Luminal Ca$^{2+}$ Regulation of Single Cardiac Ryanodine Receptors: Insights Provided by Calsequestrin and its Mutants

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The luminal Ca$^{2+}$ regulation of cardiac ryanodine receptor (RyR2) was explored at the single channel level. The luminal Ca$^{2+}$ and Mg$^{2+}$ 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 Ca$^{2+}$ regulatory mechanism(s) were identified. One is a RyR2-resident mechanism that is CSQ2 independent and does not distinguish between luminal Ca$^{2+}$ and Mg$^{2+}$. This mechanism modulates the maximal efficacy of cytosolic Ca$^{2+}$ activation. The second luminal Ca$^{2+}$ regulatory mechanism is CSQ2 dependent and distinguishes between luminal Ca$^{2+}$ and Mg$^{2+}$. It does not depend on CSQ2 oligomerization or CSQ2 monomer Ca$^{2+}$ binding affinity. The key Ca$^{2+}$-sensitive step in this mechanism may be the Ca$^{2+}$-dependent CSQ2 interaction with triadin. The CSQ2-dependent mechanism alters the cytosolic Ca$^{2+}$ sensitivity of the channel. The R33Q CSQ2 mutant can participate in luminal RyR2 Ca$^{2+}$ regulation but less effectively than wild-type (WT) CSQ2. CSQ2-L167H does not participate in luminal RyR2 Ca$^{2+}$ 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 Ca$^{2+}$ mechanism may assure that all channels respond robustly to large (>5 μM) local cytosolic Ca$^{2+}$ stimuli, whereas the CSQ2-dependent mechanism may help close RyR2 channels after luminal Ca$^{2+}$ 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 Ca$^{2+}$ influx through the L-type Ca$^{2+}$ channel and this Ca$^{2+}$ influx activates nearby RyR2 channels, initiating Ca$^{2+}$ release from the SR. This process is known as Ca$^{2+}$-induced Ca$^{2+}$ 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 Ca$^{2+}$ level (Lukyanenko et al., 1996; Gyorke and Gyorke, 1998). A drop in local intra-SR Ca$^{2+}$ has been proposed to help “turn off” RyR2-mediated SR Ca$^{2+}$ release, stabilizing CICR in cardiac muscle (Lukyanenko et al., 2001; Terentyev et al., 2002; Gyorde et al., 2004). The mechanism of this RyR2 intra-SR (luminal) Ca$^{2+}$ regulation, however, is not well understood. It may involve a Ca$^{2+}$ binding site(s) on the luminal surface of the RyR2 channel itself and/or Ca$^{2+}$ interactions with RyR2-associated intra-SR regulatory proteins like calsequestrin (CSQ). Calsequestrin is a low affinity, high capacity intra-SR Ca$^{2+}$-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 Ca$^{2+}$ 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 (Sulpisky 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 Ca$^{2+}$ buffer but as a Ca$^{2+}$-dependent regulator of RyR2 channel function (Bers, 2004; Terentyev et al., 2007).

The objective of this paper is to explore mechanisms of luminal Ca$^{2+}$ regulation of single RyR2 channels. An effective luminal RyR2 Ca$^{2+}$ regulation mechanism must first be able to distinguish between luminal Ca$^{2+}$ and

Abbreviations used in this paper: BAPTA, 1,2-bis(2-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid; CICR, Ca$^{2+}$-induced Ca$^{2+}$ 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 homozgyous 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)$_2$, CaCl$_2$, 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 0.5% 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, 5 mM NaN$_3$, 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 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 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).
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\textsuperscript{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\textsuperscript{2+} concentration was 1 μM and luminal Ca\textsuperscript{2+} was titrated from 10 μM to 10 mM. Holding potential was 0 mV and the luminal solution contained 100 mM Cs\textsuperscript{+}. (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.

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### Figure 6.
Ca\textsuperscript{2+}-dependent CSQ2 oligomerization and CSQ2–triadin interaction. (A) The Ca\textsuperscript{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\textsubscript{50} of 18.1 ± 5.23 mM and a 2.1 Hill coefficient. The curve fit to the CSQ2-R33Q data has an EC\textsubscript{50} of 16.4 ± 1.18 mM and a 3.0 Hill coefficient. Both curves were fit with V\textsubscript{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\textsuperscript{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\textsuperscript{2+} (1 mM EGTA) or 1 mM free Ca\textsuperscript{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.

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In Fig. 5 A. Single RyR2 channels associated with the R33Q mutant were sensitive to luminal Ca\textsuperscript{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\textsuperscript{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\textsuperscript{2+}. The Po at 5 and 10 mM luminal Ca\textsuperscript{2+} were 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\textsuperscript{2+} concentration varied. Indeed, the Po’s at 10 μM, 1 mM, and 10 mM luminal Ca\textsuperscript{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\textsuperscript{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\textsuperscript{2+} levels ≥3 mM and this oligomerization had similar Ca\textsuperscript{2+} dependency. Virtually no oligomerization of the CSQ2-L167H protein was observed over the Ca\textsuperscript{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

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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 Ca²⁺ 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 Ca²⁺ (EGTA) and in the presence of 1 mM Ca²⁺. In vitro binding was normalized to that in the EGTA WT condition. There was no significant Ca²⁺ sensitivity of glycosylated triadin binding to any of the CSQ2 proteins tested. There was also no significant Ca²⁺ 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 Ca²⁺. The binding of unglycosylated triadin to the CSQ2-R33Q protein was nearly significant (P > 0.06) in the presence of 1 mM Ca²⁺.

**DISCUSSION**

Several studies of luminal Ca²⁺ 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 Ca²⁺ depends on how channels are activated (they used sulmazole) and that channels solely activated by 1 μM cytosolic Ca²⁺, were sensitive to luminal Ca²⁺. They were not sensitive to changes in luminal Mg²⁺. The lack of Ca²⁺ 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 Ca²⁺ activation efficacy (not its affinity). Efficacy here refers to the maximum Po attainable by cytosolic Ca²⁺ stimulation (affinity to the cytosolic Ca²⁺ EC₅₀). Intra-SR divalent overload would then increase the maximal Po attainable by a cytosolic Ca²⁺ stimulus, explaining the excess RyR2-mediated Ca²⁺ release associated with this pathological condition. Alternatively, this mechanism may just assure that RyR2 channels respond robustly to cytosolic Ca²⁺ stimuli.

**Luminal RyR2 Ca²⁺ Regulation without CSQ2 Present**

We showed that CSQ2-free RyR2 channels, activated by 1 μM cytosolic Ca²⁺, were not sensitive to luminal Ca²⁺ (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 Ca²⁺ (Fill and Copello, 2002), enhancing the prospect of feed-through Ca²⁺ modulation. To avoid this complication, our CSQ2-free RyR2 channels were activated by 100 μM cytosolic Ca²⁺ only to assure the cytosolic Ca²⁺ activation site was saturated.

In these conditions, CSQ2-free RyR2 channels were sensitive to luminal Ca²⁺ concentrations. The Po of CSQ2-free channels doubled (~0.4 to ~0.8) when luminal Ca²⁺ was raised from 10 μM to 10 mM. The same thing happened if luminal Mg²⁺, instead of luminal Ca²⁺, was applied. Thus, a CSQ2-independent form of luminal RyR2 Ca²⁺ regulation exists and it does not distinguish between luminal Ca²⁺ and Mg²⁺. To our knowledge, the ion selectivity of luminal Ca²⁺ control mechanisms has rarely (if ever) been tested before.

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no unbound CSQ2 in the luminal 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-associated 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\(_{MAX}\) 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 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 luminal 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, luminal 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 \(~50\%\) 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.

Fliegel 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-associated 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 CPVT 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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