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Title Page

The anthracycline metabolite doxorubicinol abolishes RyR2 sensitivity to physiological changes in luminal Ca^{2+} through an interaction with calsequestrin

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Running Title Page

Running Title: Anthracyclines disrupt luminal Ca^{2+} sensitivity.

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NON-STANDARD ABBREVIATIONS.
CSQ2 – cardiac isoform of calsequestrin; calsequestrin type 2
Dox – doxorubicinol
DoxOL – doxorubicinol
DTT – dithiothreitol
I'_F – fractional mean current
NHMRC – National Health and Medical Research Council
P_o – open probability
ROS – reactive oxygen species
RyR2 – cardiac isoform of the ryanodine receptor; ryanodine receptor type 2
SERCA2A – Sarco/Endoplasmic Reticulum Ca^{2+}ATPase
SR – sarcoplasmic reticulum
T_o – open duration
T_c – closed duration

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Abstract

The chemotherapeutic anthracycline metabolite doxorubicinol (doxOL) has been shown to interact with and disrupt the function of the cardiac ryanodine receptor Ca^{2+} release channel (RyR2) in the sarcoplasmic reticulum (SR) membrane and the SR Ca^{2+} binding protein calsequestrin2 (CSQ2). Normal increases in RyR2 activity in response to increasing diastolic SR [Ca^{2+}] are influenced by CSQ2 and are disrupted in arrhythmic conditions. Therefore we explored the action of doxOL on RyR2's response to changes in luminal [Ca^{2+}] seen during diastole. DoxOL abolished the increase in RyR2 activity when luminal Ca^{2+} was increased from 0.1-1.5 mM. This was not due to RyR2 oxidation, but depended entirely on the presence of CSQ2 in the RyR2 complex. DoxOL binding to CSQ2 reduced both the Ca^{2+} binding capacity of CSQ2 (by between 48-58%) and its aggregation, and lowered CSQ2 association with the RyR2 complex by 67-77%. Each of these effects on CSQ2, and the lost RyR2 response to changes in luminal [Ca^{2+}], were duplicated by exposing native RyR2 channels to sub-physiological (≤1.0 μM) luminal [Ca^{2+}]. We suggest that doxOL and low luminal Ca^{2+} both disrupt the CSQ2 polymer and that the association of the monomeric protein with the RyR2 complex shifts the increase in RyR2 activity with increasing luminal [Ca^{2+}] away from the physiological [Ca^{2+}] range. Subsequently, these changes may render the channel insensitive to changes of luminal Ca^{2+} that occur through the cardiac cycle. The altered interactions between CSQ2, triadin and/or junctin and RyR2 may produce an arrhythmogenic substrate in anthracycline-induced cardiotoxicity.

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Introduction

The anthracycline cytotoxic antibiotics are highly effective chemotherapeutic drugs whose use is limited by the development of cardiotoxicity (Gewirtz, 1999). The onset and progression of cardiotoxic symptoms is thought to involve an oxidation mechanism, since anthracyclines readily undergo redox cycling to increase cellular levels of reactive oxygen species (ROS) (Minotti et al., 2004). We recently showed that the anthracycline doxorubicin (dox) and its metabolite doxorubicinol (doxOL) altered cardiomyocyte Ca^{2+} handling (i) by oxidation of thiol residues within the ryanodine receptor Ca^{2+} release channel (RyR2) and sarco/endoplasmic reticulum Ca^{2+} ATPase (SERCA2A) and (ii) by binding directly to RyR2 and SERCA2A (Hanna et al., 2014a).

The molecular mechanisms that underlie the oxidative and binding effects of anthracyclines on RyR2 activity have not yet been elucidated. However, altered RyR2 response to luminal Ca^{2+} is a precipitating factor in several pathological conditions including sudden cardiac death (Jiang et al., 2005), ventricular tachycardia (Jiang et al., 2005), CPVT (Fernandez-Velasco et al., 2009; Shan et al., 2012) and heart failure (Belevych et al., 2009; Kubalova et al., 2005). In these studies, altered channel sensitivity to luminal Ca^{2+} is associated with enhanced diastolic Ca^{2+} leak, spontaneous Ca^{2+} waves, and Ca^{2+} alternans, all of which create an arrhythmogenic substrate. We recently showed that changes in the redox state of RyR2 thiols alter the response of the channel to luminal Ca^{2+} (Hanna et al., 2014b). Given that anthracyclines are redox-active and promote oxidation of RyR2 thiol groups (Hanna et al., 2011; Ondrias et al., 1990), a focus of this study was the effect of anthracyclines on the luminal Ca^{2+} sensitivity of RyR2.

The mechanism of the response of RyR2 to changes in luminal Ca^{2+} remains under investigation, with evidence supporting a role for cardiac calsequestrin (CSQ2). CSQ2 is the major Ca^{2+} binding protein in the sarcoplasmic reticulum (SR) Ca^{2+} store, and communicates changes in SR store load to RyR2 (via interactions with triadin and junctin) and thus adjusts its channel activity accordingly (Dulhunty et al., 2012; Qin et al., 2009). This role for CSQ2 is supported by single channel experiments where dissociation of CSQ2 and other accessory proteins prevented RyR2 from responding to increasing luminal Ca^{2+} levels (Gyorke et al., 2004; Qin et al., 2009). Other evidence from purified RyR channels indicates that RyR2 itself also has an innate ability to respond to changes in luminal Ca^{2+} (Chen et al., 2014; Sitsapesan and Williams, 1995; Williams and Sitsapesan, 1997). This has been confirmed by a single

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point mutation in RyR2 expressed in HEK cells that abolished luminal Ca^{2+} sensitivity (Chen et al., 2014). In addition, an active role of RyR2 in regulating Ca^{2+} leak when luminal Ca^{2+} is varied within the physiological range has been demonstrated in cardiomyocytes from CSQ2-knockout mice (Knollmann et al., 2006). Finally, we have shown that single RyR2 channels isolated from sheep heart and stripped of CSQ2 remain responsive to changes in luminal Ca^{2+}. However, this response is more sensitive to luminal Ca^{2+} than CSQ2-associated channels indicating that CSQ2 dampens the response to changes in luminal [Ca^{2+}] (Dulhunty et al., 2012).

It is possible that anthracyclines could influence the regulation of RyR2 by luminal Ca^{2+} through an effect on the CSQ2/RyR2 interaction. Anthracyclines bind with micromolar affinity (Kim et al., 2005) to cardiac CSQ2 and it has been proposed that they target a binding site formed by a hydrophobic cleft in each of the three thioredoxin-like domains in CSQ2's structure (Park et al., 2005). Anthracycline binding to CSQ2 has been shown to reduce the Ca^{2+} binding capacity of CSQ2 and/or alter protein conformation with subsequent reductions in SR Ca^{2+} release and SR Ca^{2+} storage capacity (Charlier et al., 2005; Kim et al., 2005; Park et al., 2005). Therefore our aims in this study were to determine if anthracyclines alter the luminal Ca^{2+} sensitivity of RyR2, whether any effect was mediated by anthracycline binding to CSQ2 and at the same time, clarify the role of CSQ2 in setting the luminal Ca^{2+} sensitivity of RyR2.

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Materials and Methods

Materials. The Novex BOLT electrophoresis system, bis/tris gels, BOLT LDL sample buffer and electrophoresis buffer were obtained from Life Technologies (Mulgrave, Australia). The Western Blot apparatus and protein standards were obtained from Bio-Rad (Gladesville, Australia). Doxorubicinol (adriamycinol) was from Toronto Research Chemicals (Toronto, Ontario, Canada). The Pierce classic immunoprecipitation kit, rabbit polyclonal anti-CSQ2 antibody and Santa Cruz anti-mouse and anti-rabbit IgG were sourced from ThermoFisher Scientific (Scoresby, Vic, Australia). The mouse monoclonal Anti-RyR1 34C was from Developmental Studies Hybridoma Bank (Iowa City, IA, USA). Phospholipids were sourced from Avanti Polar Lipids (Alabaster, AL, USA). All other chemicals, including doxorubicin (adriamycin) were obtained from Sigma-Aldrich (Castle Hill, NSW, Australia).

SR vesicle isolation, RyR2 purification and junctional face membrane purification. Cardiac SR vesicles enriched in RyR2 were isolated from sheep heart as described previously (Laver et al., 1995). RyR2 was purified using established protocols (Beard et al., 2002; Dulhunty et al., 1999; Lai et al., 1988). Junctional face membrane was prepared according to (Costello et al., 1986) with minor change (Beard et al., 2002). The work was undertaken in accordance with the Guide for the Care and Use of Laboratory Animals adopted and promulgated by the U.S. National Institutes of Health, and was approved by the Australian National University and the University of Canberra Animal Ethics Committees.

CSQ2 isolation and purification. Sheep cardiac CSQ2 was purified using native preparative gel electrophoresis and the Ornstein–Davis buffer system (Davis, 1964; Ornstein, 1964), as previously published (Wei et al., 2008; Wei et al., 2009a). In brief, junctional face membrane was loaded onto a 7% cylindrical polyacrylamide gel, electrophoresed until the gel and fractions were eluted from the gel and collected. Fractions containing CSQ2 were identified using SDS PAGE and Western Blot. CSQ2 was washed in a *washing buffer* containing 20mM MOPS, 150mM NaCl and 1 mM CaCl2, pH 7.4.

SDS PAGE and Western Blot was performed as previously described (Laemmli, 1970; Towbin et al., 1979), with the following changes. Proteins were separated on 4-15 % SDS BOLT bis/tris polyacrylamide gels and transferred to PVDF membrane for Western Blot. PVDF membranes were exposed to primary antibodies to CSQ2, triadin, junctin and RyR2

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and secondary HRP-conjugated antibody, prior to chemiluminsence detection. Images were acquired using the Licor Odyssey® Fc Dual-Mode Imaging System.

Single channel recording and analysis. The bilayer potential, expressed relative to *cis* (cytoplasmic) solution, was voltage clamped and switched between +40 mV and -40 mV every 30 s throughout the experiment. Recording parameters have been described previously (Hanna et al., 2014a). In all bilayer studies, channel activity was recorded continuously during the entire experiment.

To test the luminal Ca^{2+} response of RyR2, *trans* [Ca^{2+}] was reduced from 1 mM to 0.1 mM by *trans* perfusion with a 0.1 mM Ca^{2+} solution. Channels were exposed to 0.1 mM Ca^{2+} for <2 min to minimize changes in CSQ2 association with the RyR2 channel complex. The *trans* [Ca^{2+}] was then increased in a stepwise manner to 0.5 mM, 1 mM and 1.5 mM, the concentration range encountered physiologically in the SR lumen during diastole. At least 4 min was allowed between each addition of $CaCl_2$ to ensure that activity stabilised to new levels and stable activity was recorded for ~3 min for analysis (see below).

We examined the effects of initial exposure to low luminal [Ca^{2+}] on RyR2 luminal Ca^{2+} sensitivity. Luminal Ca^{2+} was first lowered from 1 mM used for incorporation to 1 μM by adding 1.3 mM BAPTA to the *trans* solution for 6-10 min, then perfusing the *trans* chamber with the 250 mM Cs^+, 0.1 mM Ca^{2+} solution followed by step increases in Ca^{2+} to 1.5 mM as above. The concentrations of BAPTA required to adjust the free [Ca^{2+}] here and throughout were calculated using the program Bound and Determined (Brooks and Storey, 1992) and free [Ca^{2+}] confirmed using a Ca^{2+} electrode.

The effect of CSQ2 binding to the RyR2 complex on luminal Ca^{2+} sensitivity was examined after dissociating CSQ2 from the RyR2 complex following incorporation into the bilayer, by increasing the *trans* ionic strength with addition of 250 mM Cs^+ (total [Cs^+] is 500 mM *trans* and 250 mM *cis*). Activity was observed until there was an obvious, sustained decrease, which indicated CSQ2 dissociation from the channel (Wei et al., 2009b). The *trans* chamber was then perfused with 250 mM Cs^+ and 0.1 mM Ca^{2+} solution to prevent CSQ2 from reassociating with the channel and to restore symmetrical [Cs^+]. Luminal Ca^{2+} was then increased stepwise to 1.5 mM. Additional experiments were conducted, whereby CSQ2 was reassociated with the CSQ2-dissociated channel (above), by adding 16 μg/ml purified sheep cardiac CSQ2 to the *trans* chamber (using established protocols; (Wei et al., 2009a; Wei et

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al., 2009b)). *Trans* [Ca^{2+}] was lowered to 0.1 mM Ca^{2+} (by addition of 0.9 mM BAPTA), in the presence and absence of 2.5 mM doxOL and channel responses to stepwise luminal Ca^{2+} increase to 1.5 mM were measured.

When exploring the effects of doxOL, or DTT plus doxOL, the compounds were added and their effects on channel activity recorded before the *trans* perfusion and the luminal Ca^{2+} response tested. DoxOL was included in the 0.1 mM Ca^{2+} *trans* perfusion solution, to avoid washout of its effects. Due to the challenging nature of these experiments and time constraints, only the doxorubicin metabolite doxOL was used in bilayer experiments. DoxOL is likely to be more relevant in anthracycline induced cardiotoxicity and 2.5 μM was applied as this concentration caused the most rapid onset of effects with subsequent stabilization of channel activity (Hanna et al., 2014a) and is within the range of clinically relevant concentrations (Gewirtz, 1999).

Data analysis has been described previously (Hanna et al., 2014a). For simplicity, data obtained with open probability (P_o of single channels measured using threshold discrimination) and fractional mean current (I'_F i.e. mean current divided by maximum current in records containing more than one channel) are combined in calculations of average P_o or as P_o relative to the activity with 0.1 mM *trans* Ca^{2+}. Since we did not observe any statistically significant differences between responses recorded at +40 mV and -40 mV, measurements at the two potentials were combined in the average data.

Ca^{2+} binding capacity. The Ca^{2+} binding capacity of CSQ2 was determined using a modified $^{45}Ca^{2+}$ spin dialysis binding assay (Beard and Dulhunty, 2015; Mitchell et al., 1988). All procedures were undertaken at room temperature (22 ±°C). In brief, 200 μg of CSQ2 was incubated at a concentration of 16 μg/ml in 150 mM NaCl, 20 mM MOPS, 1 mM $CaCl_2$ (pH 7.4), 2.5 μM dox or doxOL (controls received vehicle - DMSO) for 15 min and then conjugated (without rotation) with 2.6×10−3 MBq $^{45}Ca^{2+}$ for 15 min. Unconjugated $^{45}Ca^{2+}$ was removed by centrifugation of the samples in Microcon centrifugal filter concentrators at 12000 x *g* for 10 min. $^{45}Ca^{2+}$ radioactivity of the CSQ2-$^{45}Ca^{2+}$ retentate and of an unfiltered sample aliquot were counted using a Packard 1500 Tri-Carb liquid scintillation analyser. Data is presented as nmol $^{45}Ca^{2+}$/mg CSQ2.

Aggregation. Solution turbidity, which measures CSQ2 transition from soluble to insoluble forms (or aggregation), as a function of [Ca^{2+}] was monitored spectrophotometrically in a 1

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cm path length quartz cuvette as previously described (Beard and Dulhunty, 2015; Valle et al., 2008). Three micromolar protein was suspended in a buffer containing 20 mM Tris and 100 mM KCl pH 7.4, with and without 2.5 μM dox or 2.5 μM doxOL (controls received DMSO vehicle). The absorbance (350 nm) was recorded after a series of small aliquots of $CaCl_2$ were added to the cuvette to final concentrations of 0.1 – 3 mM. After each addition, the cuvette was stirred and allowed to equilibrate at room temperature for 7.5 min, after which the absorbance was recorded at 350 nM. Absorbance values were corrected for any change in absorbance due to buffer alone. Increased absorbance at 350 nM reflects the shift towards insoluble particles (aggregated protein) (Beard and Dulhunty, 2015; Valle et al., 2008).

CSQ2 association with the RyR2 complex was tested using a CSQ2 association assay (Wei et al., 2006). All procedures were undertaken at room temperature (22 ±°C). In brief, ~200 μg junctional face membrane was resuspended at 1.5 mg/ml in *bilayer-like buffer* containing 230 mM CsMS, 20 mM CsCl, 1 mM CaCl2, 10 mM TES (pH 7.4) in the presence of *protease inhibitors* (1 mg/ml aprotinin, 1 mM benzamadine, 1 mg/ml leuopeptin, and 1 mM pepstatin A). The suspension was divided into six equivalent fractions and incubated with rotation for 45 min in 500 mM Cs^+, 1 μM Ca^{2+} (by adding 1.5 mM BAPTA), 10 μM Ca^{2+} (by adding 1.1 mM BAPTA), 100 μM Ca^{2+} (by adding 0.9 mM BAPTA) or 2.5 μM doxOL. The sixth fraction served as a control. Final protein concentration in this incubation was ~1 mg/ml. A second series of experiments involved exposure to 2.5 μM doxOL for 0, 10, 20, 30, 45 and 60 min. Fractions were then placed in an amicon ultracel (100 kDa cut off) and centrifuged following manufacturer's instruction (5000 x *g* for 30 min), to elicit dissociated/depolymerized CSQ2 in the flow through and CSQ-depleted junctional face membrane in the retentate. The retentate was washed three times in 250 μls of the *bilayer-like buffer*, sedimented as above, and the CSQ2 content of the CSQ2-deplete junctional face membrane were analyzed by SDS PAGE and Western Blot.

Co-Immunoprecipitation (Co-IP). CSQ2 associated with the RyR2 complex was also determined using a Pierce classic immunoprecipitation kit according to manufacturer's instruction, with the following changes. Two hundred μg SR vesicles were solubilised for 15 min on ice in *IP buffer* containing (mM): 1 Ca^{2+}, 20 MOPS, 150 NaCl, with 0.1% Triton X100, 5% glycerol and *protease inhibitors* (see above); final SR concentration was ~2 mg/ml. Solubilized SR vesicles were precleared by incubation with protein A/G separose at

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4 °C for 2 hr, and unbound proteins removed via sedimentation. Precleared vesicles underwent solution exchange and dilution to ~ 1mg/ml in *bilayer-like buffer* with either 500 mM Cs^+, 1 μM Ca^{2+} (by adding 1.3 mM BAPTA), 10 μM Ca^{2+} (by adding 1.1 mM BAPTA), 100 μM Ca^{2+} (by adding 0.9 mM BAPTA) or 2.5 μM doxOL. Samples were incubated for ~14-16 hr at 4 ºC with anti-RyR 34C antibody-bound protein A/G sepharose and then washed 5 times with 200 μl *bilayer-like buffer*. Samples were eluted by denaturation in 1 X BOLT LDL sample buffer at 60 °C for 10 min, and samples separated from protein A/G separose by centrifugation at 1000 x *g* for 1min.

Statistics. Data from Western Blot experiments (Figure 5 and Supplemental Figure 1) are presented as mean ± standard errors of the mean (SEM). Significance was tested using a two-way ANOVA and Tukey's post hoc test. Single channel data, Ca^{2+} binding data and turbidity is presented as mean ±SEM and significance evaluated in Figures 1-4 and Supplemental Figure 4 using a two-way ANOVA and Bonferroni post hoc test. Single channel data in Figure 6 is presented as mean ±SEM and significance evaluated using an unpaired Student's t-test. $P<0.05$ was considered statistically significant**.**

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Results

Anthracyclines abolish the response of RyR2 to changes in luminal [Ca^{2+}]. Previous reports show that native RyR2 responds to the changes in luminal [Ca^{2+}] that occur during the systolic/diastolic cycle, with RyR2 activity increasing as luminal Ca^{2+} is raised from an end systolic low of 0.1 mM to 1 or 1.5 mM (experienced at the end of diastole) (Belevych et al., 2009; Dulhunty et al., 2012; Hanna et al., 2014b); Figure 1A). We assessed the impact of anthracyclines on luminal Ca^{2+} sensitivity of RyR2 by treating channels with 2.5 μM *trans* doxOL prior to changing luminal [Ca^{2+}]. As expected, addition of *trans* 2.5 μM doxOL caused statistically significant channel activation that was followed by a statistically significant decline in RyR2 activity to a sustained level after ~10 min (Hanna et al., 2014a). After the sustained inhibition was observed, luminal [Ca^{2+}] was decreased to 0.1 mM, then increased step wise to 1.5 mM. Increasing luminal [Ca^{2+}] did not result in a statistically significant increase RyR2 activity (Figure 1B), in stark contrast to the increases in P_o observed in untreated (Dulhunty et al., 2012) or vehicle (DMSO)-treated RyR2 (Figure 1A, C-D). These data indicate that unexpectedly, exposure to doxOL abolishes the channel's response to luminal Ca^{2+}.

In the isolated lipid bilayer there are a limited number of interactions that could modify RyR2 activity. Our earlier work demonstrated that doxOL oxidizes RyR2 thiol groups in addition to interacting via binding to RyR2 (Hanna et al., 2014a). This led us to investigate whether the abolition of luminal Ca^{2+} sensing by doxOL was caused by either oxidation of RyR2 thiols, or altered doxOL binding, to either RyR2 or an accessory protein.

Anthracycline oxidation does not mediate the loss of luminal Ca^{2+} response. To determine if doxOL-induced oxidation mediated the loss of RyR2 sensitivity to luminal Ca^{2+}, channels were exposed to 1 mM DTT before being treated with 2.5 μM doxOL. DoxOL is added to the *trans* chamber, but due its high lipid solubility will cross the bilayer to the *cis* chamber. Earlier work showed that 1 mM DTT added to the *cis* chamber reliably protected channels from the drug-induced oxidation of RyR2 thiols and oxidation-induced change in channel activity (Hanna et al., 2014a; Hanna et al., 2014b). Our rationale was that if doxOL abolished luminal Ca^{2+} sensing via oxidation, protecting these thiols should prevent the loss of luminal Ca^{2+} sensing following doxOL treatment.

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DTT was added to the *cis* solution following channel incorporation and activity was recorded for 5 – 6 min. While adding this concentration of DTT does not alter RyR2 activity *per se* (Figure 2A, C; p= 0.315), it reduces RyR2 responses to luminal Ca^{2+} (Hanna et al., 2014a) and that previously published data is included here (Figure 2D, black line) for comparison. After DTT addition, doxOL was added and activity was recorded until sustained activation was observed (~ 6 – 7 min) as a result of doxOL binding to the channel complex. This activation was sustained due to the absence of oxidation-induced RyR2 inhibition (Hanna et al., 2014a). The *trans* chamber was then perfused with a solution containing 2.5 μM doxOL and 0.1 mM Ca^{2+}, and luminal [Ca^{2+}] was incrementally raised to 1.5 mM. In the presence of DTT and doxOL, the sequential increases in *trans* [Ca^{2+}] had little effect on RyR2 activity, with no statistically significant difference in average P_o between any of the luminal Ca^{2+} concentrations tested (Figure 2B and D; black dotted line, p>0.05). Notably, doxOL-induced dampening of RyR2 response to luminal [Ca^{2+}] was no different in the presence and absence of DTT, suggesting that a mechanism other than thiol oxidation underlies doxOL-induced abolition of RyR2 luminal Ca^{2+} sensing. Indeed, although like DTT, DoxOL is a robust thiol modifying agent, in the presence of DTT, doxOL is rendered incapable of modifying RyR2 thiols (Supplemental Figure 1).

DoxOL binding to CSQ2 alters RyR2 sensitivity to luminal Ca^{2+}. There is considerable evidence illustrating that CSQ2 is a major luminal Ca^{2+} sensor, communicating the level of luminal [Ca^{2+}] to RyR2 via its interactions with triadin and junctin (Dulhunty et al., 2012; Gyorke et al., 2004; Qin et al., 2009). DoxOL binds to CSQ2 (Kim et al., 2005), but not triadin and junctin (Supplemental Figure 2; Kim et al., 2005), therefore it is plausible that doxOL binding to CSQ2 may contribute to the abolition of normal RyR2 luminal Ca^{2+} sensitivity by doxOL.

If the doxOL-induced dampening of Ca^{2+} sensitivity is mediated by CSQ2 dissociation from RyR2, it follows that doxOL would not alter luminal Ca^{2+} sensitivity when CSQ2 is absent, i.e. in CSQ2-dissociated RyR2s. To test this possibility under our current experimental conditions, we first characterized the effect of CSQ2 dissociation on the luminal Ca^{2+} sensitivity of RyR2. The CSQ2 dissociation protocol was based on previous observations with RyR1 (Beard et al., 2002; Wei et al., 2009b). CSQ1 can be removed from RyR1 either by exposure to low Ca^{2+} (<100 μM) or by exposure to high ionic strength (500 mM Cs^{+}). CSQ1 depolymerizes in low Ca^{2+}, but a terminal CSQ1 monomer likely remains associated

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with RyR1 because the interaction of CSQ with junctin/triadin is stronger when Ca^{2+} is lower (Shin et al., 2000). In contrast, with high ionic strength, CSQ monomers become super-compacted, and fully dissociates from the RyR1 complex (Wei et al., 2009b). Observations with RyR2 suggest that a similar sequence of events occur in the cardiac preparation (Wei et al., 2009b).

Fully dissociating CSQ2 from the channels (with 500 mM *trans* Cs^+, CSQ(-)RyR2) resulted in an augmented sensitivity to luminal Ca^{2+}, with relative P_o being higher than native channels (CSQ(+)RyR2) at all [Ca^{2+}] (Figure 3A; $p<0.05$; Supplemental Figure 3 A,C). Of note, the relative P_o at 1.0 mM (physiological resting luminal [Ca^{2+}]) was 4.4-fold ±0.66-fold greater than activity with 0.1 mM luminal Ca^{2+} (Figure 3A; $p<0.05$). Therefore in the absence of CSQ2, RyR2 is more sensitive to luminal Ca^{2+}, confirming CSQ2s ability to influence the luminal Ca^{2+} response of RyR2 (Dulhunty et al., 2012; Gyorke et al., 2004). In a second series of experiments, channels were exposed to 2.5 μM doxOL before and during CSQ2 dissociation and the response to luminal Ca^{2+} tested. As above, sustained doxOL inhibition was established before CSQ2 was dissociated and the *trans* chamber perfused with the 250 mM Cs^+, 0.1 mM Ca^{2+} solution. In marked contrast to the results with CSQ2(+)RyR2 (Figure. 1, 3A), the response of CSQ(-)RyR2 to luminal Ca^{2+} was essentially identical in the presence and absence of doxOL, with no statistically significant difference recorded at any [Ca^{2+}] between 0.1 and 1.5 mM (Figure 3A). To ensure the differences observed in CSQ(-)RyR2 and native CSQ(+)CSQ2 was due to the removed of CSQ2, we undertook a final set of experiments. CSQ2 was dissociated from the channel by exposure to high Cs^+, and then removed from the bath by *trans* chamber perfusion. Exogenous CSQ2 (16 μg/ml) was then added to the *trans* chamber. After CSQ2-reassociation was observed (indicated by a sustained increase in RyR2 activity), the response to luminal Ca^{2+} was tested. Re-association of CSQ2 to CSQ2(-)RyR2 resulted in a modest sensitivity to luminal Ca^{2+}(Figure 3B; Supplemental Figure 3), reminiscent of the degree of sensitivity observed in native RyR2 (compare CSQ(+) RyR2 in Figure 3A and RyR-reassociated CSQ (RyR+Reass CSQ) in Figure 3B). In the presence of doxOL, CSQ-reassociated RyR2 were insensitive to changes in luminal Ca^{2+}, in a near identical manner to native RyR2 (Figure 3B; Supplemental Figure 3). Taken together, these results provide compelling evidence that CSQ2 plays the primary role in the loss of RyR2 luminal Ca^{2+} sensing caused by acute application of doxOL.

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Effects of doxOL on CSQ2 properties. The results in Figure 3 raise the question of how doxOL modifies the CSQ2/RyR2 interaction to produce a dramatic reduction in the luminal Ca^{2+} sensitivity of RyR2. It is clear from the strong response of CSQ2(-)RyR2 to luminal Ca^{2+} that the effect of doxOL is unlikely to be due to stripping CSQ2 from the RyR2/triadin/junctin complex in the same way as exposure to high ionic strength (ie by CSQ2 super-compaction). CSQ2s ability to communicate Ca^{2+} store load to RyR2 is due both to its ability to bind or buffer Ca^{2+} (Park et al., 2003), and to its Ca^{2+} dependent polymer structure (Lee et al., 2012). Dox is proposed to bind to a site on CSQ2 that interferes with CSQ2 polymerization and lowers the Ca^{2+} binding capacity of the protein (Charlier et al., 2005; Kim et al., 2005; Park et al., 2005). Therefore we hypothesized that doxOL application would reduce CSQ2 polymerization and Ca^{2+} binding capacity as occurs with exposure to low Ca^{2+} solutions (Wei et al., 2009b) and that this effect alters CSQ2 regulation of RyR2.

To explore these possibilities we first determined the Ca^{2+} binding capacity of CSQ2 as a function of [Ca^{2+}], in the presence and absence of dox and doxOL using a $^{45}Ca^{2+}$ binding assay. In the absence of anthracyclines, the Ca^{2+} binding capacity of CSQ2 increased as [Ca^{2+}] was raised from 0.1 mM to 1.5 mM (Figure 4A), as expected (Mitchell et al., 1988; Park et al., 2004). CSQ2's Ca^{2+}-dependent Ca^{2+} binding capacity was markedly different when it was incubated with either 2.5 μM dox or doxOL. Whilst there were no statistically significant changes observed at 0.1 mM Ca^{2+}, there was a statistically significant blunting of CSQ2 Ca^{2+} binding capacity at 0.5 – 1.5 mM Ca^{2+} (Figure 4A). At 1.5 mM Ca^{2+}, the effects of dox and doxOL were the most extreme, reducing Ca^{2+} binding to CSQ2 by 66±9 and 45±13 % (respectively) of that found in the absence of anthracycline.

Ca^{2+} binding capacity is dependent on CSQ2 structure and increases upon CSQ2 polymerization (Park et al., 2004). Given that dox and doxOL reduced Ca^{2+} binding to CSQ2 (Figure 4A) and dox can inhibit CSQ2 polymerization (Charlier et al., 2005; Kang et al., 2010), it follows that doxOL would also reduce CSQ2 ability to assemble into an aggregated polymer. CSQ2 aggregation can be measured as turbidity (absorbance at 350 nm), which indicates protein transition from soluble to insoluble forms, with increasing turbidity reflecting a higher proportion of insoluble particles in a sample. In the absence of anthracycline, CSQ2 turbidity increased with rising [Ca^{2+}], reaching a plateau after 1.5 mM Ca^{2+} (Figure 4B). Incubation in doxOL (2.5 μM) yielded a statistically significant 44±8 to 58±13% decrease in absorbance at [Ca^{2+}]'s of 0.5 – 3 mM (Figure 4B), disrupting the degree

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of Ca^{2+}-induced CSQ2 aggregation and indicative of a disruption in CSQ2's capacity for polymerization. The presence of dox resulted in a 77±9%, 69±7% and 73±10% decrease in absorbance at 0.5, 1.5, and 2.0 mM [Ca^{2+}], respectively (p<0.05). The decrease in CSQ2 aggregation with doxOL was statistically significantly greater than that with dox at all [Ca^{2+}]s ≤1.5 mM. Taken together these data illustrate that anthracyclines alter both the Ca^{2+} binding capacity and Ca^{2+}-induced aggregation of CSQ, with doxOL producing the stronger effects.

Do anthracyclines promote loss of CSQ2 from the RyR2 complex? To determine how these dox/doxOL–induced changes in CSQ2's Ca^{2+} binding capacity and aggregation affected CSQ2 association with RyR2, we exposed junctional face membrane (containing RyR2 with associated CSQ2 (Wei et al., 2009b)) to doxOL for 0, 10, 20, 30, 45 and 60 min. Following centrifugation to separate the insoluble pellet from the soluble fraction, the relative amount of CSQ2 remaining in the membrane pellet and presumably associated with RyR2 was detected and quantified (Figures 5A and B). The CSQ2 associated with RyR2 remained consistent as the doxOL incubation time increased to 30 min. It was not until ~45 min incubation (the approximate average overall time of doxOL exposure in bilayer experiments) that there was statistically significant loss of CSQ2 from the RyR2 complex. Thus there was considerable overlap between the biochemical exposure time and exposure during bilayer experiments. In bilayer experiments, channels were exposed to doxOL for ~10 min before luminal Ca^{2+} was lowered to 0.1 mM for 2 to 3 min and the Ca^{2+} was increased in steps to 1.5 mM over the following 30 to 40 min. Thus if doxOL-induced loss of CSQ2 occurs at the same rate in the bilayer experiment as it does in the binding experiment, it is likely that only the final increments in luminal [Ca^{2+}] would have coincided with CSQ2 dissociation. However it was equally possible that CSQ2 dissociation was more rapid in the bilayer situation, where one RyR2/triadin/junctin/CSQ2 complex is exposed to 1 ml of solution. This is in contrast to many complexes being incubated in 100 μl of solution in the binding experiment, so that CSQ2 re-association and dissociation are in a more stable equilibrium, thereby delaying CSQ2 net dissociation. To explore this possibility we analyzed a subset of bilayer experiments where the exposure to each luminal [Ca^{2+}] was briefer, with channels exposed to doxOL for a ≤30 min. As shown in Supplemental Figure 4, exposure to doxOL for ≤30 min elicits a near identical response to luminal Ca^{2+} to that seen with longer exposures, in that channels are rendered insensitive to luminal Ca^{2+} increases from 0.1 – 1.5 mM. Therefore the changes imposed by doxOL in the bilayer situation were complete within 30 min and slightly faster than CSQ2 removal in the dissociation experiments.

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To further explore the temporal difference between the dissociation and bilayer experiments, we increased the incubation volume in the association experiments to 10 ml (which is the maximal experimental volume achievable) and found that whilst data suggests that CSQ2 dissociation may happen more rapidly in a larger experimental volume, the increase in rate was not statistically significant. The relative CSQ2 binding to RyR2 after 30 min exposure of doxOL was 0.8998 ±0.094 (p=0.142) in the larger volume and was not different (p=0.142; data not shown) from that in the lower volume (CSQ2/RyR2 = 0.955 ±0.050, see Figure 5B). It remains possible that this difference may have been statistically significant if we had been able to replicate the bilayer situation of one RyR2/triadin/junctin/CSQ2 complex in a volume of 1 ml and also to reproduce the planar geometry of the bilayer. In conclusion, any time difference between the bilayer and dissociation experiments may not be surprising, given the very different techniques and different environments of the protein.

Exposure to low luminal Ca^{2+} abolishes native RyR2 luminal Ca^{2+} sensitivity. The results obtained above (Figure 5 A-B) are consistent with long term doxOL exposure inducing CSQ2 depolymerization, in a very similar manner to low Ca^{2+} treatment, which causes CSQ2 unfolding and depolymerization (Park et al., 2003), but likely leaves partially unfolded CSQ2 bound to triadin/junctin and associated with RyR2 (Wei et al., 2009b).

In assessing the effects of dox and doxOL on CSQ2 structure and function it became evident that exposure to low luminal [Ca^{2+}]s (≤100 μM) had a similar effect to doxOL (Figure 4A and B above), severely decreasing CSQ2 Ca^{2+} binding capacity and CSQ2 aggregation. This is in contrast to exposure to 500 mM Cs^+ that causes CSQ2 supercompaction and the consequent dissociation of monomers from the RyR2 complex (Wei et al., 2009b). Therefore we compared the amounts of CSQ2 associated with RyR2 after 45 min incubation in doxOL (Figure 5A,B), 500 mM Cs^+ and low Ca^{2+} solutions (Figure 5C and D). As predicted, based on our previous results with RyR1/CSQ1 (Wei et al., 2009b), the amount of CSQ2 removed from RyR2 with 500 mM Cs^+ was larger and statistically significant from that removed by treatment with low Ca^{2+}.

It was surprising however that ~13% of the CSQ2 remained associated with RyR2 after exposure to 500 mM Cs^+, although this was still less than the 41±4 - 51±5 % (p<0.05) remaining associated after exposure to low Ca^{2+}. Low Ca^{2+} (1 μM) caused an almost identical dissociation of CSQ2 to a 45 min doxOL exposure (Figure 5D). Anti-RyR2 Co-IP of junctional face membrane yielded very similar results under near identical experimental

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conditions (n=3, data not shown), confirming that the CSQ2 appearing in the Western Blots of SR vesicles was indeed bound to the RyR2 complex. These results are consistent with the postulate that doxOL, like exposure to low luminal [Ca^{2+}], depolymerizes CSQ2. Thus low luminal [Ca^{2+}] removes a statistically significant fraction of CSQ2 from the RyR2 complex but leaves terminal monomers (and perhaps dimers), associated with RyR2 and the junctional face membrane (Charlier et al., 2005; Kang et al., 2010; Wei et al., 2009b).

Exposure to low luminal Ca^{2+} (with CSQ2 depolymerization) has comparable effects on RyR2 luminal Ca^{2+} sensitivity to doxOL. Since low [Ca^{2+}]s have a similar effect on CSQ2 characteristics and association with RyR2 to doxOL, we decided to test whether the effects of the two treatments on luminal Ca^{2+} sensitivity were also comparable. The specific effect of exposing RyR2 to low luminal Ca^{2+} in the 1 μM to 10 μM range on the response of channels to subsequent changes in the physiological range of luminal Ca^{2+} (0.1 to 1.5 mM), has not previously been examined in channels incorporated into bilayers. Therefore, following incorporation and control channel recording, we exposed RyR2 channels to 1 μM luminal Ca^{2+} for 5-6 min and then raised luminal Ca^{2+} to 0.1 mM, followed by stepwise increments to 1.5 mM Ca^{2+} as above. An expected consequence of lowering luminal Ca^{2+} from 1 mM to 1 μM was the immediate increase in conductance as pore block by Ca^{2+} was removed (Figure 6A). An unexpected effect was the immediate increase in channel activity with a statistically significant 2- to 3-fold increase in both P_o and channel open duration (T_o) (Figure 6B). There were no further consistent changes in channel activity with the subsequent increase in luminal Ca^{2+} to 0.1 mM and step increases to 1.5 mM, and in fact trends towards a decline in activity at 1 and 1.5 mM luminal Ca^{2+}. Like exposure to doxOL (Figure 2), initial exposure to 1 μM luminal Ca^{2+} abolished RyR2 sensitivity to luminal Ca^{2+} within the 0.1 to 1.5 mM range (Figure 6). The effect of exposure to low luminal Ca^{2+} on the RyR2 response to changing luminal Ca^{2+} within this physiological range is markedly different from the effect of exposure to high ionic strength (500 mM Cs^{+}) in increasing sensitivity to luminal Ca^{2+} (Figure 3).

It is notable that a failure of channels to respond to increasing luminal Ca^{2+} through the relatively narrow physiological range of 0.1 and 1.0 mM following exposure to sub-physiological luminal Ca^{2+} (i.e. 1.0 μM luminal Ca^{2+} in Figure 6A and B) is reminiscent of previous results. Recombinant RyR2 channels lacking CSQ2, triadin or junctin (Chen et al., 2014; Sitsapesan and Williams, 1995) responded with a dramatic increase (i.e. 1000-fold) as luminal Ca^{2+} is increased from 1 nM to 50 or 100 μM, but did not increase further with

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further increases in luminal [Ca^{2+}]. In RyR2 incorporated into bilayers using *trans* solutions either lacking luminal Ca^{2+} or containing nM [Ca^{2+}]s, there is in fact a decline in activity with further increases to ≥1 mM [Ca^{2+}] (Laver, 2007; Walweel et al., 2014).

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Discussion

Overview. In this paper, we present important and novel findings regarding the effects of doxOL on cardiac SR Ca^{2+} handling. The principle findings are firstly and unexpectedly, that doxOL abolishes the luminal Ca^{2+} sensitivity of RyR2 within the physiological range of luminal Ca^{2+} concentrations. Secondly, that this loss of sensitivity is mediated by a direct effect of doxOL on CSQ2, and not via doxOL-induced oxidation of RyR2 or doxOL binding to RyR2. Thirdly, that doxOL reduces CSQ2 Ca^{2+} binding affinity and its ability to aggregate and likely to form polymers, thereby reducing amounts of CSQ2 bound to the RyR2 complex. Finally, that the effect of doxOL on CSQ2 is comparable to exposing RyR2 to $[Ca^{2+}]$s (≤100 μM) in that both prevent CSQ2 aggregation and abolish RyR2 sensitivity to changes in luminal $[Ca^{2+}]$ in the 0.1 to 1.5 mM range.

Anthracyclines abolish RyR2 luminal Ca^{2+} sensing within the physiological range of luminal $[Ca^{2+}]$. The sensitivity of RyR2 to activation by luminal Ca^{2+} is an area of intense study, due to its purported role in heart failure, CPVT and atrial fibrillation (Belevych et al., 2009; Kim et al., 2005; Park et al., 2003; Stevens et al., 2009; Wei et al., 2006). These conditions are associated with changes in Ca^{2+} handling in cardiomyocytes, including increased diastolic Ca^{2+} leak and generation of spontaneous Ca^{2+} waves. The leak is thought to result from an increased sensitivity of RyR2 to activation by luminal Ca^{2+} during diastole. While the cause of heightened sensitivity is controversial, enhanced oxidation is thought to be important. Since anthracyclines are powerful oxidizing agents and induce similar phenotypic effects to the arrhythmogenic cardiac conditions, it follows that doxOL could enhance the response of RyR2 to luminal Ca^{2+}. That doxOL actually abolished the luminal Ca^{2+} sensing ability of RyR2 within the physiological range of luminal $[Ca^{2+}]$ was therefore unexpected.

RyR2 oxidation by doxOL does not mediate drug-induced loss of luminal Ca^{2+} sensing. The failure of DTT to restore the normal Ca^{2+} response ruled out doxOL-induced oxidation in mediating the loss of luminal Ca^{2+} sensitivity. DTT blocked the inhibitory effects of doxOL-induced RyR2 oxidation on channel activity at a constant luminal $[Ca^{2+}]$ of 1 mM (Hanna et al., 2014a) and would presumably prevent other functional effects that depended on doxOL modification of these thiols. Thus we can infer that the oxidative effects of doxOL are separate from its ability to modulate luminal Ca^{2+} sensing.

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CSQ2 dissociation restores luminal Ca^{2+} sensing to doxOL-treated RyR2. Since oxidation did not mediate doxOL-induced abolition of luminal Ca^{2+} sensitivity, we hypothesized that doxOL acted by binding to either RyR2 or CSQ2, since both proteins are doxOL binding targets (Park et al., 2005; Saeki et al., 2002). The contribution of CSQ2 was validated when CSQ2 removal by exposure to 500 mM Cs^{+} restored luminal Ca^{2+} sensing to levels similar to those observed in the absence of doxOL (i.e. in CSQ2-stripped RyR2). As doxOL was present in all solutions after CSQ2 dissociation, doxOL binding to RyR2 could not have been responsible for removing luminal Ca^{2+} sensitivity.

The role of CSQ2 in luminal Ca^{2+} sensing by RyR2 is controversial. CSQ2 is important in SR Ca^{2+} handling, acting as a SR Ca^{2+} buffer and communicating SR store load to RyR2, likely via anchoring proteins triadin and/or junctin (Gyorke et al., 2004; Qin et al., 2009; Stevens et al., 2009; Wei et al., 2006). However, CSQ2 dissociation experiments in the absence of doxOL suggest that while CSQ2 modifies the channel's response to luminal Ca^{2+}, RyR2 possesses an innate sensitivity to luminal Ca^{2+}. This is supported by studies in CSQ2 knockout mice where a response to changes in SR load is preserved, despite the absence of CSQ2 (Knollmann et al., 2006). Innate RyR2 luminal Ca^{2+} sensitivity is observed in purified RyR2 (Sitsapesan and Williams, 1995), and was found to depend on RyR2 E4872 in the membrane-spanning helical bundle (Chen et al., 2014). However an innate Ca^{2+} sensitivity does not preclude a role for other factors (such as CSQ2), in modifying RyR2 response to luminal Ca^{2+} changes. Purified RyR2 luminal Ca^{2+} sensitivity is strongest between 10 nM and 0.1 mM luminal [Ca^{2+}] and appears to saturate in the physiological range between 0.1 and 1 mM (Chen et al., 2014). Our results suggest that considerable Ca^{2+} sensitivity in this range in native channels depends on the normal association of regulatory proteins, including CSQ2 polymers, with RyR2.

That CSQ2 is necessary for normal RyR2 luminal Ca^{2+} sensitivity, is highlighted in studies on the CPVT-linked CSQ2 mutant L167H, which reduced Ca^{2+} binding capacity and abolishes luminal Ca^{2+} sensing by RyR2, and CSQ2 Ca^{2+}-dependent polymerization (di Barletta et al., 2006; Qin et al., 2008). The authors attributed this loss of response to a change in the interaction between CSQ2 and triadin. Anthracycline binding to CSQ2 also lowers CSQ2 Ca^{2+} storage capacity and inhibits Ca^{2+} -dependent polymerization of CSQ2 (this study and (Charlier et al., 2005; Kang et al., 2010), which would reduce polymerisation and the amount of CSQ2 bound to junctin/triadin. Interestingly, doxOL treatment partially depleted

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RyR2 of CSQ2, approximately 40% of native CSQ2 remained bound to the channel, likely to be in mononer or dimer formation. The residual CSQ2 bound to RyR2 imparted a very different functional effect to the channel from that of polymerized CSQ2, and instead was comparable to exposure to low luminal [Ca^{2+}]. Both low luminal Ca^{2+} and doxOL reduced CSQ2 Ca^{2+} binding capacity and aggregation, and blunted the ability of RyR2 to respond to subsequent changes in luminal [Ca^{2+}] in the physiological range. We propose that like low ($\leq$1μM) luminal Ca^{2+}, long term doxOL exposure depolymerizes CSQ2, and as a result, reduces the luminal Ca^{2+} sensitivity of the channel in the 0.1 to 1.5 mM range. This was observed in channels exposed to doxOL for 30 or more minutes, yet this exposure time was not sufficient to remove a statistically significant fraction of CSQ2 from RyR2 in the CSQ2 association and RyR2 Co-IP experiments. Thus we postulate that CSQ2 depolymerization proceeds more rapidly in the bilayer situation.

Implications for effects of doxOL in the intact cell. It is difficult to predict how the loss of luminal Ca^{2+} sensing caused by doxOL in single channels would translate to the whole cell. The results indicate that doxOL could effectively block RyR2 luminal Ca^{2+} sensitivity in a cellular environment. While whole cell studies confirm an increase in luminal Ca^{2+} sensitivity in gain-of-function arrhythmogenic heart models (Guo et al., 2007), there are few studies that have quantified such changes in loss-of-function situations. In one example, RyR2 channels carrying a mutation associated with catecholaminergic idiopathic ventricular fibrillation lost the ability to respond to activation by luminal Ca^{2+} (Jiang et al., 2005). Disruption of the communication between CSQ2 and triadin has been suggested to account for the loss of luminal Ca^{2+} sensitivity in RyR2 complex containing a CPVT-linked CSQ2 mutant (Qin et al., 2008). It holds that doxOL may also induce arrhythmia by interrupting the CSQ2 triadin/junctin interaction as discussed above. In addition the maintained luminal Ca^{2+} sensitivity that we see in CSQ2 stripped channels (Figure 3) is consistent with the observation that CSQ2 null myocytes are sensitive to changes in luminal [Ca^{2+}] (Knollmann et al., 2006).

Conclusion

We show that doxOL has a dramatic CSQ2-dependent effect on the RyR2 channel complex, that renders the channel insensitive to the changes in luminal [Ca^{2+}] that occur in the systolic/diastolic cycle (by analogy with some CPVT mutations), and which could account for doxOL-induced arrhythmia. We further show that doxOL reduces the amount of CSQ2 associated with RyR2 and interacts directly with CSQ2 to lower its Ca^{2+} binding capacity and

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induce structural changes consistent with depolymerization. DoxOL's actions closely parallel the effects of exposure to low (≤100 μM) luminal Ca^{2+} on CSQ2 and its association with the RyR2 complex. These actions differ markedly from effects of CSQ2 dissociation from RyR2 following super-compaction in high ionic strength solutions and from CSQ2 removal during RyR2 purification.

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Authorship Contributions

Participated in research design: A.D.H, A.F.D, N.A.B

Conducted experiments: A.D.H, A.L, C.T, H.W, A.F.D, N.A.B

Performed data analysis: A.D.H, A.F.D, N.A.B.

Wrote or contributed to the writing of the manuscript: A.D.H, A.F.D, N.A.B.

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References

Beard, NA, and AF Dulhunty. 2015. C-terminal residues of skeletal muscle calsequestrin are essential for calcium binding and for skeletal ryanodine receptor inhibition. *Skel Mus J*. **5**:6-10.

Beard, NA, MM Sakowska, AF Dulhunty, and DR Laver. 2002. Calsequestrin is an inhibitor of skeletal muscle ryanodine receptor calcium release channels. *Biophys J*. **82**:310-320.

Belevych, AE, D Terentyev, S Viatchenko-Karpinski, R Terentyeva, A Sridhar, Y Nishijima, LD Wilson, AJ Cardounel, KR Laurita, CA Carnes, GE Billman, and S Gyorke. 2009. Redox modification of ryanodine receptors underlies calcium alternans in a canine model of sudden cardiac death. *Cardiovas Res*. **84**:387-395.

Brooks, SP, and KB Storey. 1992. Bound and determined: a computer program for making buffers of defined ion concentrations. *Anal Biochem*. **201**:119-126.

Charlier, HA, Jr., RD Olson, CM Thornock, WK Mercer, DR Olson, TS Broyles, DJ Muhlestein, CL Larson, BJ Cusack, and SE Shadle. 2005. Investigations of calsequestrin as a target for anthracyclines: comparison of functional effects of daunorubicin, daunorubicinol, and trifluoperazine. *Mol Pharmacol*. **67**:1505-1512.

Chen, W, R Wang, B Chen, X Zhong, H Kong, Y Bai, Q Zhou, C Xie, J Zhang, A Guo, X Tian, PP Jones, ML O'Mara, Y Liu, T Mi, L Zhang, J Bolstad, L Semeniuk, H Cheng, J Zhang, J Chen, DP Tieleman, AM Gillis, HJ Duff, M Fill, LS Song, and SR Chen. 2014. The ryanodine receptor store-sensing gate controls Ca^{2+} waves and Ca^{2+}-triggered arrhythmias. *Nature med*. **20**:184-192.

Costello, B, C Chadwick, A Saito, A Chu, A Maurer, and S Fleischer. 1986. Characterization of the junctional face membrane from terminal cisternae of sarcoplasmic reticulum. *J Cell Biol*. **103**:741-753.

Davis, BJ. 1964. Disc Electrophoresis. Ii. Method and Application to Human Serum Proteins. *Ann N Y Acad Sci*. **121**:404-427.

di Barletta, MR, S Viatchenko-Karpinski, A Nori, M Memmi, D Terentyev, F Turcato, G Valle, N Rizzi, C Napolitano, S Gyorke, P Volpe, and SG Priori. 2006. Clinical phenotype and functional characterization of CASQ2 mutations associated with catecholaminergic polymorphic ventricular tachycardia. *Circulation*. **114**:1012-1019.

Dulhunty, AF, DR Laver, EM Gallant, MG Casarotto, SM Pace, and S Curtis. 1999. Activation and inhibition of skeletal RyR channels by a part of the skeletal DHPR II-III loop: effects of DHPR Ser687 and FKBP12. *Biophys J*. **77**:189-203.

Dulhunty, AF, E Wium, L Li, AD Hanna, S Mirza, S Talukder, NA Ghazali, and NA Beard. 2012. Proteins within the intracellular calcium store determine cardiac RyR channel activity and cardiac output. *Clin Exp Pharmacol Physiol.* . **39**:477-484.

Fernandez-Velasco, M, A Rueda, N Rizzi, JP Benitah, B Colombi, C Napolitano, SG Priori, S Richard, and AM Gomez. 2009. Increased Ca^{2+} sensitivity of the ryanodine receptor mutant RyR2R4496C underlies catecholaminergic polymorphic ventricular tachycardia. *Circ Res*. **104**:201-209, 212p following 209.

Gewirtz, DA. 1999. A critical evaluation of the mechanisms of action proposed for the antitumor effects of the anthracycline antibiotics adriamycin and daunorubicin. *Biochem Pharmacol*. **57**:727-741.

Guo, T, X Ai, TR Shannon, SM Pogwizd, and DM Bers. 2007. Intra-sarcoplasmic reticulum free Ca^{2+} and buffering in arrhythmogenic failing rabbit heart. *Circ Res*. **101**:802-810.

Gyorke, I, NA Hester, LR Jones, and S Gyorke. 2004. The Role of Calsequestrin, Triadin, and Junctin in Conferring Cardiac Ryanodine Receptor Responsiveness to Luminal Calcium. *Biophys J*. **86**:2121-2128.

Hanna, AD, M Janczura, E Cho, AF Dulhunty, and NA Beard. 2011. Multiple actions of the anthracycline daunorubicin on cardiac ryanodine receptors. *Mol Pharmacol*. **80**:538-549.

Hanna, AD, A Lam, S Tham, AF Dulhunty, and NA Beard. 2014a. Adverse Effects of Doxorubicin and its Metabolic Product on Cardiac RyR2 and SERCA2A. *Mol Pharmacol*. **86**:438-449.

Hanna, AD, A Lam, C Thekkedam, EM Gallant, NA Beard, and AF Dulhunty. 2014b. Cardiac ryanodine receptor activation by high Ca^{2+} store load is reversed in a reducing cytoplasmic redox environment. *J Cell Sci*. **127**:4531-4541.

Jiang, D, R Wang, B Xiao, H Kong, DJ Hunt, P Choi, L Zhang, and SR Chen. 2005. Enhanced store overload-induced Ca^{2+} release and channel sensitivity to luminal Ca^{2+} activation are common defects of RyR2 mutations linked to ventricular tachycardia and sudden death. *Circ Res* **97**:1173-1181.

Kang, C, MS Nissen, EJ Sanchez, KS Lam, and H Milting. 2010. Potential adverse interaction of human cardiac calsequestrin. *Eur J Pharmacol*. **646**:12-21.

Kim, E, M Tam, WF Siems, and C Kang. 2005. Effects of drugs with muscle-related side effects and affinity for calsequestrin on the calcium regulatory function of sarcoplasmic reticulum microsomes. *Mol Pharmacol*. **68**:1708-1715.

Knollmann, BC, N Chopra, T Hlaing, B Akin, T Yang, K Ettensohn, BE Knollmann, KD Horton, NJ Weissman, I Holinstat, W Zhang, DM Roden, LR Jones, C Franzini-Armstrong, and K Pfeifer. 2006. Casq2 deletion causes sarcoplasmic reticulum volume increase, premature Ca^{2+} release, and catecholaminergic polymorphic ventricular tachycardia. *J Clin Invest.* **116**:2510-2520.

Kubalova, Z, D Terentyev, S Viatchenko-Karpinski, Y Nishijima, I Gyorke, R Terentyeva, DN da Cunha, A Sridhar, DS Feldman, RL Hamlin, CA Carnes, and S Gyorke. 2005. Abnormal intrastore calcium signaling in chronic heart failure. *Proc Natl Acad Sci USA*. **102**:14104-14109.

Laemmli, UK. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature*. **227**:680-685.

Lai, FA, HP Erickson, E Rousseau, QY Liu, and G Meissner. 1988. Purification and reconstitution of the calcium release channel from skeletal muscle. *Nature*. **331**:315-319.

Laver, DR. 2007. Ca^{2+} stores regulate ryanodine receptor Ca^{2+} release channels via luminal and cytosolic Ca^{2+} sites. *Clin Exp Pharmacol Physiol*. **34**:889-896.

Laver, DR, LD Roden, GP Ahern, KR Eager, PR Junankar, and AF Dulhunty. 1995. Cytoplasmic Ca^{2+} inhibits the ryanodine receptor from cardiac muscle. *J Membr Biol*. **147**:7-22.

Lee, KW, JS Maeng, JY Choi, YR Lee, CY Hwang, SS Park, HK Park, BH Chung, SG Lee, YS Kim, H Jeon, SH Eom, C Kang, H Kim do, and KS Kwon. 2012. Role of Junctin protein interactions in cellular dynamics of calsequestrin polymer upon calcium perturbation. *J Biol Chem.* **287**:1679-1687.

Minotti, G, P Menna, E Salvatorelli, G Cairo, and L Gianni. 2004. Anthracyclines: molecular advances and pharmacologic developments in antitumor activity and cardiotoxicity. *Pharmacol Rev*. **56**:185-229.

Mitchell, RD, HK Simmerman, and LR Jones. 1988. Ca^{2+} binding effects on protein conformation and protein interactions of canine cardiac calsequestrin. *J Biol Chem.* **263**:1376-1381.

Ondrias, K, L Borgatta, DH Kim, and BE Ehrlich. 1990. Biphasic effects of doxorubicin on the calcium release channel from sarcoplasmic reticulum of cardiac muscle. *Circ Res*. **67**:1167-1174.

Ornstein, L. 1964. Disc Electrophoresis. I. Background and Theory. *Ann N Y Acad Sci*. **121**:321-349.

Park, H, IY Park, E Kim, B Youn, K Fields, AK Dunker, and C Kang. 2004. Comparing skeletal and cardiac calsequestrin structures and their calcium binding: a proposed mechanism for coupled calcium binding and protein polymerization. *J Biol Chem.* **279**:18026-18033.

Park, H, S Wu, AK Dunker, and C Kang. 2003. Polymerization of calsequestrin. Implications for Ca^{2+} regulation. *J Biol Chem.* **278**:16176-16182.

Park, IY, E Kim, H Park, K Fields, KA Dunker, and C Kang. 2005. Interaction Between Cardiac Calsequestrin and Drugs with Known Cardiotoxicity. *Mol Pharmacol.* **67**:94-104.

Qin, J, G Valle, A Nani, H Chen, J Ramos-Franco, A Nori, P Volpe, and M Fill. 2009. Ryanodine receptor luminal Ca^{2+} regulation: swapping calsequestrin and channel isoforms. *Biophys J*. **97**:1961-1970.

Qin, J, G Valle, A Nani, A Nori, N Rizzi, SG Priori, P Volpe, and M Fill. 2008. Luminal Ca^{2+} regulation of single cardiac ryanodine receptors: insights provided by calsequestrin and its mutants. *J Gen Physiol*. **131**:325-334.

Saeki, K, I Obi, N Ogiku, M Shigekawa, T Imagawa, and T Matsumoto. 2002. Doxorubicin directly binds to the cardiac-type ryanodine receptor. *Life sciences*. **70**:2377-2389.

Shan, J, W Xie, M Betzenhauser, S Reiken, BX Chen, A Wronska, and AR Marks. 2012. Calcium leak through ryanodine receptors leads to atrial fibrillation in 3 mouse models of catecholaminergic polymorphic ventricular tachycardia. *Circ Res*. **111**:708-717.

Shin, DW, J Ma, and DH Kim. 2000. The asp-rich region at the carboxyl-terminus of calsequestrin binds to Ca^{2+} and interacts with triadin. *FEBS Lett*. **486**:178-182.

Sitsapesan, R, and AJ Williams. 1995. The gating of the sheep skeletal sarcoplasmic reticulum Ca^{2+}- release channel is regulated by luminal Ca^{2+}. *J Memb Biol*. **146**:133-144.

Stevens, SC, D Terentyev, A Kalyanasundaram, M Periasamy, and S Gyorke. 2009. Intra-sarcoplasmic reticulum Ca^{2+} oscillations are driven by dynamic regulation of ryanodine receptor function by luminal Ca^{2+} in cardiomyocytes. *J Physiol* **587**:4863-4872.

Towbin, H, T Staehelin, and J Gordon. 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. . *Proc Natl Acad Sci U S A.* . **76**:4350-4354.

Valle, G, D Galla, A Nori, SG Priori, S Gyorke, V de Filippis, and P Volpe. 2008. Catecholaminergic polymorphic ventricular tachycardia-related mutations R33Q and L167H alter calcium sensitivity of human cardiac calsequestrin. *Biochem J*. **413**:291-303.

Walweel, K, L Li, P Molenaar, MS Imtiaz, CG dos Remedios, NA Beard, AF Dulhunty, DF van Helden, and DR Laver. 2014. Regulation of human RyR2 by Ca^{2+} and Mg^{2+} in the cytoplasm and in the lumen of the sarcoplasmic reticulum. *J Gen Physiol*. **144**:263-271.

Wei, L, YA Abdellatif, D Liu, T Kimura, M Coggan, EM Gallant, NA Beard, PG Board, and AF Dulhunty. 2008. Muscle-specific GSTM2-2 on the luminal side of the sarcoplasmic reticulum modifies RyR ion channel activity. *Int J Biochem Cell Biol*. **40**:1616-1628.

Wei, L, EM Gallant, AF Dulhunty, and NA Beard. 2009a. Junctin and triadin each activate skeletal ryanodine receptors but junctin alone mediates functional interactions with calsequestrin. *Int J Biochem Cell Biol*. **41**:2214-2224.

Wei, L, AD Hanna, NA Beard, and AF Dulhunty. 2009b. Unique isoform-specific properties of calsequestrin in the heart and skeletal muscle. *Cell Calcium*. **45**:474-484.

Wei, L, M Varsanyi, AF Dulhunty, and NA Beard. 2006. The conformation of calsequestrin determines its ability to regulate skeletal ryanodine receptors. *Biophys J.* **91**:1288-1301.

Williams, AJ, and R Sitsapesan. 1997. Regulation of current flow through ryanodine receptors by luminal Ca^{2+}. *J Memb Biol*. **159**:179-185.

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Footnotes

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Legends for Figures

Figure 1. DoxOL abolishes the RyR2 response to changes in *trans* Ca^{2+}. (A) - (B) 3s records of single channel activity, where channel opening is upward from zero current (c, solid line) to maximum open conductance (o, dashed line) at +40 mV. Baseline activity in the presence of vehicle (DMSO) **(A)** or 2.5 μM doxOL **(B).** Following *trans* perfusion c*is* and *trans* [Ca^{2+}] are 1 μM and 0.1 mM, respectively. For all channels, *trans* [Ca^{2+}] was sequentially increased to 0.5 mM, 1 mM and then 1.5 mM. Individual P_o values are listed below the trace. **(C)** Average data for P_o measured in the presence (black line) or absence (grey line; vehicle) of 2.5 μM doxOL. **(D)** Average data for P_o relative to activity measured with 0.1 mM *trans* Ca^{2+} with vehicle (grey line) or 2.5 μM doxOL (black line). Average data with a statistically significant difference from baseline activity recorded with 0.1 mM *trans* Ca^{2+} are indicated by * and that different from activity recorded in the absence of doxOL at the same [Ca^{2+}] by # (p<0.05). Graphs in **(C)** and **(D)** show mean±SEM; n = 17 – 24. All channels were exposed to changes in luminal [Ca^{2+}].

Figure 2. DTT pre-treatment does not restore RyR2 Ca^{2+} sensing in the presence of doxOL (A) – (B) 3 s records of single channel activity, where channel is opening downward from zero current (c, solid line) to maximum open conductance (o, dashed line) at -40 mV. **(A)** Control activity (top trace), addition of 1 mM DTT to the *cis* chamber (middle trace), followed by addition of 2.5 μM doxOL to the *trans* chamber (bottom trace). One μM *cis* [Ca^{2+}] and 1 mM *trans* [Ca^{2+}] are held constant throughout the recording. **(B)** RyR2 responses to increasing [Ca^{2+}] in channels treated with both DTT and doxOL (same channel as in **(A)**. RyR2 single channel response to sequential addition of *trans* [Ca^{2+}] from 0.1, 0.5, 1 to 1.5 mM as labelled. *Cis* [Ca^{2+}] is held constant at 1μM. Individual P_o values are listed next to the trace. **(C)** Average P_o in the presence and absence of 1 mM DTT (with 1 mM *trans* Ca^{2+}). **(D)** Average data for P_o relative to baseline activity (with 0.1 mM *trans* Ca^{2+}) in the presence of 1 mM DTT and 2.5 μM doxOL (black dotted line), and of 1 mM DTT alone (black solid line). All channels were exposed to changes in luminal [Ca^{2+}]. Errors bars for average data from RyR2 + DTT are small and are found within the marker. The RyR + DTT data presented have been published in another form previously (Hanna et al., 2014a) and are included here for comparison. Graphs in **(C)** and **(D)** show mean±SEM n=11-16. Average data with a statistically significant difference from baseline activity with 0.1 mM *trans* Ca^{2+}

are indicated by * ($P < 0.05$). Data for untreated channels (grey solid line) and doxOL treated channels (grey dotted line) are included for reference.

Figure 3. CSQ2 dissociation restores luminal Ca^{2+} sensing in the presence of doxOL
Average data (mean±SEM) for relative P_o in CSQ2 dissociated channels (CSQ(-), black lines) in the absence (•) or presence (▲) of 2.5 μM doxOL. All channels were exposed to changes in luminal [Ca^{2+}]. The response of all native channels to increasing *trans* Ca^{2+} in the presence and absence of 2.5 μM doxOL (grey lines) are included for comparison. Average data with a statistically significant difference from baseline activity with 0.1 mM *trans* Ca^{2+} in doxOL treated CSQ2(-) channels is indicated by *. Average data of doxOL treated CSQ2(-) channels which were statistically significantly different to the matched concentration in doxOL treated native channels is indicated by # ($P < 0.05$; n = 7 – 10. (B) In these data, native RyR2 have been subject to a 500 mM Cs^{+} wash (to dissociate CSQ2, chamber perfusion (to removed dissociated CSQ) and been incubated in 16 μg/ml CSQ2, after which CSQ2 reassociated with the RyR2 complex (RyR+Reass CSQ). Average data for P_o relative to activity measured with 0.1 mM *trans* Ca^{2+}, measured in the presence (grey triangle) or absence (grey circle; vehicle) of 2.5 μM doxOL. Average data with a statistically significant difference from baseline activity recorded with 0.1 mM *trans* Ca^{2+} are indicated by * and that different from activity recorded in the absence of doxOL at the same [Ca^{2+}] by # ($p<0.05$; n = 10). All channels were exposed to changes in luminal [Ca^{2+}].

Figure 4. Doxorubicin and doxOL reduce CSQ2 Ca^{2+} binding capacity and CSQ2 aggregation. (A). The $^{45}Ca^{2+}$ binding in a spin dialysis binding assay was carried out using 200 μg protein (at a concentration of 1 mg/ml) in 150 mM NaCl, 20 mM MOPS, 100 μM to 3 mM $CaCl_2$, and 2.6 kBq $^{45}Ca^{2+}$. Each data point is the mean $^{45}Ca^{2+}$ bound, in nmol Ca^{2+}/mg CSQ, and the bars are ±S.E.M at [Ca^{2+}] from 0 – 1.5 mM Ca^{2+}. A statistically significant difference in average data from that of CSQ2 in the presence of vehicle (milliQ water) at the same [Ca^{2+}] is indicated by *, while a statistically significant difference in average data from that of CSQ2 in the presence of doxOL at the same [Ca^{2+}] is indicated by # ($p<0.05$; n = 5 - 9). **(B)** CSQ turbidity, an indicator of polymerization and aggregation is measured as absorbance at 350 nm. Experiments were carried out in a buffer containing 20 mM MOPS, 100 mM KCl, and 0-3 mM $CaCl_2$. Data is presented as mean ± SEM for each condition. A statistically significant difference in average data from that of CSQ2 in the presence of vehicle at the same [Ca^{2+}] is indicated by * ($p<0.05$; n = 5 - 9).

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Figure 5. CSQ2 association with the RyR2 macromolecular complex. Junctional face membrane was exposed to 2.5 μM doxOL in bilayer-like buffer (230 mM CsMS, 20 mM CsCl, 1mM CaCl2, 10 mM TES (pH 7.4)) for different times to determine effects on the amount of CSQ2 associated with the RyR2 complex. **(A)** After SDS PAGE and Western Blot, the blots were probed with antibodies to RyR2 (top) and to CSQ2 (bottom). Incubation times are shown for each lane. **(B)** Relative association of CSQ2 with RyR2. Band densities of target protein (CSQ2) are normalized to RyR2 in each lane and expressed relative to normalized band densities in the absence of doxOL (0 min). Data is presented as mean ± SEM for each condition and * indicate average data with a statistically significant difference ($P < 0.05$; n = 6) from the 0 min incubation. **(C) – (D)** repeat of **(A)** and **(B),** except after incubation for 45 min in bilayer-like buffer under conditions listed below each lane. Lane 1 is a control experiment (doxOL vehicle (veh) - DMSO). Band densities of target protein (CSQ2) are normalized to RyR2 in each lane and expressed relative to normalized band densities in the presence of vehicle. Data is presented as mean ± SEM for each condition and average data with a statistically significant difference from that in the presence of vehicle are indicated by * ($p<0.05$; n = 6).

Figure 6. Effect of low luminal Ca^{2+} pre-treatment on RyR2 luminal Ca^{2+}-sensitivity. Native RyR2 incorporated into lipid bilayers in the presence of 1 mM luminal (*trans*) Ca^{2+} with cytoplasmic Ca^{2+} held constant at 1 μM. After control recording, *trans* Ca^{2+} was lowered to 1 μM for 5-6 min, before being adjusted to 0.1 mM and then raised in steps as indicated to 1.5 mM. **(A)** Single channel recordings at -40 mV at each *trans* [Ca^{2+}] indicated. Channel opening is down from the closed (solid line, c) to the maximum current (broken line, o) levels. **(B)** Graphs show mean ± SEM for average data (n=20) for P_o (upper), open duration (T_o) (middle) and closed duration (T_c) (lower) under each condition as indicated. All channels were exposed to changes in luminal [Ca^{2+}]. Average data with a statistically significant difference from activity recorded with 1 mM *trans* Ca^{2+} are indicated by * ($p<0.05$).

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Figure 1

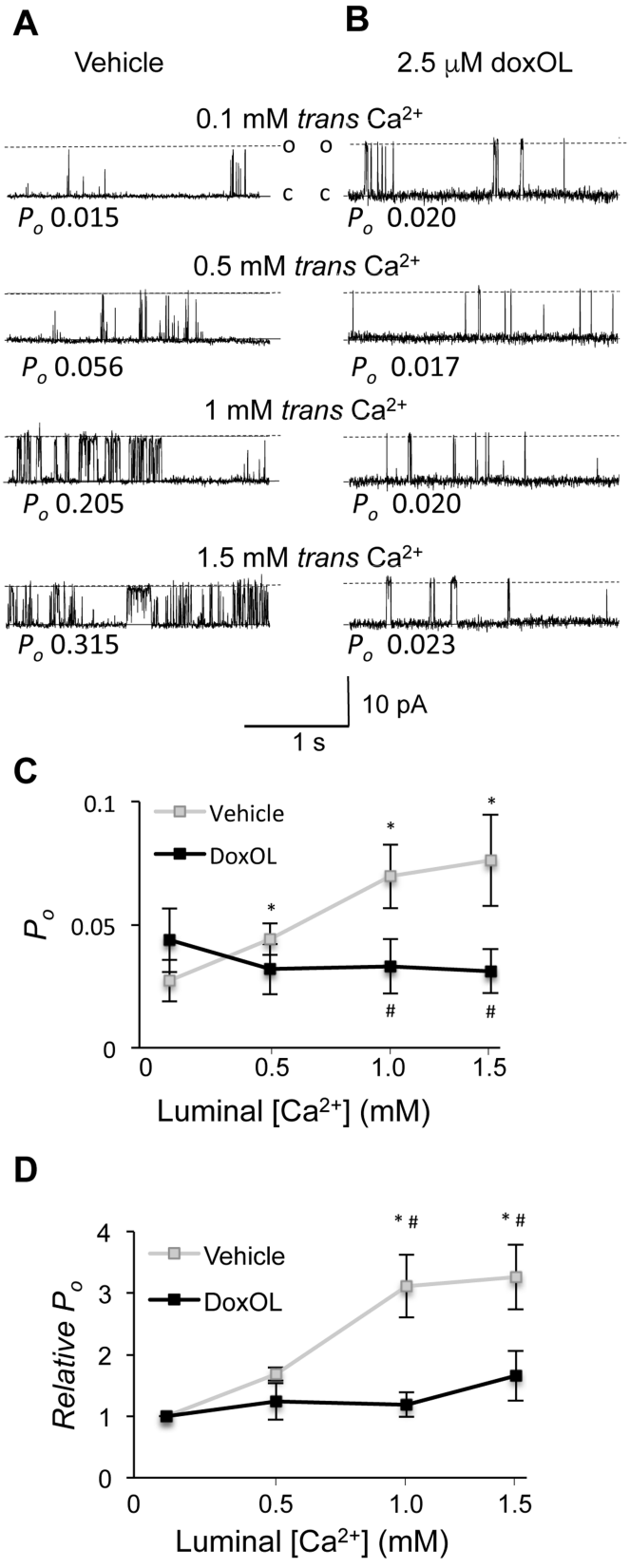

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Figure 2

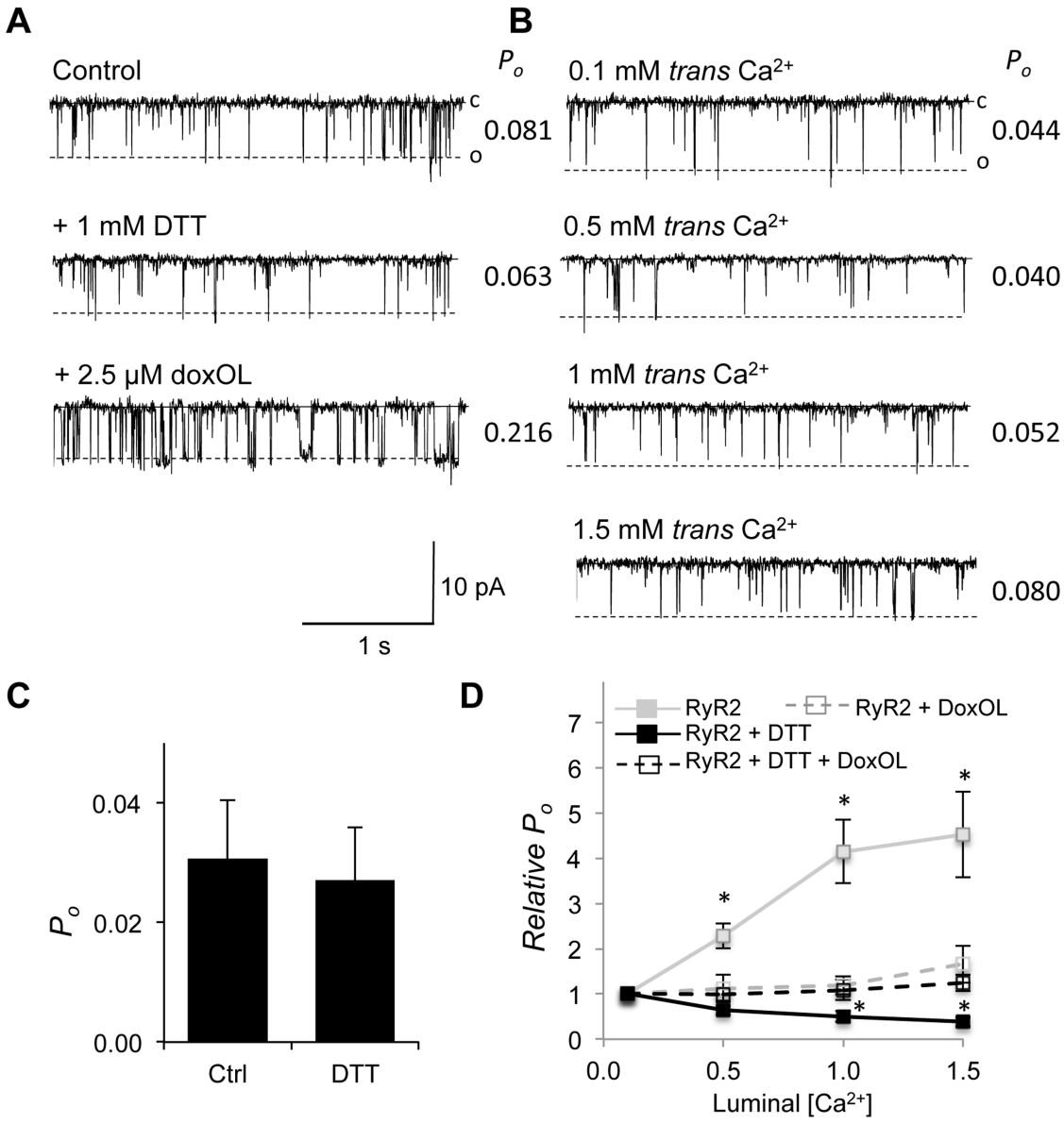

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Figure 3

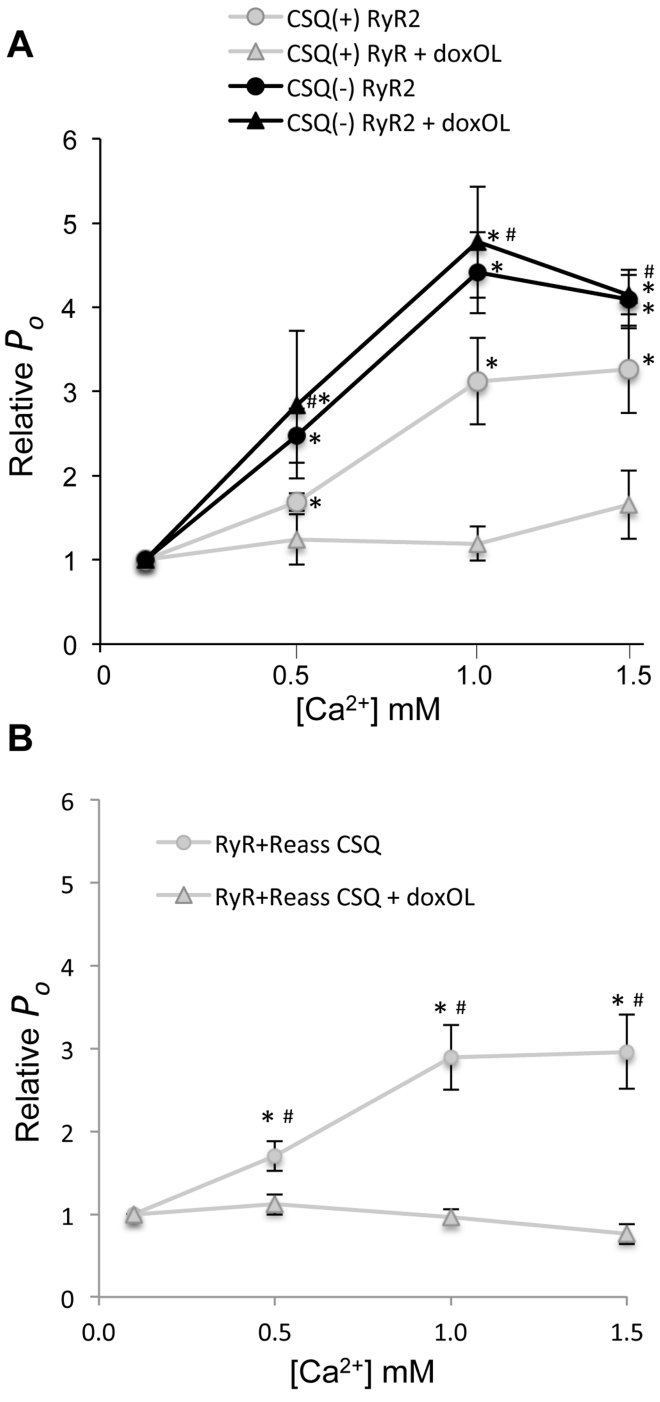

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Figure 4

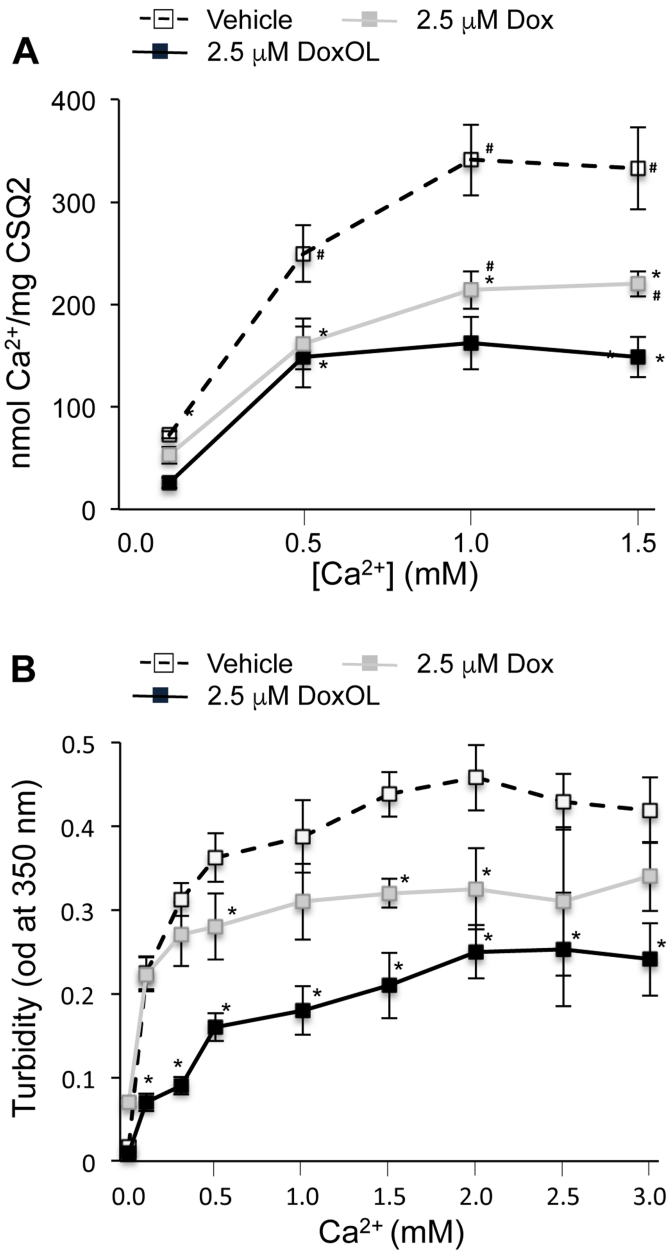

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Figure 5

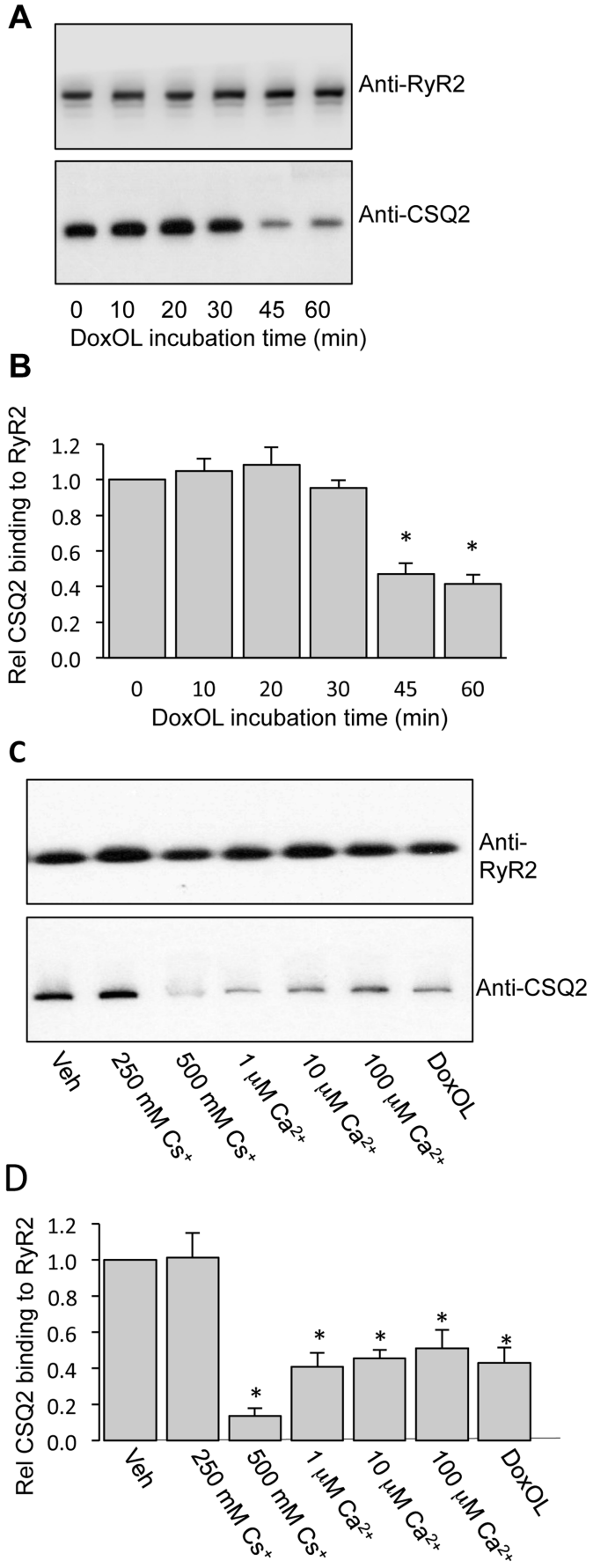

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Figure 6