Mechanism of calsequestrin regulation of single cardiac ryanodine receptor in normal and pathological conditions

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Release of Ca^{2+} from the sarcoplasmic reticulum (SR) drives contractile function of cardiac myocytes. Luminal Ca^{2+} regulation of SR Ca^{2+} release is fundamental not only in physiology but also in physiopathology because abnormal luminal Ca^{2+} regulation is known to lead to arrhythmias, catecholaminergic polymorphic ventricular tachycardia (CPVT), and/or sudden cardiac arrest, as inferred from animal model studies. Luminal Ca^{2+} regulates ryanodine receptor (RyR)2-mediated SR Ca^{2+} release through mechanisms localized inside the SR; one of these involves luminal Ca^{2+} interacting with calsequestrin (CASQ), triadin, and/or junctin to regulate RyR2 function.

CASQ2-RyR2 regulation was examined at the single RyR2 channel level. Single RyR2s were incorporated into planar lipid bilayers by the fusion of native SR vesicles isolated from either wild-type (WT), CASQ2 knockout (KO), or R33Q-CASQ2 knock-in (KI) mice. KO and KI mice have CPVT-like phenotypes. We show that CASQ2(WT) action on RyR2 function (either activation or inhibition) was strongly influenced by the presence of cytosolic MgATP. Function of the reconstituted CASQ2(WT)–RyR2 complex was unaffected by changes in luminal free $[Ca^{2+}]$ (from 0.1 to 1 mM). The inhibition exerted by CASQ2(WT) association with the RyR2 determined a reduction in cytosolic Ca^{2+} activation sensitivity. RyR2s from KO mice were significantly more sensitive to cytosolic Ca^{2+} activation and had significantly longer mean open times than RyR2s from WT mice. Sensitivity of RyR2s from KI mice was in between that of RyR2 channels from KO and WT mice. Enhanced cytosolic RyR2 Ca^{2+} sensitivity and longer RyR2 open times likely explain the CPVT-like phenotype of both KO and KI mice.

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INTRODUCTION

Release of Ca^{2+} from the SR drives contractile function of cardiac myocytes. The cardiac RyR2 Ca^{2+} release channel mediates SR Ca^{2+} release. Single RyR2 channel activity is governed by a myriad of cellular factors including cytosolic Ca^{2+}, Mg^{2+}, and ATP, as well as the local intra-SR (luminal) Ca^{2+} concentration (Fill and Copello, 2002). Luminal Ca^{2+} regulation on SR Ca^{2+} release is fundamental in normal cardiac function and has recently garnered a great deal of attention because abnormal luminal Ca^{2+} regulation is known to lead to arrhythmias, catecholaminergic polymorphic ventricular tachycardia (CPVT), and/or sudden cardiac arrest (Priori and Chen, 2011), as inferred from animal model studies.

Luminal Ca^{2+} regulates RyR2-mediated SR Ca^{2+} release in different ways. Luminal Ca^{2+} may pass through an open RyR2 channel and act on cytosolic Ca^{2+} regulatory sites on that same channel (Laver, 2007). However, RyR2s are largely immune to this auto-RyR2 Ca^{2+} feed-through regulation (Liu et al., 2010). The immunity may arise because Ca^{2+} is already occupying the cytosolic activation site(s) of the open RyR2 and thus the fluxed Ca^{2+} can have little effect (Liu et al., 2010). Note that cytosolic Ca^{2+} activation sites on nearby RyR2s may not be occupied. If the Ca^{2+} fluxing through one RyR2 activates a neighboring RyR2, the result is inter-RyR2 Ca^{2+}-induced Ca^{2+} release (CICR). Because single RyR2 Ca^{2+} flux amplitude varies with luminal Ca^{2+} concentration (i.e., the trans-SR Ca^{2+}-driving force), the likelihood of inter-RyR2 CICR will be luminal Ca^{2+} dependent. Indeed, single RyR2 Ca^{2+} flux regulation of inter-RyR2 CICR is a significant factor in the luminal Ca^{2+} control of SR Ca^{2+} release (Guo et al., 2012; Gillespie and Fill, 2013; Laver et al., 2013). Luminal Ca^{2+} also directly regulates RyR2-mediated SR Ca^{2+} release through mechanisms localized inside the SR. One of these mechanisms entails luminal Ca^{2+} binding directly to the luminal side of the RyR2 protein (Jiang et al., 2004; Qin et al., 2008;

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Abbreviations used in this paper: CASQ, calsequestrin; CICR, Ca^{2+}-induced Ca^{2+} release; CPVT, catecholaminergic polymorphic ventricular tachycardia; EC_{50}, 50% effective concentration; JC, junctin; KI, knock-in; KO, knockout; MOT, mean open time; Po, open probability; TRD, triadin.

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Dulhunty et al., 2012). Another involves luminal Ca^{2+} interacting with calsequestrin (CASQ), triadin (TRD), and/or junctin (JC) to regulate RyR2 function.

Either CASQ mutation or ablation generates SR Ca^{2+} release abnormalities, leading to the CPVT phenotype (Knollmann et al., 2006; Priori and Chen, 2011). This not only highlights the significance of CASQ-dependent RyR2 regulation but also justifies the effort to understand the underlying CASQ-based mechanism. Progress has been slowed by the complexity of the CASQ–RyR interaction. In cardiac muscle cells, only one CASQ isoform is expressed (CASQ2; Lahat et al., 2001), whereas skeletal muscle contains CASQ2 and the skeletal muscle CASQ1 isoform (Paolini et al., 2007). CASQ is a low affinity, high capacity intra-SR Ca^{2+}-binding protein (MacLennan and Wong, 1971; Fliegel et al., 1987; Scott et al., 1988; Choi and Clegg, 1990; Arai et al., 1992). Several Ca^{2+} ions (20–80) bind to CASQ with a K_d of ~1 mM (di Barletta et al., 2006). Ca^{2+} binding to CASQ induces a significant protein conformational change (Slupsky et al., 1987; Mitchell et al., 1988), as CASQ transforms from its disordered monomeric state into a polymer (Park et al., 2003). CASQ is closely associated with the RyR2 channel, as are TRD and JC (Györke et al., 2004). It is thought that CASQ2 serves two functions: one as a local intra-SR Ca^{2+} buffer, and another as part of an intra-SR Ca^{2+}-dependent RyR2 regulatory complex (Bers, 2004; Terentyev et al., 2007).

Here, we examine CASQ2-RyR2 regulation at the single RyR2 channel level. Single RyR2s were incorporated into planar lipid bilayers by the fusion of native SR vesicles. Native SR vesicles were isolated from either WT, CASQ2 knockout (KO), or R33Q-CASQ2 knock-in (KI) mice and thus contain the corresponding and variable complements of CASQ2, JC, and TRD. KO and KI mice have CPVT-like phenotypes (Knollmann et al., 2006; Rizzi et al., 2008). We show that CASQ2(WT) action on RyR2 function (either activation or inhibition) is determined by the presence of cytosolic MgATP. In the presence of cytosolic MgATP, the addition of CASQ2(WT) inhibited rat and mouse RyR2s that had been stripped of endogenous CASQ2. Function of the reconstituted CASQ2(WT)–RyR2 complex was unaffected by changes in luminal Ca^{2+} (from 0.1 to 1 mM). The inhibition exerted by the addition of CASQ2(WT) resulted in a reduction in cytosolic Ca^{2+} activation sensitivity. We also tested the function of mouse RyR2s from WT, KO, and KI SR vesicles that were never subjected to stripping and adding back of CASQ2. WT, KO, or KI RyR2 function was also insensitive to luminal Ca^{2+} (from 0.1 to 1 mM). However, KO RyR2s were significantly more sensitive to cytosolic Ca^{2+} activation and had significantly longer mean open times (MOTs) than WT channels. The cytosolic Ca^{2+} sensitivity of KI RyR2 was in between that of KO and WT RyR2 channels. The enhanced cytosolic RyR2 Ca^{2+} sensitivity and longer open time likely explain the CPVT-like phenotype of the KO and KI mice. Western blotting shows that KO and KI SR vesicles contain substantially reduced levels of TRD and JC. The stripping of CASQ2(R33Q) from KI RyR2s resulted in KO-like function. The addition of CASQ2(WT) to KO RyR2s did not entirely restore WT-like function, consistent with normal CASQ2 action on RyR2 function requiring a normal complement of TRD/JC to be present.

MATERIALS AND METHODS

Animals

All animal procedures were approved by the Animal Care and Use Committee of the Rush University Medical Center (rat) and of University of Padova (mouse).

Heavy SR microsomes

Heavy SR microsomes were prepared from either rat or mouse ventricular muscles. Rat SR microsomes were isolated using the method described by Chamberlain and Fleischer (1988). As for mice, 8-wk-old male animals were used: 31 for WT, 44 for KO, and 37 for KI. Transgenic homozygous KI, transgenic homozygous KO, and C57BL6 control WT mice were described previously (Rizzi et al., 2008; Denegri et al., 2012). Mouse SR microsomes were prepared from WT, KO, and KI mouse ventricular muscle as follows: frozen tissue was homogenized in five volumes of 0.3 M sucrose, 0.5 mM DTT, 3 mM NaN3, and 10 mM imidazole, pH 7.4, in the presence of protease inhibitors (PMSF and benzamidine), with a homogenizer (Polytron; Kinematica) at 21,500 rpm for 15 s, followed by 40 s at 24,000 rpm. The homogenate was centrifuged for 25 min at 3,000 *g*. Supernatant was saved, and pellet was rehomogenized and spun again at 3,000 *g*. Combined supernatants were centrifuged at 20,000 *g* for 25 min. After centrifugation, supernatant was filtered and then centrifuged at 120,000 *g* for 120 min. The pellet (SR microsomes) was resuspended in the homogenization buffer. Protein was quantified according to Lowry et al. (1951).

Single-channel recording

Planar lipid bilayers (100-µm diameter) were composed of a 5:4:1 mixture (50 mg/ml in decane) of bovine brain phosphatidylethanolamine, phosphatidylserine, and phosphatidylcholine. The solution in the compartment (cis) on one side of the bilayer was virtually grounded and initially contained a HEPES-Tris solution (250 mM HEPES and 120 mM Tris, pH 7.4). The solution in the compartment (trans) bathed on the other side of the bilayer was initially filled with a HEPES-Ca^{2+} solution (250 mM HEPES and 1–10 mM $Ca(OH)_2$, pH 7.4). Heavy SR microsomes (5–15 µg) were added to the cis solution along with 500 mM CsCl and 2 mM CaCl to promote microsome fusion. Microsome fusion resulted in the cytosolic side of the RyR2 channel facing the cis compartment (Tu et al., 1994), and thus the cis solution is referred to as cytosolic. The trans solution is the intra-SR surface of the RyR2 and thus is referred to as luminal. Immediately upon observing single RyR2 activity, the cytosolic and luminal solutions were replaced to establish the various recording conditions used in our studies (see figure legends for specific solution compositions). The various recording solutions contained multiple RyR2-permeable cations (Ca^{2+} and Cs^+ or Ca^{2+}, Mg^{2+}, and Cs^+). The single RyR2 currents were net currents with Cs^+ acting as the primary charge carrier. All recordings were done at room temperature with current sampled at 50 µs/pt and filtered at 1 kHz. No correction for missing events was made. Single-channel analysis was done using pCLAMP9 software (Molecular Devices).

Native and stripped channels

Single RyR2 studies were done using native RyR2 or CASQ-stripped RyR2 channels. Stripped channels were cleared of associated CASQ by exposing the luminal side of the channel to 20 mM Ca^{2+} for more than 10 min (Qin et al., 2008). This stripping process is analogous to that applied by Györke et al. (2004) and Beard et al. (2005). In some cases, pre-isolated CASQ(WT) (see Qin et al., 2008) was added back (at 10 µg/ml) to the luminal side of the stripped RyR2. Native RyR2s are those that were not subjected to the stripping protocol and are referred throughout this paper without any adjective. The luminal side of such native RyR2s was never subjected to free Ca^{2+} concentrations >1 mM.

Western blot

Equal amounts of SR microsomes (100 µg) were analyzed by SDS-PAGE (Rizzi et al., 2008). After transfer to membranes, immunoblots were revealed with the corresponding primary antibodies specific for CASQ2 (Thermo Fisher Scientific), TRD or JC (provided by I. Marty [Grenoble Institut des Neurosciences, La Tronche, France] and S. Cala [Wayne State University, Detroit, MI], respectively), and secondary antibodies conjugated with either alkaline phosphatase or horseradish peroxidase. In the latter case, visualization was achieved using ECL Western Blotting substrate (Thermo Fisher Scientific). Intensity of each band was determined by Scion Image software. Protein-signal densities were normalized to the corresponding actin-signal densities within a linear relationship of antigen concentration versus signal density.

RESULTS

Györke et al. (2004) reported that the addition of CASQ2 inhibited single RyR2 channels when cytosolic Mg^{2+} and ATP are present. However, Beard et al. (2005) showed that CASQ2 activated RyR2s when cytosolic ATP, but no Mg^{2+}, was present. Qin et al. (2008) showed that CASQ2 activated stripped RyR2s when no cytosolic ATP or Mg^{2+} was present. We have suggested (Qin et al., 2008) that different cytosolic regulatory states of RyR2 used in these different studies might account for the discrepancies. Here, we directly tested this premise. Fig. 1 compares single rat stripped RyR2 function before and after 10 µg/ml CASQ2(WT) was added to its luminal side. Fig. 1 A shows single stripped RyR2 recordings in the absence of cytosolic Mg^{2+} and ATP, and that the addition of CASQ2(WT) increases channel activity.

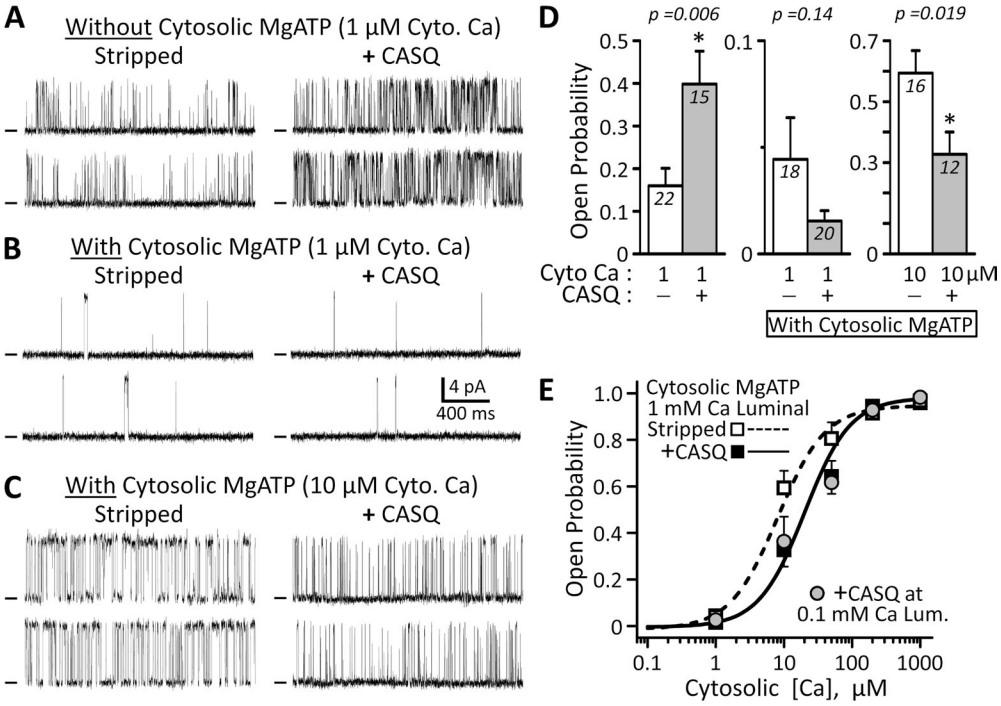


Figure 1. Cytosolic MgATP alters how CASQ2 influences rat RyR2 Po. Rat heavy SR microsomes were fused into bilayers. Single RyR2 channel recordings were done at +40 mV. Open events are upward deflections from marked zero current level. The luminal recording solution contained 0.1 or 1 mM of free Ca^{2+}, 200 mM cesium methanesulfonate, and 10 mM HEPES, pH 7.4. The cytosolic recording solution contained 0.1–1,000 µM of free Ca^{2+}, 0.5 mM EGTA, and 10 mM HEPES, pH 7.4, with or without MgATP (1 mM of free Mg^{2+} and 5 mM of total ATP). Only segments of the overall cytosolic Ca^{2+} concentration range could be tested on any individual channel or bilayer. (A) Sample single RyR2 recordings of stripped (endogenous CASQ removed) and +CASQ (10 µg/ml CASQ added back to the luminal solution) conditions with 1 µM of cytosolic free Ca^{2+} and no MgATP present. There was 1 mM of luminal Ca^{2+} present. (B) Sample single RyR2 recordings of stripped and +CASQ conditions with 1 µM of cytosolic free Ca^{2+} and MgATP present. There was 1 mM of luminal Ca^{2+} present. (C) Sample single RyR2 recordings of stripped and +CASQ conditions with 10 µM of cytosolic free Ca^{2+} and MgATP present. There was 1 mM of luminal Ca^{2+} present. (D) Summary of rat RyR2 Po results. Means (±SEM) are plotted. The number of RyR2s tested is indicated inside bars. White and gray bars are the stripped and +CASQ conditions, respectively. The addition of CASQ significantly increased RyR2 Po (*t* test; $P < 0.05$) when no MgATP was present. The addition of CASQ significantly reduced Po when MgATP was present. There was always 1 mM of luminal Ca^{2+} present. Note that the Po scales for the three plots shown are different. (E) Cytosolic free Ca^{2+} dose–response (Po) of RyR2s in the stripped (open square) and +CASQ (closed square) conditions with cytosolic MgATP present. Gray circles represent the +CASQ condition with 0.1 mM of luminal Ca^{2+} present (instead of 1 mM). The Po in the +CASQ condition was not significantly different with 1 or 0.1 mM of luminal Ca^{2+} present. The dashed line was fit (Hill equation) to the stripped RyR2 data and has a Ca^{2+} EC_{50} of 8.1 µM (Hill coefficient of 1.24). The solid line was fit to the +CASQ (1 mM of luminal Ca^{2+}) RyR2 data and has a Ca^{2+} EC_{50} of 20.1 µM (Hill coefficient of 1.29). Not depicted is a line fit to the +CASQ (0.1 mM of luminal Ca^{2+}) RyR2 data (EC_{50} = 21.3 µM; Hill coefficient of 1.27).

Fig. 1 B shows recordings with 1 mM of cytosolic free Mg^{2+} and ATP present. Even though the open probability (Po) is low (because of the inhibitory action of the Mg^{2+}), the addition of CASQ2(WT) decreased channel activity. To better visualize the action of CASQ2(WT) with both cytosolic Mg^{2+} and ATP present, Fig. 1 C shows sample recording at 10 µM of cytosolic free Ca^{2+}. The addition of CASQ2(WT) clearly decreased RyR2 Po in the presence of cytosolic Mg^{2+} and ATP. A summary of Po data is shown in Fig. 1 D. The addition of CASQ2(WT) to a stripped RyR2 significantly increased Po when no cytosolic Mg^{2+} or ATP was present. The addition of CASQ2(WT) significantly decreased Po with cytosolic Mg^{2+} and ATP present.

In cardiac muscle, the RyR2 function, in both normal and pathological conditions, is driven by cytosolic Ca^{2+} in the presence of cytosolic Mg^{2+} and ATP. Any change in single RyR2 function will influence either the RyR2's cytosolic Ca^{2+} 50% effective concentration (EC_{50}) and/or its Po level at a particular cytosolic Ca^{2+} concentration. This has been demonstrated for various RyR2 regulatory factors including CASQ2, luminal Ca^{2+}, cytosolic ATP, cytosolic Mg^{2+}, and caffeine (Györke and Györke, 1998; Qin et al., 2008, 2009; Porta et al., 2011). The cytosolic Ca^{2+} sensitivity of rat RyR2 channels in the presence of cytosolic Mg^{2+} and ATP is shown in Fig. 1 E. With 1 mM of luminal Ca^{2+}, the cytosolic Ca^{2+} EC_{50} of stripped RyR2s (Fig. 1 E, open squares) was 8.1 µM and shifted to 20.1 µM after 10 µg/ml CASQ2(WT) was added to the luminal solution (closed squares). Lowering the luminal Ca^{2+} level from 1 to 0.1 mM had no effect on single RyR2 activity (cytosolic Ca^{2+} EC_{50} or maximal Po) when CASQ2(WT) was present (Fig. 1 E, gray circles). The cytosolic Ca^{2+} EC_{50} with 0.1 mM of luminal Ca^{2+} and CASQ2(WT) present was 21.3 µM. In the absence of cytosolic Mg^{2+} and ATP, the same luminal Ca^{2+} change significantly alters single rat RyR2 activity (Qin et al., 2008). Thus, the action of luminal Ca^{2+} on CASQ2-associated RyR2 varies with the cytosolic regulatory status of the channel. Single RyR2 function in the presence of both Mg^{2+} and ATP is more likely to reflect that occurring in vivo.

The CASQ2 modulation of RyR2 function is frequently explored using genetically engineered mice. Fig. 2 A shows single WT mouse RyR2 recordings with cytosolic Mg^{2+} and ATP present at 10 µM of cytosolic Ca^{2+}. The recordings shown are with endogenous CASQ2 present (Fig. 2 A, control), after endogenous CASQ2 was stripped off (stripped), and after the addition of 10 µg/ml CASQ2(WT) to the stripped channel (+CASQ). Stripping of endogenous CASQ increased RyR2 activity, and adding CASQ back reduced it. Fig. 2 B shows summary results comparing three conditions: control, stripped, and added back. Thick lines are Hill equation fits to the mouse RyR2 stripped and CASQ add-back data and have cytosolic Ca^{2+} EC_{50} values of 9.7 and 30.9 µM, respectively. The EC_{50} of mouse control data was 73.8 µM (fit not depicted). The thin curves represent the rat RyR2 stripped and CASQ add-back data presented in Fig. 1 E. Stripping and adding back CASQ had similar actions on rat and mouse RyR2 channels (i.e., CASQ addition reduced Po). The addition of CASQ to the stripped RyR2 reduced Po at all cytosolic Ca^{2+} levels but not all the way to the control level. In other words, stripping and adding back CASQ2 did not reproduce control RyR2 where endogenous CASQ2 was present. This suggests that either the stripping and add-back process altered how CASQ2 interacts with the RyR2 channel, or that not enough CASQ2 was added back.

Autosomal recessive CPVT is associated with CASQ2 mutations, either point mutations or splicing mutations, leading to a near absence of CASQ2 (Priori and Chen,

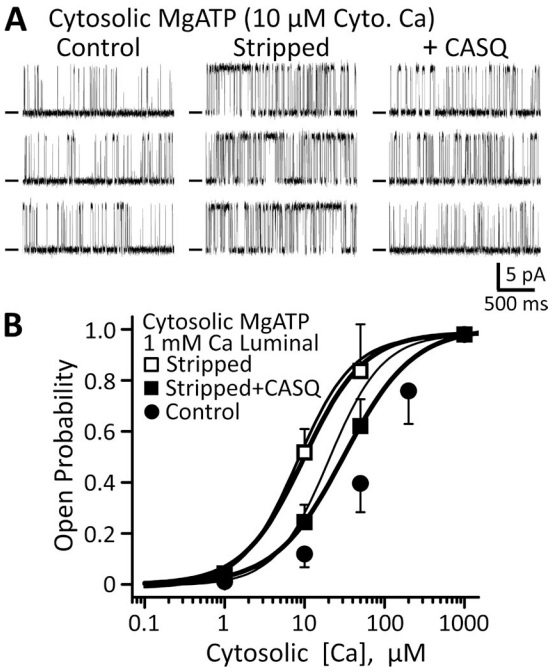
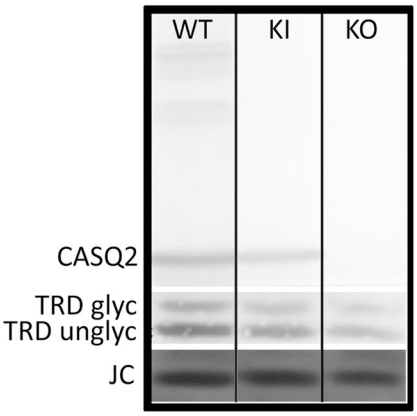


Figure 2. Action of CASQ2 addition on mouse RyR2 Po. Mouse-heavy SR microsomes were fused into bilayers. Single RyR2 channel recordings were done at +40 mV. Open events are upward deflections from marked zero current level. The luminal recording solution contained 1 mM of free Ca^{2+}, 200 mM cesium methanesulfonate, and 10 mM HEPES, pH 7.4. The cytosolic recording solution contained 0.1–1,000 µM of free Ca^{2+}, 0.5 mM EGTA, 10 mM HEPES, pH 7.4, and MgATP (1 mM of free Mg^{2+} and 5 mM of total ATP). (A) Sample single RyR2 recordings before (control) and after endogenous CASQ was stripped, as well as when 10 µg/ml CASQ was added back to stripped channels. There was 10 µM of cytosolic free Ca^{2+} present. (B) Cytosolic free Ca^{2+} dose–response (Po) of RyR2s in the control (closed circles), stripped (open square), and +CASQ (closed square) conditions. The Po in the stripped and +CASQ conditions (at 10 µM of cytosolic Ca^{2+}) was significantly different (*t* test; $P < 0.05$). Thick lines were fit (Hill equation) to the stripped or +CASQ data and had Ca^{2+} EC_{50}s of 9.7 and 30.9 µM, respectively. Not depicted is a line fit to the control data (EC_{50} = 73.8 µM). Thin lines are fits to our rat RyR2 data (stripped and +CASQ).

2011). It was reported previously that KO and R33Q-CASQ2 KI mice share CPVT-like phenotypes (Knollmann et al., 2006; Rizzi et al., 2008). Fig. 3 shows protein profiles of cardiac SR microsomes obtained from WT, KI, or KO hearts. KI microsomes have a lower R33Q-CASQ2 content compared with CASQ2 content in WT microsomes, and reduced expression of both isoforms of TRD and of JC, as reported previously in total heart homogenates (Rizzi et al., 2008), whereas KO microsomes are characterized by a more drastic reduction of both TRD and JC compared with both WT and KI, as judged by densitometric analysis (Fig. 3).

RyR2s from WT, KO, or KI cardiac SR microsomes were incorporated into bilayers. Fig. 4 A shows single RyR2 recordings from each type of microsomes at two luminal Ca^{2+} concentrations (0.1 and 1 mM) in the presence of cytosolic Mg^{2+} and ATP. The cytosolic Ca^{2+} concentration was 10 µM. The Po of endogenous RyR2s from KO and KI microsomes was higher compared with that of WT microsomes. The change in luminal Ca^{2+} concentration had no noticeable action on WT, KO, or KI RyR2 function. Fig. 4 B shows a summary of Po results. There was no detectable Po change of WT, KO, or KI channels when luminal Ca^{2+} was changed from 0.1 to 1 mM. This insensitivity of mouse RyR2s to luminal Ca^{2+}, when both cytosolic Mg^{2+} and ATP are present, is consistent with our rat RyR2 results (Fig. 1 E), as well as with recently published rat results (Tencerová et al., 2012).



	WT	KI	KO
CASQ2	100%	54.5%	0%
TRD glyc	100%	56.5%	27.3%
TRD unglyc	100%	40.8%	24.7%
JC	100%	70.6%	28.5%

Figure 3. Expression of CASQ2, TRD, and JC in cardiac microsomes from WT, R33Q-CASQ2 KI, or CASQ2 KO mice. Mouse-heavy SR microsomes were isolated from WT, KO, and KI mice. Identical amounts (100 µg/lane) of microsomes were analyzed by SDS-PAGE and immunoblotted and probed with anti-CASQ2, anti-TRD, or anti-JC antibodies. The average percentages of the amount of R33Q-CASQ2, two forms of TRD and JC in KI and KO microsomes compared with that of WT, are reported in the bottom section.

The cytosolic Ca^{2+} sensitivities of RyR2 channels from mouse WT (closed circles), KI (open squares), and KO (open circles) microsomes, with cytosolic Mg^{2+} and ATP present, are compared in Fig. 4 C. In all cases, the luminal Ca^{2+} concentration was 1 mM. Thick solid lines are Hill equation fits to the WT, KI, and KO results and have EC_{50}s of 73.8, 29.4, and 15.8 µM, respectively. The Po of KO and KI RyR2s was higher compared with that of WT, at all cytosolic Ca^{2+} levels tested. The very low RyR2 Po at 100 nM of free cytosolic Ca^{2+} (which is approximately the diastolic cytosolic Ca^{2+} level), in the presence of cytosolic Mg^{2+} and ATP, makes accurate Po measurements difficult. To predict RyR2 Po at 100 nM, the Hill equation fits in Fig. 4 C are extrapolated to 100 nM of cytosolic Ca^{2+} on an expanded scale (Fig. 4 D). The KO channels do not have any form of CASQ associated with them. Thus, we added 10 µg/ml CASQ(WT) to the KO RyR2 channels (Fig. 4 C, gray circles). The dotted line is a Hill equation fit to the KO plus CASQ results and had a cytosolic Ca^{2+} EC_{50} of 24.6 µM, which is not substantially different than the KO RyR2 EC_{50} (i.e., CASQ absent). The lack of effect of CASQ2 on KO channels is likely caused by the altered complement of both TRD and JC, as shown in Fig. 3. The KI channels had R33Q-CASQ2 associated with them. The R33Q-CASQ2 was stripped from the KI RyR2 channels (Fig. 4 C, gray squares). The dashed line is a Hill equation fit to the KI-CASQ (i.e., stripped) results and had a cytosolic Ca^{2+} EC_{50} of 21.2 µM, which also is not substantially different than the KO EC_{50}.

During diastole, the likelihood that a random single RyR2 opening evokes the inter-RyR CICR that underlies Ca^{2+} spark/waves depends on the duration of the RyR2 openings, not simply their frequency or the overall Po. Because experiments were difficult to perform at the diastolic cytosolic Ca^{2+} concentration (100 nM), we quantified mouse RyR2 open times at 1 µM of cytosolic Ca^{2+} in the presence of Mg^{2+} and ATP. Continuous single RyR2 recordings at 1 µM of cytosolic Ca^{2+} are shown in Fig. 5 A for WT and KO microsomes. The luminal Ca^{2+} concentration was 1 mM, as it would be during late diastole. Openings are infrequent in both WT and KO, but there are some relatively long openings in the RyR2 recording from KO microsomes. Duration of open and closed events was measured in four to five different WT, KO, and KI channels and compiled into composite histograms. Fig. 5 B compares the composite closed-time histograms of WT, KO, and KI RyR2s. The thick line in Fig. 5 B is the best fit with three exponential components. The dashed line in Fig. 5 B in the left-most histograms shows the fits with either one or two exponential components. The WT, KO, and KI closed-time distributions were not markedly different. Fig. 5 C compares

the arithmetic mean closed times of the WT, KO, and KI channels. The mean closed time of the KO RyR2 was about half that of WT, but this apparent difference was not significant. Fig. 5 D compares the composite open-time histograms of WT, KO, and KI RyR2s. In contrast to the closed-time histograms, the distributions of mouse KO and KI open times had many more long openings than did WT channels. Fig. 5 E compares the arithmetic MOT of the mouse WT, KO, and KI RyR2s (open bars). The KO RyR2 MOT was significantly longer than the WT MOT. For comparison, the MOT of rat RyR2s in the same recording conditions with 1 (Fig. 5 E, R1, gray bar) and 0.1 µM (R2, black bar) of cytosolic free Ca^{2+} is also shown.

DISCUSSION

Physiological CASQ2–RyR2 interaction

Qin et al. (2008) showed that single RyR2 channels are activated when luminal Ca^{2+} is elevated from 0.1 to 1 mM, the putative physiological range. This activation occurred without cytosolic Mg^{2+} or ATP present and required the presence of CASQ2. Here, we show that single CASQ2-associated RyR2 channels were not sensitive to luminal Ca^{2+} in the 0.1–1-mM range. The difference may be explained by the different cytosolic activation status of the RyR2. Cytosolic Mg^{2+} and ATP were present here so the RyR2's cytosolic Ca^{2+} EC_{50} was near 10 µM, lower than that reported in Qin et al. (2008). This might explain why early works reported that luminal Ca^{2+} regulation required the presence of cytosolic ATP (Lukyanenko et al., 1996) or sulmazole (Sitsapesan and Williams, 1994), both RyR2-activating ligands. The complexities of the RyR2–CASQ2 regulatory interaction were nicely reviewed by Gaburjakova et al. (2012), and our results fit into their hypotheses. Moreover, Tencerová et al. (2012) reported that luminal Ca^{2+} levels up to 1 mM do not substantially activate RyR2 when physiological Mg^{2+} and ATP levels are present at a diastole-like cytosolic Ca^{2+} level (100 nM), consistent with our results. Tencerová et al. (2012) attributed this to cytosolic Mg^{2+} inhibition masking the action of luminal Ca^{2+}. Our results suggest that this masking may also extend to higher cytosolic Ca^{2+} levels used in our study. Because the cytosol of living cells contains both Mg^{2+} and ATP, we suggest that single-channel luminal RyR2 Ca^{2+} regulation studies are best done with both of these agents present to best reflect cellular reality.

Luminal Ca^{2+} did not modulate CASQ2-associated RyR2s, but removing (or adding back) CASQ2 did significantly alter RyR2 function. In the absence of cytosolic

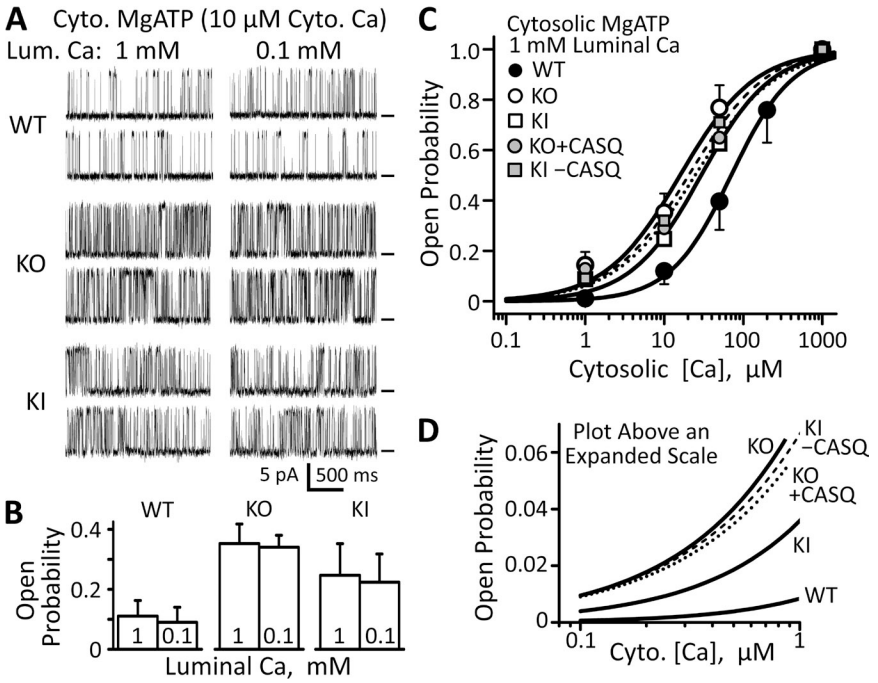


Figure 4. Comparison of mouse WT, CASQ2 KO, and R33Q-CASQ2 KI RyR2 Po. Mouse-heavy SR microsomes were isolated from WT, KO, and KI mice. These microsomes were then fused into bilayers to record single RyR2 channel function (+40 mV). Open events are upward deflections from marked zero current level. The luminal recording solution contained 0.1 or 1 mM of free Ca^{2+}, 200 mM cesium methanesulfonate, and 10 mM HEPES, pH 7.4. The cytosolic recording solution contained 0.1–1,000 µM of free Ca^{2+}, 0.5 mM EGTA, 10 mM HEPES, pH 7.4, and MgATP (1 mM of free Mg^{2+} and 5 mM of total ATP). (A) Sample recordings of WT, KO, and KI RyR2 channels with 1 or 0.1 mM of luminal Ca^{2+} present. Cytosolic free Ca^{2+} was 10 µM. (B) Summary Po results from WT (n = 8), KO (n = 18), and KI (n = 10) RyR2 channels. Luminal Ca^{2+} was either 0.1 or 1 mM. Cytosolic free Ca^{2+} was 10 µM. The Po was not significantly different with 1 or 0.1 mM of luminal Ca^{2+} present in any of the tested RyR2s (WT, KO, and KI). (C) Cytosolic free Ca^{2+} dose–response (Po) of WT RyR2s (closed circles), KO RyR2s (open circles), and KI RyR2s (open squares) with 1 mM of luminal Ca^{2+} present. Gray circles represent KO RyR2 after 10 µg/ml CASQ was added to the luminal solution. Gray squares represent KI RyR2 after the CASQ present was stripped off (see Materials and methods). Error bars (±SEM) are only shown for WT and KO datasets for clarity and roughly represent error level of the other results shown. Solid lines are fits (Hill equation) to the WT, KO, and KI data and have Ca^{2+} EC_{50}s of 73.8, 15.8, and 29.4 µM, respectively. Dotted line is a fit of KO plus CASQ data and has a Ca^{2+} EC_{50} of 24.6 µM. Dashed line is a fit to the KI-CASQ data and has a Ca^{2+} EC_{50} of 21.2 µM. The Po of WT and KO RyR2s at 10 µM of cytosolic Ca^{2+} is significantly different (t test; $P < 0.05$). (D) A portion of the plot presented in C, with an expanded scale to better illustrate curves for cytosolic free Ca^{2+} levels present during diastole in cells.

Mg^{2+} and in the presence of ATP, the addition of CASQ2 increased RyR2 Po at 1 µM of cytosolic Ca^{2+}. This cytosolic Ca^{2+} level is approximately the RyR2's Ca^{2+} EC_{50} in these experimental conditions. Without CASQ2 and in the presence of cytosolic Mg^{2+} and ATP, the addition of CASQ2 decreased RyR2 Po at 1 and 10 µM of cytosolic Ca^{2+}. The RyR2's Ca^{2+} EC_{50} with cytosolic Mg^{2+} and ATP is ~10 µM. Thus, CASQ association/dissociation appears to be the salient RyR2 regulatory event in vivo. Because CASQ conformation and polymerization vary depending on luminal Ca^{2+}, changes in luminal Ca^{2+} may modulate RyR2 function by defining the CASQ2-RyR2 association state. In the presence of Mg^{2+} and ATP, Györke et al. (2004) reported that elevating luminal Ca^{2+} from 0.02 to 5 mM increased the Po of dog RyR2s when CASQ2, TRD, and JC were present. TRD and JC were likely present in our RyR2 studies, and to our knowledge, no species-specific RyR2 function differences exist. A luminal Ca^{2+} response is absent in the present study because we limited our high luminal Ca^{2+} level to 1 mM. Higher luminal Ca^{2+} levels (i.e., 5 mM) are known to dissociate CASQ2 from RyR2 channels (Beard et al., 2005). Thus, the 5 mM of luminal Ca^{2+} applied in Györke et al. (2004) had likely dissociated CASQ2 from the RyR2 regulatory complex. Indeed, we show here that stripping (or dissociation) of CASQ2 increases Po of both rat (Fig. 1) and mouse (Fig. 2) WT RyR2 channels. Thus, the present results suggest that the Po increase observed by Györke et al. (2004) occurred because 5 mM of luminal Ca^{2+} had stripped (or dissociated) CASQ2 from RyR2.

CASQ2–RyR2 interaction in KO and KI mice: Evidence for the molecular mechanism leading to diastolic Ca^{2+} release and CPVT

The CASQ2–RyR2 functional interaction involves TRD and JC (Györke et al., 2004; Dulhunty et al., 2012). CASQ2, TRD, and JC expression patterns vary considerably in mice that have had CASQ2 genetically ablated or mutant CASQ2 inserted (Györke et al., 2004;

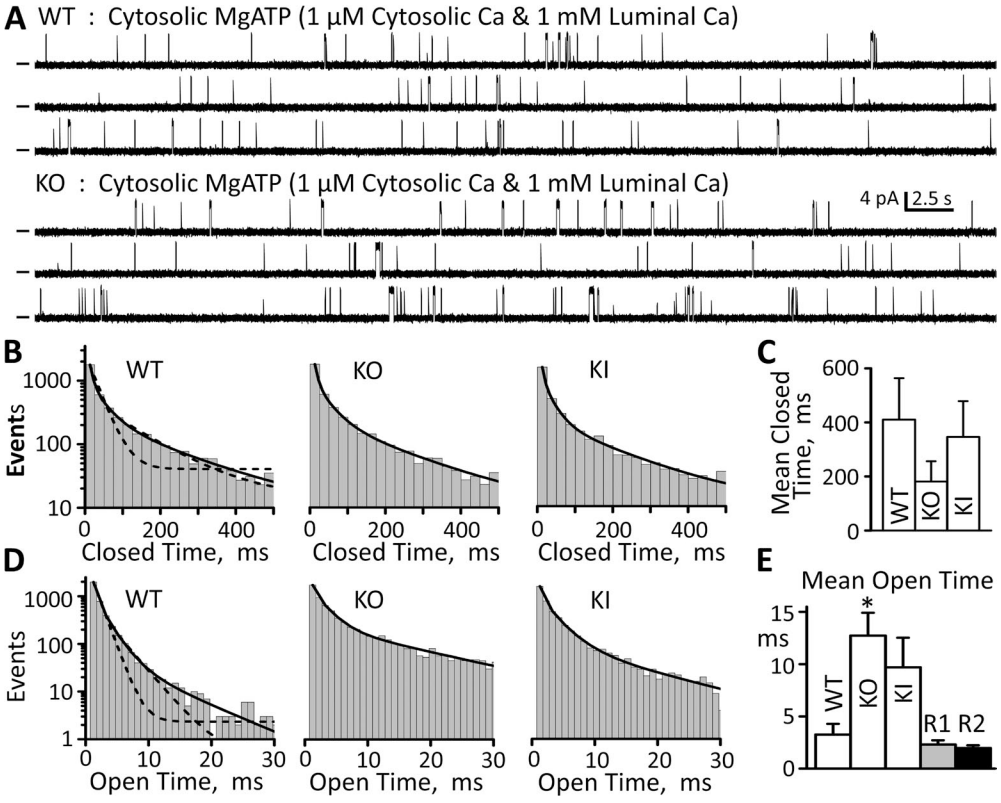


Figure 5. Comparison of mouse WT, CASQ2 KO, and CASQ2-R33Q KI RyR2 open times. Mouse-heavy SR microsomes were fused into bilayers. Single RyR2 channel recordings were done at +40 mV. Open events are upward deflections from marked zero current level. The luminal recording solution contained 1 mM of free Ca^{2+}, 200 mM cesium methanesulfonate, and 10 mM HEPES, pH 7.4. The cytosolic recording solution contained 1 µM of free Ca^{2+}, 0.5 mM EGTA, 10 mM HEPES, pH 7.4, and MgATP (1 mM of free Mg^{2+} and 5 mM of total ATP). (A) Sample recordings of WT and KO RyR2 channel activity. The average Po of WT and KO RyR2s were 0.011 ± 0.006 (n = 8) and 0.104 ± 0.052 (n = 14), respectively. (B) Closed-time histograms each compiled from four to five different channels. The WT histogram represents 3,974 events over 961 s of recording from four different WT RyR2s and was best fit by an exponential function with three time constants (solid line). The best-fit WT closed-time constants (percentage of areas) were 6 (80%), 28 (17%), and 170 ms (3%). Fits with one and two time constants are shown as dashed lines in left-most histograms. The KO histogram represents 7,300 events over 1,269 s of recording from five different KO RyR2s. The best-fit KO closed-time constants were 8 (86%), 46 (12%), and 217 ms (2%). The KI histogram represents 4,788 events over 1,221 s of recording from five different KI RyR2s. The best-fit KI closed-time constants were 8 (81%), 38 (16%), and 183 ms (3%). (C) Mean closed times of the WT, KO, and KI RyR2s. (D) Open-time histograms compiled from four to five different channels. The best-fit (three time constants; solid line) WT open-time constants were 0.6 (83%), 2.0 (16%), and 7.7 ms (1%). Fits with one and two time constant open/closed times of the WT, KO, and KI RyR2s (open bars). For comparison, the MOTs (E) of rat RyRs in the some recording conditions with 1 (R1, gray bar) and 0.1 µM (R2, black bar) of cytosolic free Ca^{2+} are shown. Asterisk indicates a statistical difference (t test; $P < 0.05$) compared with WT channels.

Jiang et al., 2004; di Barletta et al., 2006; Cerrone et al., 2007; Priori and Chen, 2011). Most of these genetically engineered mice display a CPVT-like phenotype. Both dominant and recessive forms of CPVT are generated by abnormal SR Ca^{2+} release during diastole: abnormal Ca^{2+} release from RyR2 induces cell-wide Ca^{2+} waves, delayed after-depolarizations, and triggered activity, all of which are arrhythmogenic (Priori and Chen, 2011). Here, we have explored single RyR2 function in cardiac microsomes derived from WT, KO, and KI mice in diastolic-like recording conditions. Diastolic cytosolic free Ca^{2+} level is normally ~100 nM, and consequently, single RyR2 Po is very low (~0.00016; Porta et al., 2011). Such a low Po level represents a substantial obstacle for single-channel studies. Some previous studies have overcome this obstacle by applying cytosolic ATP without cytosolic Mg^{2+} present (Jiang et al., 2007; Laver, 2007; Hilliard et al., 2010). This experimental maneuver elevates single RyR2 Po at ~100 nM of cytosolic Ca^{2+} to measurable levels but also establishes a very nonphysiological "diastolic" regulatory condition. Consequently, we applied a different approach here. We measured RyR2 Po at several cytosolic free Ca^{2+} concentrations (1 µM to 1 mM) with both Mg^{2+} and ATP present. We then used standard Hill equation fits to extrapolate Po levels at 100 nM of cytosolic Ca^{2+} (see Fig. 4 D). The predicted Po of endogenous RyR2s from WT microsomes (at 100 nM of cytosolic Ca^{2+}) was 0.00064, which is reasonably close to our previous direct rat RyR2 measurement (~0.00016; Porta et al., 2011).

Compared with WT, cardiac SR microsomes from KO and KI mice had ~75 and 50% less TRD, and 72 and 30% less JC, respectively. Cardiac SR from KO mice had obviously no CASQ2, whereas SR from KI mice had ~50% less CASQ than WT counterpart. The predicted Po's of endogenous KO and KI RyR2s (at 100 nM Ca^{2+}) were 0.0095 and 0.0039, respectively. These are 14.8 and 6.1 times the WT Po value. In vivo, CPVT is caused by diastolic intracellular Ca^{2+} waves that are likely initiated by abnormal diastolic RyR2 openings. Higher diastolic RyR2 Po would explain the frequent waves in CASQ2 KO and R33Q-CASQ2 KI mice (Knollmann et al., 2006; Rizzi et al., 2008). The present results suggest that the underlying RyR2 disregulation may involve luminal Ca^{2+}-dependent CASQ2 association with the RyR2, not luminal Ca^{2+} changes modulating the static CASQ2–JC–TRD–RyR2 complex. Specifically, the longer RyR2 openings of KO and KI RyR2s (see Fig. 5) increase the likelihood of the inter-RyR CICR that underlies diastolic Ca^{2+} waves. Guo et al. (2012) also recently showed that the likelihood of the inter-RyR2 CICR will also inherently increase with local SR load because single RyR2 Ca^{2+} current amplitude increases with load (i.e., trans-SR Ca^{2+} driving force), consistent with several mathematical models of CICR local control (Rice et al., 1999; Hinch, 2004; Ramay et al., 2011; Sato and Bers, 2011; Williams et al., 2011). Thus, as local load increases, longer openings would combine with the larger current to amplify the probability of inter-RyR CICR, explaining the CPVT-like phenotype in both KO and KI mice. This view implies that WT hearts are in effect CPVT resistant because diastolic RyR2 openings are normally relatively short. This is consistent with recent reports showing that flecainide (Watanabe et al., 2009; Hilliard et al., 2010) and the nonspecific β blocker carvedilol (Zhou et al., 2011) prevent CPVT by shortening RyR2 MOT.

Conclusions

By comparing the behavior of single RyR2s channels derived from native cardiac SR vesicles obtained from WT, KI, or KO mice, we have provided new insight into the molecular CASQ2-dependent mechanism that promotes inter-RyR2 CICR and diastolic (spontaneous) Ca^{2+} release that ultimately leads to after-depolarizations and triggered activity. This mechanism alters cytosolic RyR2 Ca^{2+} sensitivity and is mediated by a multiple protein complex that includes RyR2, CASQ, TRD, and JC proteins. Evidence is provided that either CASQ2 ablation or CASQ2 point mutation results in a profound molecular adaptation of this protein complex. The ablation or point mutation not only causes the primary expected change in CASQ2/CASQ2-R33Q expression but also results in secondary changes in TRD and JC expression. Analysis of the molecularly adapted RyR2s shows that MOT is likely the key electrophysiological parameter whose change in both KI and KO mice promotes the cascade of events leading to the CPVT phenotype.

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