM *trans* Cs^+ (with 1 mM *trans* Ca^{2+}) or high (13 mM) luminal Ca^{2+} can disrupt charge-charge interactions between CSQ and junctin/triadin (Zhang et al., 1997; Kawasaki and Kasai, 1994; Ikemoto et al., 1989) and thus may dissociate CSQ, while leaving other RyR coproteins in place (Fig. 1, lane 2). On the other hand, many proteins in addition to CSQ, including triadin and junctin, are dissociated from the SR by incubation with EGTA and CHAPS (Kawasaki and Kasai, 1994; this study). Dissociation of an anchoring protein would account for the failure of CSQ to inhibit channel activity after incubation with EGTA or CHAPS.

The phosphorylation status of CSQ is important in determining the effect of CSQ on CHAPS purified RyRs (Szegedi et al., 1999). Dephosphorylated CSQ was able to induce an almost fivefold P_o increase, whereas the phosphorylated CSQ did not activate the RyR. Furthermore, Varsanyi and Heilmeyer (1980) report that CSQ is isolated in its phosphorylated form. Thus it is likely that a fraction of the CSQ used in this study is phosphorylated; some purified RyRs examined were unaffected by CSQ (i.e., the bound CSQ may have been phosphorylated), whereas others showed threefold activation (CSQ may not have been phosphorylated).

Regulation of RyRs by luminal [Ca^{2+}]

The level of Ca^{2+} loading of the SR is important in regulating Ca^{2+} release. Increasing or decreasing SR Ca^{2+} load increases or decreases (respectively) the sensitivity of Ca^{2+} release in response to depolarization (Bassani et al., 1995; Lamb et al., 2001), cytoplasmic Ca^{2+} (Copello et al., 1997), caffeine (Lamb et al., 2001), ATP (Donoso et al., 1995), and polylysine (Saiki and Ikemoto, 1999). Moreover, Lamb et al. (2001) find that endogenous levels of SR Ca^{2+} loading in fast (extensor digitorum longus (EDL)) and slow twitch (soleus) muscle is the primary factor determining the difference in caffeine sensitivities in the two fiber types. How-

ever, it is not clear how the Ca^{2+} load in the SR alters Ca^{2+} release.

These effects of SR Ca^{2+} load on Ca^{2+} release are also reflected in single RyR channel studies. Luminal Ca^{2+} increases channel activity in both purified (Tripathy and Meissner, 1996) and native RyRs (Sitsapesan and Williams, 1995; Beard et al., 2000). This increase in activity has been attributed to Ca^{2+} flowing from the luminal solution, through the channel, and binding to Ca^{2+} activation sites on the cytoplasmic side of the RyR (Tripathy and Meissner, 1996). An alternative hypothesis is that luminal Ca^{2+} is sensed by regulatory sites on the luminal side of the RyR (Sitsapesan and Williams, 1995; Ching et al., 2001). Because these bilayer studies were performed under conditions where CSQ (and possibly triadin and junctin) were not likely to have been present, regulation by CSQ bound to triadin/junctin can be discounted. Triadin and junctin were not present when RyRs were purified using CHAPS solublization (Tripathy and Meissner, 1996). Evidence suggests that CSQ may not have been present when native RyRs were incorporated into bilayers in the absence of luminal Ca^{2+} (Kawasaki and Kasai, 1994; Sitsapesan and Williams, 1995; Wang et al., 1998; Ching et al., 2001).

It has been proposed in other studies that CSQ is involved in RyR regulation by luminal Ca^{2+} (for review, see Donoso et al., 1995). SR Ca^{2+} release is most sensitive to luminal Ca^{2+} over the same range of Ca^{2+} (0–2 mM) that induces substantial, reversible conformational changes in CSQ (Ikemoto et al., 1989; He et al., 1993). We present evidence that high levels of luminal [Ca^{2+}] can activate RyRs by at least two mechanisms, one that is independent of calsequestrin and another that involves CSQ dissociation from the RyR complex. Raising [Ca^{2+}] from 1 to 13 mM dissociates CSQ from the JFM (Fig. 7), inducing a 2.4-fold rise in RyR activity, which could be fully reversed by adding back CSQ (Fig. 8). Wang et al. (2001) also found that 10 mM Ca^{2+} induces a 2.1-fold increase in p_o of cardiac RyRs in bilayers. These, and our studies are consistent with previous findings that high luminal [Ca^{2+}] disrupts the charge-charge interactions binding triadin, junctin, and CSQ (Mitchell et al., 1988; Zhang et al., 1997). Presumably the CSQ-independent mechanism is due to a direct activation of the RyR by luminal Ca^{2+} seen in single channel studies (see above).

The role of CSQ in RyR function in vivo is not clear. CSQ might regulate RyRs via dissociation of CSQ, although, it is not likely that the dissociation of CSQ from the JFM would be caused by normal physiological changes in luminal [Ca^{2+}] and so may not be an important mechanism in vivo. 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. Whether this occurs due to CSQ dissociation from the JFM or another mechanism remains to be investigated. It is also possible that luminal Ca^{2+} can also regulate RyR activity via Ca^{2+}-induced changes in CSQ conformation within the physiological range of luminal Ca^{2+}. These changes in RyR activity may be more subtle than the gross changes that we see with CSQ dissociation. Thus, like calmodulin, which modifies RyR regulation in a manner dependent on cytoplasmic [Ca^{2+}] (Tripathy and Meissner, 1995), CSQ may be an important coprotein, modifying RyR function in a luminal [Ca^{2+}]-dependent way. Finally, given that triadin binds to both CSQ and to the cytoplasmic side of the RyRs (Groh et al., 1999), it is possible that triadin forms part of the signaling pathway by which CSQ can communicate information about the level of luminal Ca^{2+} to the RyR.

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