Luminal Ca²⁺ Regulation of Single Cardiac Ryanodine Receptors: Insights Provided by Calsequestrin and its Mutants

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The luminal Ca²⁺ regulation of cardiac ryanodine receptor (RyR2) was explored at the single channel level. The luminal Ca²⁺ and Mg²⁺ sensitivity of single CSQ2-stripped and CSQ2-associated RyR2 channels was defined. Action of wild-type CSQ2 and of two mutant CSQ2s (R33Q and L167H) was also compared. Two luminal Ca²⁺ regulatory mechanism(s) were identified. One is a RyR2-resident mechanism that is CSQ2 independent and does not distinguish between luminal Ca²⁺ and Mg²⁺. This mechanism modulates the maximal efficacy of cytosolic Ca²⁺ activation. The second luminal Ca²⁺ regulatory mechanism is CSQ2 dependent and distinguishes between luminal Ca²⁺ and Mg²⁺. It does not depend on CSQ2 oligomerization or CSQ2 monomer Ca²⁺ binding affinity. The key Ca²⁺-sensitive step in this mechanism may be the Ca²⁺-dependent CSQ2 interaction with triadin. The CSQ2-dependent mechanism alters the cytosolic Ca²⁺ sensitivity of the channel. The R33Q CSQ2 mutant can participate in luminal RyR2 Ca²⁺ regulation but less effectively than wild-type (WT) CSQ2. CSQ2-L167H does not participate in luminal RyR2 Ca²⁺ regulation. The disparate actions of these two catecholaminergic polymorphic ventricular tachycardia (CPVT)–linked mutants implies that either alteration or elimination of CSQ2-dependent luminal RyR2 regulation can generate the CPVT phenotype. We propose that the RyR2-resident, CSQ2-independent luminal Ca²⁺ mechanism may assure that all channels respond robustly to large (>5 µM) local cytosolic Ca²⁺ stimuli, whereas the CSQ2-dependent mechanism may help close RyR2 channels after luminal Ca²⁺ falls below ∼0.5 mM.

INTRODUCTION

In cardiac muscle, the type-2 ryanodine receptor (RyR2) channel plays a central role in excitation–contraction coupling. The cardiac action potential triggers a small Ca²⁺ influx through the L-type Ca²⁺ channel and this Ca²⁺ influx activates nearby RyR2 channels, initiating Ca²⁺ release from the SR. This process is known as Ca²⁺-induced Ca²⁺ release (CICR). How CICR is controlled in cells remains an open question.

The open probability (Po) of single RyR2 channels is sensitive to changes in the intra-SR Ca²⁺ level (Lukyanenko et al., 1996; Gyorke and Gyorke, 1998). A drop in local intra-SR Ca²⁺ has been proposed to help "turn off" RyR2-mediated SR Ca²⁺ release, stabilizing CICR in cardiac muscle (Lukyanenko et al., 2001; Terentyev et al., 2002; Gyorke et al., 2004). The mechanism of this RyR2 intra-SR (luminal) Ca²⁺ regulation, however, is not well understood. It may involve a Ca²⁺ binding site(s) on the luminal surface of the RyR2 channel itself and/or Ca²⁺ interactions with RyR2-associated intra-SR regulatory proteins like calsequestrin (CSQ). Calsequestrin is a low affinity, high capacity intra-SR Ca²⁺-binding protein (Fliegel et al., 1987; Scott et al., 1988; Choi and Clegg, 1990; Fujii et al., 1990; Arai et al., 1991; Treves et al., 1992). Cardiac muscle contains only one CSQ isoform

(CSQ2; Lahat et al., 2001) whereas skeletal muscle contains two (CSQ1 and CSQ2; Paolini et al., 2007, and references therein). The two CSQ isoforms are quite similar but the C terminus of CSQ2 possesses variable lengths of acidic residues and two consensus phosphorylation sites (Yano and Zarain-Herzberg, 1994). Several Ca²⁺ ions (20–80) bind to CSQ with a K_D around 2 mM (di Barletta et al., 2006). Calcium binding induces a significant conformational change in the CSQ protein (Slupsky et al., 1987; Mitchell et al., 1988) prerequisite of the CSQ oligomerization process (Park et al., 2003). The RyR and CSQ are closely associated and this association is thought to involve other integral SR proteins, triadin and junctin (Gyorke et al., 2004). It is now commonly believed that CSQ2 acts not only as a local intra-SR Ca²⁺ buffer but as a Ca²⁺-dependent regulator of RyR2 channel function (Bers, 2004; Terentyev et al., 2007).

The objective of this paper is to explore mechanisms of luminal Ca²⁺ regulation of single RyR2 channels. An effective luminal RyR2 Ca²⁺ regulation mechanism must first be able to distinguish between luminal Ca²⁺ and

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Abbreviations used in this paper: BAPTA, 1,2-bis(2-aminophenoxy)ethane-*N,N,N′,N′*-tetraacetic acid; CICR, Ca²⁺-induced Ca²⁺ release; CPVT, catecholaminergic polymorphic ventricular tachycardia; RyR2, type-2 ryanodine receptor; WT, wild-type.

Mg^{2+} (because luminal Mg^{2+} is likely always present at millimolar levels). Second, it should operate between 0.2 and 1 mM, the putative range over which local intra-SR Ca^{2+} likely varies in cells. Third, it should substantially reduce RyR2 open probability (Po) as luminal Ca^{2+} decreases if its role is to help terminate CICR. The CSQ2-dependent luminal RyR2 Ca^{2+} regulation mechanism delineated here appears to meet these criteria.

Molecular insight into the CSQ2-dependent regulatory mechanism was attained not only using native and recombinant CSQ2 but also using two CSQ2 mutants linked to recessive forms of catecholaminergic polymorphic ventricular tachycardia (CPVT). CPVT is a familial arrhythmogenic disorder characterized by adrenergically mediated polymorphic ventricular tachyarrhythmias, leading to syncope and sudden cardiac death in individuals with otherwise structurally normal hearts. The tachyarrhythmia is typically triggered by physical exercise or emotional stress (Leenhardt et al., 1995). A recessive form of CPVT is associated with homozygous mutations in the gene encoding CSQ2 (Kontula et al., 2005). Two of these CPVT-linked CSQ2 point mutations are R33Q (Terentyev et al., 2006) and L167H (di Barletta et al., 2006). Terentyev et al. (2006) showed that the R33Q mutant abnormally regulated single RyR2 channels and demonstrated that R33Q overexpression (on top of the endogenous CSQ2 already present) promoted abnormal spontaneous diastolic Ca^{2+} release events (waves and sparks) in cardiomyocytes. Using the same approach, di Barletta et al. (2006) found that overexpression of the L167H mutant did not substantially alter Ca^{2+} release compared with control myocytes (i.e., it was as if no CSQ2 overexpression had occurred). The action of L167H on single RyR2 channels was not tested.

Here, we show that the R33Q mutant reduced RyR2 Po at submillimolar luminal Ca^{2+} concentrations but less effectively than wild-type (WT) CSQ2. Whereas, the action of the L167H mutant was as if no CSQ2 were present. Our results also indicate that Ca^{2+}-dependent signaling between CSQ2 and triadin is important to RyR2 luminal Ca^{2+} regulation.

MATERIALS AND METHODS

Chemicals and Drugs
BAPTA (1,2-bis(2-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid),5,5'-Dibromo-BAPTA(1,2-bis(2-amino-5-bromophenoxy)ethane-N,N,N',N'- tetraacetic acid), Ca(OH)$_2$, CsCl, and HEPES were obtained from Fluka. CaCl$_2$ standard for calibration was from World Precision Instruments Inc. Phospholipids were obtained from Avanti Polar Lipids and decane from Sigma-Aldrich. All other drugs and chemicals were either from Fluka or Sigma-Aldrich and were reagent grade.

Production and Purification of Recombinant Calsequestrin
CSQ2 constructs were generated as previously described (di Barletta et al., 2006; Terentyev et al., 2006). Expression and induction of recombinant CSQ2 proteins were according to Terentyev

et al. (2006). Purification was done by phenyl-sepharose purification either in column or in batch. Proteins were quantified according to standard procedures (Lowry et al., 1951). Native CSQ2 protein was also isolated from adult rat hearts using established procedures (Kobayashi et al., 2000).

In Vitro Binding Assay Using a T7-Affinity Column
Vesicles of the heavy SR fraction, prepared from rabbit hearts previously described (Saito et al., 1984), were solubilized in a buffer containing 3% CHAPS, 1 M NaCl, 20 mM Tris-HCl, pH 7.5, 1 mM DTT, and protease inhibitors. Solubilized membranes were centrifuged at 105,000 g in a Beckman Airfuge for 1 h. The supernatant was precleared with T7-affinity beads for 2 h at 4°C to eliminate nonspecific binding and then incubated with the T7-CSQ2 affinity beads in 0.3% CHAPS, 20 mM Tris-HCl pH 7.5, 0.15 M NaCl, 1 mM DTT for 20 h at 4°C in the presence of either 1 mM EGTA or 1 mM CaCl$_2$. Bound proteins were eluted by boiling in the SDS sample buffer and subjected to SDS-PAGE (Laemmli, 1970) in 10% polyacrylamide gels. After electrophoretic separation, proteins were either stained with Coomassie staining or transferred onto nitrocellulose membranes. Western blots with the Sh33 anti-Triadin antibody (Guo et al., 1996) were performed using polyclonal antibodies (gift from K.P. Campbell, The University of Iowa, Iowa City, IA). Densitometric analysis was performed with Image for Windows software (version Beta 4.0.2; Scion).

Turbidity Measurements
Experiments were performed in a double-beam Model Lambda-2 spectrophotometer (Perkin-Elmer), with a 1-cm path length quartz cell at room temperature. Turbidity measurements of CSQ2-WT, CSQ2-R33Q, and CSQ2-L167H (100 µg/ml protein) were performed by adding 1–2-µl aliquots of concentrated CaCl$_2$ solution (0.1–1 M) in 20 mM MOPS, pH 7.2, 100 mM CsCl. At each addition, protein samples were stirred and equilibrated for 2 min before measuring absorbance at 350 nm. Data were corrected for sample dilution and expressed as absorbance at 350 nm.

Sarcoplasmic Reticulum Preparation
Sarcoplasmic reticulum microsomes were prepared from rat heart, according to published methods (Chamberlain et al., 1984). In brief, ventricles were cut into 5–10-mm cubes before 30–32-g portions were homogenized in 5 volumes (vol/wt) of sucrose, 0.5 mM dithiothreitol, 3 mM NaN3, and 10 mM imidazole-HCl, pH 6.9. The homogenate was centrifuged 15 min at 5,000 rpm (3,800 g). The supernatant was filtered and centrifuged again at 13,500 rpm for 15 min (27,900 g). After filtration through cheesecloth, the supernatant was centrifuged for 2 h at 32,000 rpm (119,200 g). The pellet was resuspended and samples quick frozen in liquid nitrogen. Stored aliquots were quickly defrosted, kept on ice, and used within 5 h.

Single-Channel Recording
Planar lipid bilayers were formed from a 5:3:2 mixture of bovine brain phosphatidylethanolamine, phosphatidylserine, and phosphatidylcholine (50 mg/ml in decane) across a 100-µm hole in a 12-micron-thick Teflon partition. This hole separated two aqueous compartments. One compartment (trans) was filled with HEPES-Ca^{2+} (10 mM Ca^{2+}, pH 7.4) and virtually grounded through a patch-clamp amplifier. The trans compartment always contains the luminal side of the RyR2 channel (Tu et al., 1994). The other compartment (cis) was filled with HEPES-TRIS solution (114 mM TRIS, pH 7.4). Subsequently, 500–1000 mM Cs-methanesulfonate, 2 mM CaCl$_2$, and then 5–15 µg of the cardiac SR microsome preparation were added to the cytosolic compartment while stirring. Once channel activity was observed, the solutions in both compartments were exchanged at a rate of 4 ml/min (for 5 min) to establish the desired test conditions. Unless otherwise specified,

the holding potential was always constant at 0 mV and all recordings were made at room temperature (20–22°C).

In many experiments, luminal Ca^{2+} or Mg^{2+} was varied from 0.01 to 10 mM. The luminal solution also contained 100 mM Cs^+ to assure ample charge carrier was ever present. Consequently, the net unit current (always in the lumen-to-cytosol direction) was carried by a mixture of ions (Cs^+ and either Ca^{2+} or Mg^{2+}). The fraction of the net current carried by the divalent varied with the ionic conditions present. A published RyR permeation model was used to estimated the unidirectional Ca^{2+} current from net current (Gillespie et al., 2005; Xu et al., 2006). In our experimental conditions, this Ca^{2+} current was <0.01, ~0.2, or ~1 pA when 0.01, 1, or 10 mM luminal Ca^{2+} was present, respectively.

Single-channel currents were digitized at 10 kHz and filtered at 1–2 kHz using an A/D converter and amplifier (Axon CMS Molecular Devices). Acquired data were analyzed using pClamp (Axon CMS Molecular Devices). Open probability determinations were made from recordings lasting 120–240 s.

### Single-Channel CSQ2 Stripping and CSQ2 Replacement

Single RyR2 channel were reconstituted in planar lipid bilayer from native SR vesicles as described above. After their incorporation into the bilayer, the luminal side of some channels were subjected to a >10 min 10 mM Ca^{2+} solution prewash to promote dissociation of CSQ2 (if present) from the channel. This process is analogous to that applied by Gyorke et al. (2004) and Beard et al. (2005). We refer to channels subjected to this procedure as CSQ2 stripped channels. In some cases, CSQ2 (WT or mutant) protein was added to the luminal side at 5 μg/ml of previously CSQ2 stripped RyR2 channels. The added CSQ2 can then associate with the channels and we call these CSQ2-replaced channels.

Note that the cytosolic side of the RyR2 channel was not subjected to the high salt wash and thus this treatment could not have "salted off" cytosolic RyR2–protein partners like FKBP. Since the high salt wash was done at the single-channel level (not at the SR vesicle level), it was not possible to biochemically confirm CSQ2 association/dissociation before/after the salt wash.

RESULTS

The channels tested were pharmacologically identified as RyR channels consistent with our previous studies (Mejia-Alvarez et al., 1999; Kettlun et al., 2003). They were inhibited by 2 mM cytosolic Mg^{2+} or 10 mM cytosolic Ca^{2+}. They were activated by either 1 μM cytosolic Ca^{2+}, 5 mM ATP, or 10 mM caffeine. Their gating/conductance was characteristically modified by 10 μM ryanodine and their permeation characteristics consistent with being RyR2 channels (Fill and Copello, 2002).

Fig. 1 A (left) shows sample single RyR2 channel recordings of a control channel at various luminal Ca^{2+} levels. Control channels are those that were never exposed to high luminal Ca^{2+} level and thus could have endogenous CSQ associated with them. Fig. 1 A (right) shows the luminal Mg^{2+} sensitivity of a CSQ2-associated channel. Fig. 1 B shows summary open probability (Po) data collected from experiments on several different channels. The activity of CSQ2-associated channels showed no luminal Mg^{2+} sensitivity (Fig. 1 B, open circles). Control channels responded to luminal Ca^{2+} as illustrated by the filled circles (Fig. 1 B). The Po of control channels increased from ~0.02 to 0.2 when the luminal
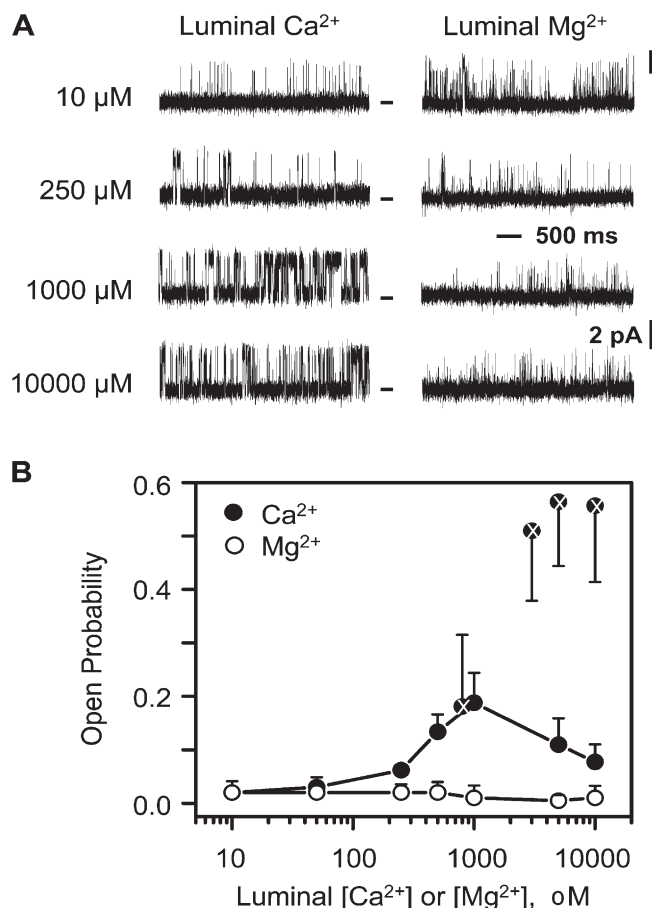
Figure 1. Luminal Ca^{2+} and Mg^{2+} sensitivity of RyR2 channels. Channels were incorporated into bilayers by fusing heavy native SR microsomes. Cytosolic free Ca^{2+} concentration was constant (1 μM). The luminal Ca^{2+} or Mg^{2+} concentration was titrated from 10 μM to 10 mM. Holding potential was 0 mV. The luminal solution contained 100 mM Cs^+ and net unit current was always in the lumen-to-cytosolic direction. (A) Example single channel recordings with zero current level marked. Recordings at left are from a control channel that was never exposed to the CSQ2 stripping process with no added CSQ2 in the luminal bathing solution. Recordings at right are from a channel that was first CSQ2 stripped before 0.5 μg/ml CSQ2-WT was added to the luminal solution. (B) Summary Po results (mean ± SEM) where the filled circles represent the Ca^{2+} sensitivity of 11 different control channels and the open circles represent the Mg^{2+} sensitivity of 10 different channels. The X-marked circles represent data collected from six different CSQ2-associated control channels in the presence of 3 mM cytosolic diBromoBAPTA.

Ca^{2+} level was elevated from 10 to 1000 μM. The Po decreased at higher Ca^{2+} levels. These luminal Ca^{2+}-dependent Po changes in control channels were reversible.

As described in methods, exposure of the luminal side of the channel to a >10-min 10 mM Ca^{2+} prewash dissociates (strips) CSQ2 from the channel. The control channel data shown Fig. 1 B (filled circles) were never subjected to this prewash. The control channel data, however, includes Po data collected at 10 mM luminal Ca^{2+} that is consistent with CSQ2 still being associated with the channel. This is possible because these control

channel recordings at 10 mM were relatively short (~2 min). In our hands, CSQ2 dissociation required at least a 4-min exposure to a 10 mM Ca^{2+} solution. Dissociation of CSQ2 from a control channel was considered to be coincident with sustained Po reduction. Attempts to better define this phenomenon were unsuccessful (due to variability and a relatively low n).

It is known that large Ca^{2+} currents in the lumen-to-cytosol direction can change local cytosolic Ca^{2+} levels sufficiently to alter the Po of single RyR2 channels (Xu and Meissner, 1998). This is called feed-through Ca^{2+} modulation. To assess if feed-through Ca^{2+} modulation influences our observations, luminal Ca^{2+} sensitivity of control channels was also measured in the presence of 3 mM cytosolic diBromoBAPTA (Fig. 1 B, X-marked filled circles). With this fast Ca^{2+} buffer present, the luminal Ca^{2+} sensitivity of CSQ2-associated channels became more sigmoidal with an EC_{50} of 687 ± 37 µM. This implies that Ca^{2+} passing through the channel when high luminal Ca^{2+} levels (5 and 10 mM) are present feeds back and inhibits the channel, generating the observed "bell-shaped" luminal Ca^{2+} sensitivity. This is consistent with the large fraction of the net current being carried by Ca^{2+} when 5 and 10 mM Ca^{2+} are present (see Materials and methods).
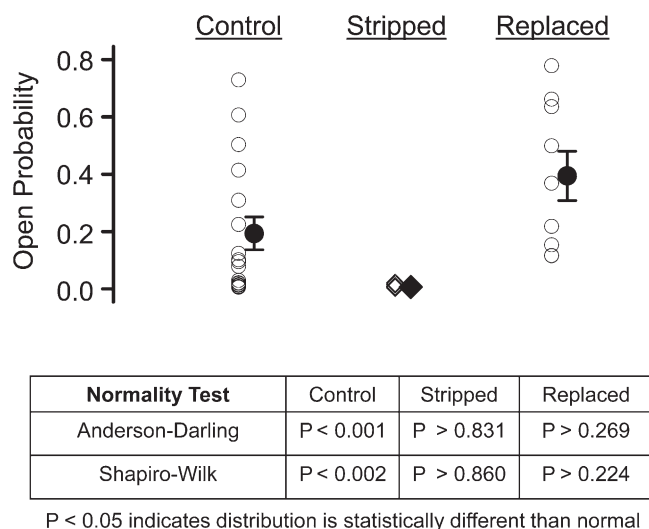
Example single channel recordings illustrating the luminal Ca sensitivity of a CSQ2 stripped channel are shown in Fig. 2 A (left). Summary Po results from several CSQ stripped channels are shown in Fig. 2 B (open diamonds). The Po of stripped channels did not change over the tested luminal Ca^{2+} range. The lost luminal Ca^{2+} sensitivity following the stripping procedure suggests the luminal Ca^{2+} sensitivity of control channels (see Fig. 1 B) was CSQ2 dependent. This was tested by adding recombinant purified CSQ2 (0.5 µg/ml) to the luminal side of previously CSQ2-stripped channels. Sample recordings from CSQ2-replaced channels are shown in Fig. 2 A (right). The average luminal Ca^{2+} sensitivity of several CSQ2-replaced channels is shown in Fig. 2 B (filled circles). The dotted line in Fig. 2 B represents the control channel data presented in Fig. 1 B. The CSQ2-replaced and control channels have analogous luminal Ca^{2+} sensitivities. However, there is a clear difference in the peak Po reached at 1000 µM luminal Ca^{2+}. The reason for this may be that not all of the control channels had CSQ2 associated with them and this possibility is examined further below.

The results shown in Fig. 3 were collected in the presence of 1 µM cytosolic Ca^{2+} and 1 mM luminal Ca^{2+} in three different experimental situations. The first situation is labeled control where channels had never been exposed to the CSQ2 stripping procedure. The second is stripped where channels were exposed to the stripping procedure. The third is replaced where CSQ2 (0.5 µg/ml) was added back to previously stripped channels. In Fig. 3 (top), the mean (filled symbols) and

A

B
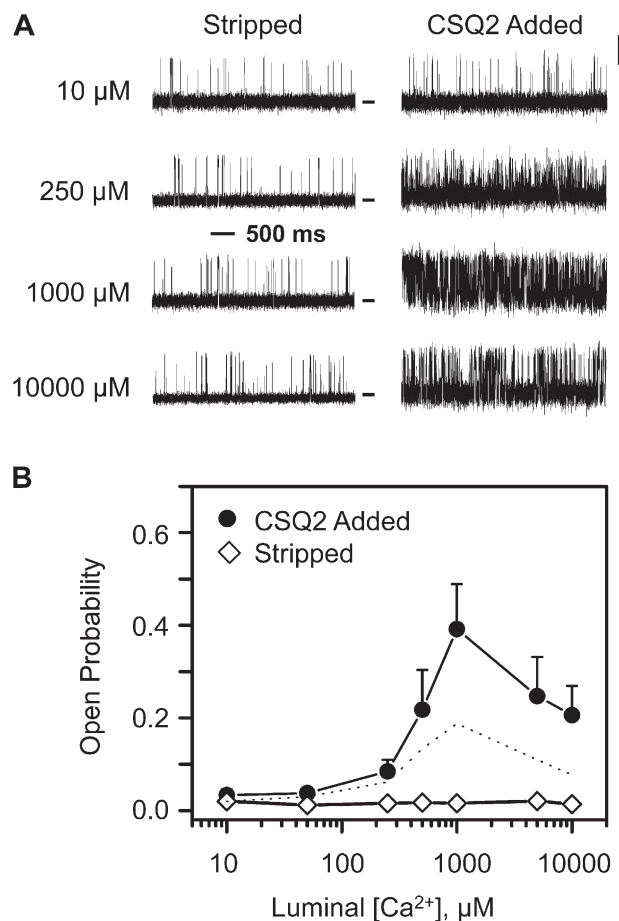
Figure 2. Luminal Ca^{2+} of CSQ2-stripped and CSQ2-added RyR2 channels. The CSQ2-added channels here refer to channels that were first CSQ2 stripped before 0.5 µg/ml CSQ2-WT was added to the luminal solution. Cytosolic free Ca^{2+} concentration was constant (1 µM). The luminal Ca^{2+} or Mg^{2+} concentration was titrated from 10 µM to 10 mM. Holding potential was 0 mV. The luminal solution contained 100 mM Cs^+ and net unit current was always in the lumen-to-cytosolic direction. (A) Example single channel recordings from a CSQ2-stripped channel (left) and a CSQ2-added channel (right). Zero current levels are marked. Bar, 2 pA. (B) Summary Po results. Luminal Ca^{2+} sensitivity of the CSQ2-added data (black circles) was collected from 14 different channels. Luminal Ca^{2+} sensitivity of the CSQ2-stripped data (open diamonds) was collected from six different channels. Dotted line represents control Po result presented in Fig. 1 B.

corresponding individual determinations (open symbols) for each situation are plotted. The normality of the distributions about their means was tested using the Anderson-Darling and Shapiro-Wilk normality tests. These tests indicate a distribution is statistically different than normal if the statistic P is less than 0.05. These tests indicated that the control channel data population was the only one that was statistically different than normal (Fig. 3, bottom). This supports the contention that the control channel population contains two classes of channels (i.e., those with and without CSQ2 attached).

Normality Test	Control	Stripped	Replaced
Anderson-Darling	P < 0.001	P > 0.831	P > 0.269
Shapiro-Wilk	P < 0.002	P > 0.860	P > 0.224

P < 0.05 indicates distribution is statistically different than normal

Figure 3. Normality testing. Data collected in the presence of 1 µM cytosolic Ca^{2+} and 1 mM lumenal Ca^{2+} in three different experimental situations. Control represents channels that were not subjected to the CSQ2 stripping process. Stripped represents channels that underwent the stripping process. Replaced represents stripped channels after CSQ2 (0.5 µg/ml) was added to the luminal bath. Open symbols are individual determinations. Filled symbols are means (±SEM). Outcome of the Anderson-Darling and Shapiro-Wilk normality tests are shown at the bottom.

To this point, RyR2 luminal Ca^{2+} sensitivity has been defined in the presence of a constant 1 µM cytosolic bath Ca^{2+} concentration. Fig. 4 A shows how CSQ2 affects RyR2 cytosolic Ca^{2+} sensitivity at a constant luminal Ca^{2+} concentration (1 mM). The cytosolic Ca^{2+} sensitivity of CSQ2-stripped (Fig. 4 A, open diamonds) and CSQ2-replaced channels (Fig. 4 A, filled circles) is compared. The EC_{50} of cytosolic Ca^{2+} activation was 2.01 ± 0.34 µM (maximum Po ∼0.6) when no CSQ2 was associated with the channel. It was 1.04 ± 0.17 µM (maximum Po ∼0.8) when CSQ2 was present. An unpaired T test was used to determine if mean Po's of the CSQ2-replaced and stripped channels at each of the different cytosolic Ca^{2+} levels were statistical different. The Po was significantly different at all cytosolic Ca^{2+} levels >0.5 µM. Note that the lumen-to-cytosolic Ca^{2+} flux was essentially constant in these experiments because luminal Ca^{2+} was always 1 mM. Thus, this CSQ2-dependent change in cytosolic RyR2 Ca^{2+} sensitivity was not the result of feed-through Ca^{2+} modulation.

The dashed line in Fig. 4 A summarizes the cytosolic Ca^{2+} sensitivity of CSQ2-stripped channels in the presence of 10 mM luminal Ca^{2+}, instead of 1 mM (Fig. 4 A, open diamonds). These data show that luminal Ca^{2+} (in absence of CSQ2) changes the maximal Po level, not the cytosolic Ca^{2+} affinity, of the channel. This is a form of CSQ2-independent luminal RyR2 Ca^{2+} regulation. Fig. 4 B further explores this CSQ2-independent regulation. The luminal divalent cation titrations shown (Fig. 4 B) were done with 100 µM cytosolic Ca^{2+} present.
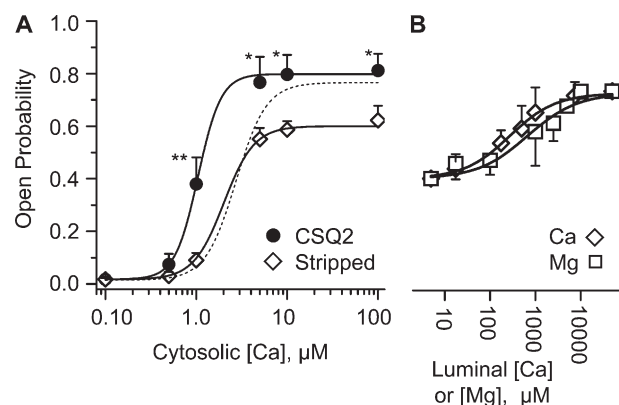
Figure 4. CSQ2 shifted the cytosolic Ca^{2+} sensitivity of single RyR2 channels. Holding potential was 0 mV and the luminal solution contained 100 mM Cs^+. (A) Summary Po results from CSQ2-stripped (open circles; n = 8) and CSQ2-replaced (filled circles; n = 6) channels. The CSQ2-replaced channels were associated with CSQ2-WT (0.5 µg/ml in luminal chamber). Luminal free Ca^{2+} concentration was 1 mM and cytosolic Ca^{2+} was titrated from 0.1 to 100 µM. The curve fit to the filled circles has an EC_{50} of 1.04 ± 0.17 µM and a 3.4 Hill coefficient. The curve fit to the CSQ2-stripped data has an EC_{50} of 2.01 ± 0.34 µM and a 2.6 Hill coefficient. An unpaired t test was used to determine if the Po between CSQ2-replaced and stripped channels at each Ca^{2+} concentration was statistically different (**, P < 0.01; *, P < 0.05). Dotted curve represents the cytosolic Ca^{2+} sensitivity of CSQ2-stripped channels when 10 mM luminal Ca^{2+} was present. (B) Luminal Ca^{2+} and Mg^{2+} sensitivity of CSQ2-stripped channels. These stripped channels were maximally activated by high cytosolic Ca^{2+} (100 µM) and then luminal Ca^{2+} (open diamond; n = 13) or Mg^{2+} (open square; n = 16) was varied. The curve fit to the Ca^{2+} data has an EC_{50} of 379 ± 247 µM and a 0.70 Hill coefficient. The curve fit to the Mg^{2+} data has an EC_{50} of 972 ± 208 µM and a 0.77 Hill coefficient.

This is more than 10-fold higher than the measured EC_{50} of cytosolic RyR2 Ca^{2+} activation and thus assures there is no (or very little) feed-through Ca^{2+} activation here. The Po of these CSQ2-free channels was ∼0.4 when luminal Ca^{2+} was 10 µM. It rose to ∼0.8 when luminal Ca^{2+} was increased to 10 mM (Fig. 4 B, open diamonds). The EC_{50} of this CSQ2-independent luminal Ca^{2+} activation was 379 ± 247 µM. Interestingly, very similar results were obtained if Mg^{2+}, instead of Ca^{2+}, was applied to the luminal side of the channel (Fig. 4 B, open squares). The EC_{50} of this luminal Mg^{2+} activation was 972 ± 208 µM. No significant differences in Po at any luminal divalent concentrations were found. This indicates that the CSQ2-independent mechanism does not discriminate well between luminal Ca^{2+} and Mg^{2+}.

Several mutants of CSQ2 are linked to the tachyarrhythmic disorder CPVT. How two of these CSQ2 mutants (R33Q and L167H) regulate single RyR2 channels is shown in Fig. 5. Single RyR2 channels were incorporated into the bilayer and then stripped of any endogenous CSQ2 present. With 1 µM cytosolic Ca^{2+} always present, the luminal Ca^{2+} concentration was titrated in the presence of either the R33Q or L167H mutant (0.5 µg/ml). Sample single channel recordings are shown
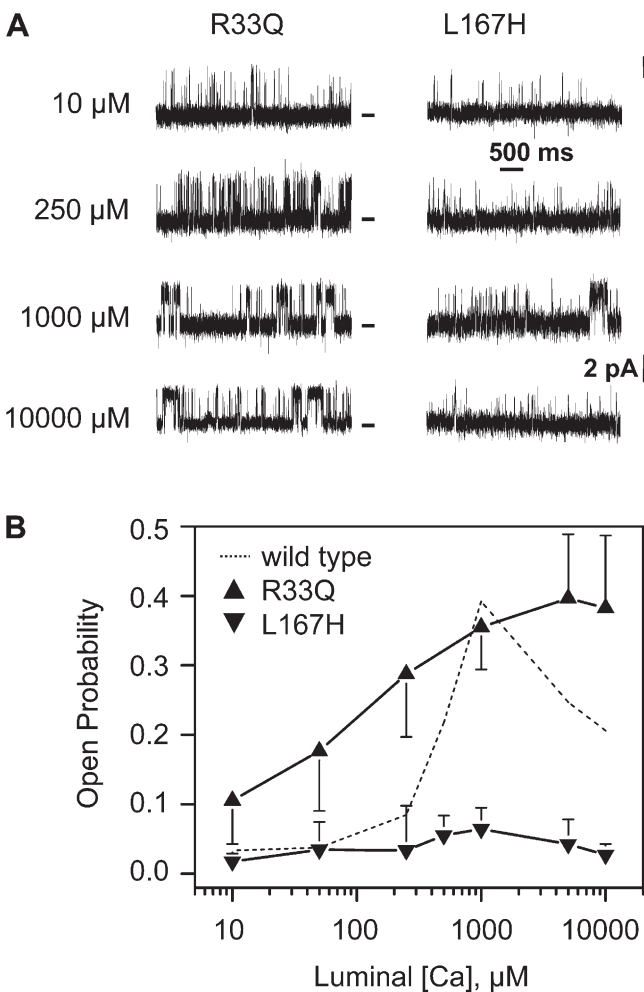
Figure 5. Luminal Ca^{2+} regulation of RyR2 channels by the CSQ2-R33Q and CSQ2-L167H mutants. Mutant CSQ2 (0.5 µg/ml) was added to the luminal side of previously CSQ2-stripped channels. Cytosolic free Ca^{2+} concentration was 1 µM and luminal Ca^{2+} was titrated from 10 µM to 10 mM. Holding potential was 0 mV and the luminal solution contained 100 mM Cs^+. (A) Example channel recordings with CSQ2-R33Q (left) or CSQ2-L167H (right) present are shown (zero current level marked). (B) Summary Po results. The CSQ2-R33Q data (triangle) was collected on eight different channels. The CSQ2-L167H data (inverted triangles) was collected on eight different channels as well. Dotted line represents CSQ2-WT result presented in Fig. 2 B.

in Fig. 5 A. Single RyR2 channels associated with the R33Q mutant were sensitive to luminal Ca^{2+}. Channels associated with the L167H mutant were not. Fig. 5 B shows summary results collected from many different channels. The dashed line represents the luminal Ca^{2+} sensitivity of channels associated with WT CSQ2 (from Fig. 2 B). Channels associated with the R33Q mutant (filled triangles) had significantly higher Po (t test P < 0.05) at luminal ≤250 µM compared with those channels associated with WT CSQ2. However, their Po was similar to channels associated with WT CSQ2 at 1 mM luminal Ca^{2+}. The Po at 5 and 10 mM luminal Ca^{2+} were
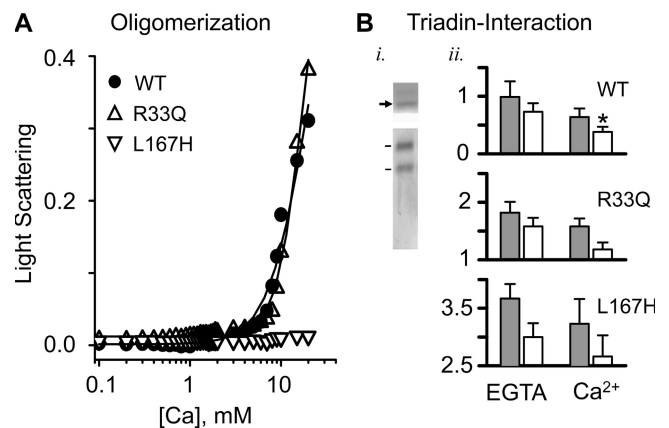
Figure 6. Ca^{2+}-dependent CSQ2 oligomerization and CSQ2–triadin interaction. (A) The Ca^{2+} sensitivity of light (350 nm) scattering of CSQ2-WT (filled circles), CSQ2-R33Q (triangles), and CSQ2-L167H (inverted triangles) proteins in presence of 100 mM CsCl. Samples were stirred for 2 min before measurement. The curve fit to the CSQ2-WT data has an EC_{50} of 18.1 ± 5.23 mM and a 2.1 Hill coefficient. The curve fit to the CSQ2-R33Q data has an EC_{50} of 16.4 ± 1.18 mM and a 3.0 Hill coefficient. Both curves were fit with V_{MAX} arbitrarily fixed at 0.6. (B) At left, top panel (i) depicts the Coomassie blue–stained SDS-PAGE of purified, recombinant CSQ2-WT (arrow, MW of ~52,000). Bottom panel (i) depicts the Western blot with anti-triadin antibodies, revealing two bands having MW of ~45,000 (glycosylated form) and 40,000 (unglycosylated form), respectively. At right (ii), the Ca^{2+} sensitivity of the interaction of glycosylated and unglycosylated triadin with CSQ2-WT, CSQ2-R33Q, and CSQ2-L167H was measured with either very low Ca^{2+} (1 mM EGTA) or 1 mM free Ca^{2+} present, and data are shown as means ± SEM (n = 5). Filled bars represent glycosylated triadin, open bars represent unglycosylated triadin. Asterisk indicates P < 0.05 using an unpaired Student's t test.

not statistically different (t test P > 0.1) between the CSQ2-R33Q and CSQ2-WT datasets. In the presence of the L167H mutant (Fig. 5 B, filled inverted triangles), the Po did not change as the luminal Ca^{2+} concentration varied. Indeed, the Po's at 10 µM, 1 mM, and 10 mM luminal Ca^{2+} were not statistically different (t test P > 0.1) when these data were compared with the stripped (CSQ2-free) channel data.

The differences in CSQ2-dependent RyR2 function (Fig. 5 B) could conceivably arise due to mutant vs. WT-dependent differences in CSQ2 oligomerization and/or the CSQ2–RyR2 interaction. Light scattering was used to measure the Ca^{2+} dependence of CSQ2 oligomerization in conditions (100 mM CsCl) similar to those used for bilayer experiments (Fig. 6 A). Increased light scattering here reflects more CSQ2 oligomerization. The CSQ2-WT and CSQ2-R33Q proteins oligomerized at Ca^{2+} levels >3 mM and this oligomerization had similar Ca^{2+} dependency. Virtually no oligomerization of the CSQ2-L167H protein was observed over the Ca^{2+} concentration range tested.

The CSQ2–RyR2 functional interaction most likely involves the Triadin protein (Gyorke et al., 2004; Terentyev et al., 2007). In cardiac muscle, there is one isoform of

Triadin (Marty, 2004) that runs on SDS gels as an ∼40-kD doublet comprised of glycosylated and unglycosylated molecules. The glycosylation site of triadin is at asparagine residue 75 (Kobayashi et al., 2000). The relative proportions of glycosylated and unglycosylated triadin in cardiac muscle cells varies among mammalian species. In human and rabbit, both are present but there is proportionally more unglycosylated triadin. The functional significance of this is not yet clear.

CSQ2 binding to these two forms of cardiac triadin was measured. Fig. 6 Bi shows an example triadin-CSQ2-WT immune pull-down. The top panel shows the Coomassie blue–stained SDS-PAGE of CSQ2-WT. The bottom panel shows the classical triadin doublet revealed by Western blot with anti-triadin antibodies. Data like these were used to evaluate the Ca^{2+} sensitivity of glycosylated (filled bars) and unglycosylated (open bars) cardiac triadin binding to either the WT, R33Q, or L167H CSQ2 proteins. In Fig. 6 Bii, CSQ2–triadin binding was measured in the virtual absence of Ca^{2+} (EGTA) and in the presence of 1 mM Ca^{2+}. In vitro binding was normalized to that in the EGTA WT condition. There was no significant Ca^{2+} sensitivity of glycosylated triadin binding to any of the CSQ2 proteins tested. There was also no significant Ca^{2+} sensitivity of unglycosylated triadin binding to the CSQ2-L167H protein. However, binding of unglycosylated triadin to the CSQ2-WT protein was significantly reduced (P < 0.05) in the presence of 1 mM Ca^{2+}. The binding of unglycosylated triadin to the CSQ2-R33Q protein was nearly significant (P > 0.06) in the presence of 1 mM Ca^{2+}.

DISCUSSION

Several studies of luminal Ca^{2+} regulation of single RyR1 and RyR2 channels have been done, yielding variable and sometimes confusing results (Sitsapesan and Williams, 1995; Lukyanenko et al., 1996; Tripathy and Meissner, 1996; Gyorke and Gyorke, 1998; Xu and Meissner, 1998; Ching et al., 2000; Beard et al., 2002; Laver et al., 2004; Beard et al., 2005). For example, Sitsapesan and Williams (1995) concluded that regulation of RyR2 channels by luminal Ca^{2+} depends on how channels are activated (they used sulmazole) and that channels solely activated by micromolar cytosolic Ca^{2+} were not sensitive to luminal Ca^{2+} changes. Xu and Meissner (1998) demonstrated luminal Ca^{2+} feeding through caffeine-activated channels can modulate RyR2 activity, implying that a luminally localized regulatory process may not exist. More recently, Gyorke et al. (2004) argues that luminal Ca^{2+} feed-through is not significant but that the CSQ2–triadin complex somehow acts as a luminal Ca^{2+} regulatory sensor. This is consistent with recent studies exploring the molecular defects underlying CPVT, which suggest that a CSQ2 regulatory mechanism exists (Terentyev et al., 2006). Indeed, the commonly accepted view is that there

is a CSQ2-dependent mechanism that helps to stabilize RyR2-mediated CICR in cardiac muscle.

Here, we explore this view using no cytosolic activators (such as sulmazole, caffeine, or ATP) and report absolute Po (not normalized or relative Po) values.

Luminal RyR2 Ca^{2+} Regulation without CSQ2 Present

We showed that CSQ2-free RyR2 channels, activated by 1 µM cytosolic Ca^{2+}, were not sensitive to luminal Ca^{2+} (Fig. 2 B, open diamonds). This may be what some previous single channel studies observed and may explain why many previous investigators were compelled to apply cytosolic channel activators. Many of the selected activators are known to make the channel hypersensitive to cytosolic Ca^{2+} (Fill and Copello, 2002), enhancing the prospect of feed-through Ca^{2+} modulation. To avoid this complication, our CSQ2-free RyR2 channels were activated by 100 µM cytosolic Ca^{2+} only to assure the cytosolic Ca^{2+} activation site was saturated.

In these conditions, CSQ2-free RyR2 channels were sensitive to luminal Ca^{2+} concentrations. The Po of CSQ2-free channels doubled (∼0.4 to ∼0.8) when luminal Ca^{2+} was raised from 10 µM to 10 mM. The same thing happened if luminal Mg^{2+}, instead of luminal Ca^{2+}, was applied. Thus, a CSQ2-independent form of luminal RyR2 Ca^{2+} regulation exists and it does not distinguish between luminal Ca^{2+} and Mg^{2+}. To our knowledge, the ion selectivity of luminal Ca^{2+} control mechanisms has rarely (if ever) been tested before.

The lack of Ca^{2+} specificity makes the physiological relevance of this CSQ2-independent regulation arguable. This regulatory pathway could conceivably become important in certain pathological conditions. For example, cardiac SR overload could conceivably raise the total intra-SR divalent concentration sufficiently to be sensed by this mechanism. If so, then our results suggest this mechanism would modulate cytosolic Ca^{2+} activation efficacy (not its affinity). Efficacy here refers to the maximum Po attainable by cytosolic Ca^{2+} stimulation (affinity to the cytosolic Ca^{2+} EC_{50}). Intra-SR divalent overload would then increase the maximal Po attainable by a cytosolic Ca^{2+} stimulus, explaining the excess RyR2-mediated Ca^{2+} release associated with this pathological condition. Alternatively, this mechanism may just assure that RyR2 channels respond robustly to cytosolic Ca^{2+} stimuli.

Luminal RyR2 Ca^{2+} Regulation with CSQ2 Present

We showed that CSQ2-associated RyR2 channels, activated by 1 µM cytosolic Ca^{2+}, were sensitive to luminal Ca^{2+}. They were not sensitive to changes in luminal Mg^{2+}. Thus, the CSQ2-dependent luminal RyR2 Ca^{2+} regulation mechanism distinguishes between these ions. It does not require the presence of another cytosolic activator (ATP or sulmazole). It does not require the presence of additional free CSQ2 in the luminal bath as illustrated by Fig. 1 B (filled circles) where regulation occurs with

no unbound CSQ2 in the lumenal bath. This means CSQ2-dependent regulation does not involve CSQ2 association/dissociation and that made it impractical to define the CSQ2 dose dependency. We considered examining the dose dependency of CSQ2 reassociation over a set interval but the physiological importance of this parameter is not entirely clear. Instead, we simply elected to define function at a set bath CSQ2 concentration, a concentration like that used successfully by other groups (Gyorke et al., 2004; Beard et al., 2005).

Our results also suggest that the CSQ2-dependent mechanism alters the cytosolic Ca^{2+} sensitivity of the channel. At a constant luminal Ca^{2+} (1 mM), CSQ2-free and CSQ2-assocaited channels had different cytosolic Ca^{2+} activation affinity and efficacy (Fig. 4 A, diamonds vs. circles). Comparing this and the data presented in Fig. 2 B (circles; where 1 µM cytosolic Ca^{2+} is always present) suggests that luminal Ca^{2+} alters Po by changing the cytosolic Ca^{2+} sensitivity of CSQ2-associated channels. If so, then our data indicates that a local luminal Ca^{2+} reduction (1 mM to 100 µM), in the presence of a constant cytosolic Ca^{2+} stimulus (1 µM), would turn off the channel. This is consistent with the work of Gyorke and Gyorke (1998), who suggested that a change in luminal Ca^{2+} (20 µM to 5 mM) seemed to make ATP-activated RyR2 channels more sensitive to cytosolic Ca^{2+} activation.

The CSQ2-dependent luminal Ca^{2+} regulatory mechanism also depended on CSQ2 structure–function. Two different CSQ2 mutants (R33Q and L167H) were tested here. The L167H CSQ2 mutant did not support CSQ2-dependent regulation. The R33Q mutant did but abnormally. The Po of WT and R33Q-associated channels at 1 mM was similar. However, channels associated with R33Q were not "turned off" as effectively when luminal Ca^{2+} was reduced <1 mM (compared with channels associated with WT CSQ2). This is quite consistent with the observations reported in Terentyev et al. (2006).

How are changes to luminal Ca^{2+} sensed by the CSQ2–RyR2 complex? This could involve Ca^{2+}-dependent CSQ2 polymerization, Ca^{2+} binding to the CSQ2 monomer, and/or some sort of Ca^{2+}-dependent CSQ2–RyR2 interaction. Calcium-dependent CSQ2 polymerization does not seem to play a part because the observed regulation does involve CSQ2 association/dissociation (see discussion above). There also seems to be little (or no) correlation between CSQ2 Ca^{2+} binding properties and the observed CSQ2-dependent RyR2 luminal Ca^{2+} regulation. This latter point is based on the known Ca^{2+} binding properties of the CSQ2s tested here (WT, R33Q, and L167H). di Barletta et al. (2006) reported that the Ca^{2+} affinities and B_{MAX}s of these CSQ2s were not statistically different (t test P > 0.45). Kim et al. (2007) reported that the Ca^{2+} binding capacity of the L167H monomer is ~50% less (compared with WT) and that R33Q had reduced Ca^{2+} binding capacity at high Ca^{2+} levels. This apparent discrepancy between these studies
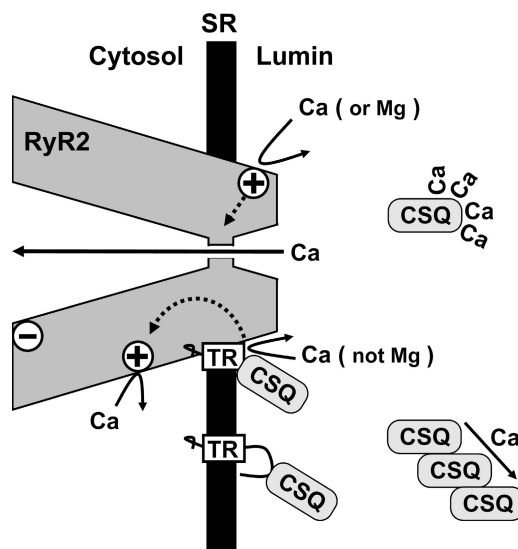
Figure 7. Summary cartoon of RyR2 luminal Ca^{2+} regulation. Triadin is labeled TR. Calcium binding sites that activate channel are indicated with a plus. The cytosolic Ca^{2+} inhibitory site is marked with a minus. Proteins are not drawn to scale.

is probably due to the methodological differences. In any event, there seems to be little (or no) correlation between CSQ2 Ca^{2+} binding properties and the CSQ2-dependent regulation measured here.

Terentyev et al. (2007) recently showed that a decoy peptide corresponding to the CSQ2 binding domain of triadin interferes with CSQ2's capacity to regulate single RyR2 channels. Thus, we elected to explore the possible role of triadin in CSQ2-dependent RyR2 regulation. We compared the Ca^{2+} sensitivity of the triadin interaction with WT, R33Q, and L167H. These CSQ2s all bind to triadin but only the binding of WT and R33Q with unglycosylated triadin was Ca^{2+} sensitive. Thus, the Ca^{2+} sensitivity of the triadin–CSQ2 interaction, not triadin–CSQ2 binding per se, could explain the luminal RyR2 Ca^{2+} regulation observed here. If so, then L167H may have bound to the triadin–RyR2 complex but did not regulate because the triadin–CSQ2 (L167H) interaction is not Ca^{2+} sensitive. Whereas with R33Q bound, regulation would be abnormal because the Ca^{2+} sensitivity of the triadin–CSQ2 (R33Q) interaction is abnormal.

Fig. 7 illustrates our overall working interpretation. In this cartoon, Ca^{2+} binding to the CSQ2 monomer and Ca^{2+}-dependent CSQ2 oligomerization do not regulate the RyR2 channel. A CSQ2 monomer bound to the triadin–RyR2 complex is the key regulating entity. The Ca^{2+} sensitivity of CSQ2–triadin interaction is the key lumenal Ca^{2+} sensing step and it discriminates between Ca^{2+} and Mg^{2+}. Lumenal Ca^{2+} alters the cytosolic Ca^{2+} sensitivity of the channel through the RyR2–triadin–CSQ2 interaction. There is also a RyR2-resident, CSQ2-independent, lumenal Ca^{2+} regulatory pathway that does not discriminate between Ca^{2+} and Mg^{2+}.

The Cellular Context

The intra-SR free Ca^{2+} concentration in a resting cardiac myocyte is likely close to 1 mM (Bers, 2004). In response to a single cardiac action potential, the intra-SR free Ca^{2+} concentration may fall to $\sim$50% of its normal resting value (Shannon et al., 2000). High frequency stimulation or larger SR Ca^{2+} releases may drive intra-SR Ca^{2+} levels to even lower levels. Our results suggest that such changes in luminal Ca^{2+} inhibit the channel by reducing its cytosolic Ca^{2+} sensitivity and thus help terminate the SR Ca^{2+} release process. As intra-SR Ca^{2+} levels are replenished, the cytosolic Ca^{2+} sensitivity of the channel would return to its resting condition.

di Barletta et al. (2006) showed that overexpression of CSQ2-WT, not CSQ2-L167H, increased the amplitude of intracellular Ca^{2+} sparks/transients. In that study, the new CSQ2 was expressed on top of the normal complement of CSQ2-WT. Our observation that L167H has no RyR2 regulatory action is then quite consistent with di Barletta et al. (2006). Our R33Q results are also consistent because they show R33Q was substantially less effective in turning off R33Q-assocaited channels. Thus, cells containing R33Q should have RyR2 channels with a greater propensity to open during diastole, promoting increased SR Ca^{2+} leak and frequency of spontaneous SR Ca^{2+} release events (as observed by Terentyev et al., 2006).

Our results show that two CPVT-linked CSQ2 mutants have very different actions on single RyR2 luminal Ca^{2+} regulation. Since several RyR2 channel mutations also generate CPVT phenotypes (with WT CSQ2 present), it is becoming clear that CVPT can result from any of a number of defects that modify or abolish normal RyR2 luminal Ca^{2+} regulation. Although no triadin-linked forms of CPVT have been identified yet, it would not be surprising if one was in the near future. Lastly, our studies of CSQ2-dependent RyR2 regulation were done under stationary experimental conditions in bilayers. This must be considered when extrapolating our data to the cellular situation. In cells, CSQ2-dependent RyR2 regulation operates in a dynamic complex regulatory environment that is simply not present in our studies. Defining the kinetics of CSQ2 modulation of RyR2 function in a more physiological context will likely be an important, albeit challenging, focus of future studies that could change our view of how CSQ2 regulates the RyR2 channel.

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