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Essential role of Calmodulin in RyR inhibition by dantrolene

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Running Title: Dantrolene inhibition requires calmodulin

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ABSTRACT

Dantrolene is the first line therapy of malignant hyperthermia. Animal studies suggest that dantrolone also protects against heart failure and arrhythmias caused by spontaneous Ca release. While dantrolene inhibits Ca^{2+} release from the sarcoplasmic reticulum (SR) of skeletal and cardiac muscle preparations, its mechanism of action has remained controversial, because dantrolene does not inhibit single ryanodine receptor (RyR) Ca^{2+} release channels in lipid bilayers. Here we test the hypothesis that calmodulin (CaM), a physiological RyR binding partner that is lost during incorporation into lipid bilayers, is required for dantrolene inhibition of RyR channels. In single channel recordings (100 nM cytoplasmic [Ca^{2+}] + 2 mM ATP), dantrolene caused inhibition of RyR1 (rabbit skeletal muscle) and RyR2 (sheep) with a maximal inhibition of P_o (E_{max}) to 52 ± 4 % of control only after adding physiological [CaM] = 100 nM. Dantrolene inhibited RyR2 with an IC_{50} of 0.16 ± 0.03 μM. Mutant N98S-CaM facilitated dantrolene inhibition with an IC_{50} = 5.9 ± 0.3 nM. In mouse cardiomyocytes, dantrolene had no effect on cardiac Ca^{2+} release in the absence of CaM, but reduced Ca^{2+} wave frequency (IC_{50} = 0.42 ± 0.18 μM, E_{max}= 47 ± 4 %) and amplitude (IC_{50} = 0.19 ± 0.04 μM, E_{max}=66 ± 4 %) in the presence of 100 nM CaM. We conclude that CaM is essential for dantrolene inhibition of RyR1 and RyR2. Its absence explains why dantrolene inhibition of single RyR channels has not been previously observed.

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INTRODUCTION

Dantrolene is a well-known inhibitor of Ca^{2+} release in skeletal muscle (Hainaut and Desmedt, 1974) that has been used clinically as the treatment for malignant hyperthermia (MH). MH is a potentially fatal inherited disorder of skeletal muscle in which mutations in the proteins involved in excitation-contraction coupling (e.g. RyR1 and DHPR) (Jung et al., 2012; McCarthy et al., 1990; Monnier et al., 1997) cause uncontrolled SR calcium release and muscle contracture in the presence of volatile anesthetics. Notably, mutations in the cardiac RyR isoform (RyR2) that correspond to the MH mutations in RyR1 cause catecholaminergic polymorphic ventricular tachycardia (CPVT) (Yano, 2005). Recent *in vitro* and animal studies suggest that dantrolene has antiarrhythmic effects in CPVT and possibly also in heart failure (Jung et al., 2012; Kobayashi et al., 2009; Kobayashi et al., 2010; Maxwell et al., 2012).

Dantrolene acts on skeletal and cardiac muscle by inhibiting Ca^{2+} release from the SR (Hainaut and Desmedt, 1974; Kobayashi et al., 2005; Uchinoumi et al., 2010). Assays of Ca^{2+} release in intact myocytes and cell homogenates containing SR vesicles (Fruen et al., 1997) suggest that dantrolene inhibits the SR Ca^{2+} release channel with a half-inhibiting concentration (IC_{50}) of 0.3 μM (Kobayashi et al., 2009). Even though a dantrolene binding site has been identified in the DP1 regions in RyR1 and RyR2 (Kobayashi et al., 2009; Parness and Palnitkar, 1995; Paul-Pletzer et al., 2002; Paul-Pletzer et al., 2005), there has been only one direct observation of RyR inhibition by dantrolene in bilayer-based single channel recordings (Nelson et al., 1996). Studies since then find no effect of dantrolene in single channel recordings (Cherednichenko et al., 2008; Diaz-Sylvester et al., 2008; Szentesi et al., 2001; Wagner et al., 2014). Hence, it is not clear if dantrolene acts directly on the RyR or some other protein involved in excitation-contraction coupling such as the DHPR (Chou et al., 2014; Salata et al., 1983).

Calmodulin (CaM) is known to regulate the activity of RyR1 and RyR2 (Balshaw et al., 2001; Tripathy et al., 1995). CaM inhibits RyR2 directly by binding to residues 3583-3603 of each RyR2

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subunit (Huang et al., 2013) with high affinity (K_d 20-100 nM) (Guo et al., 2011). Similarly, CaM may either increase RyR1 activity at resting cytoplasmic [Ca^{2+}] or decrease activity at higher [Ca^{2+}] (Tripathy et al., 1995). Fruen and colleagues (Fruen et al., 1997; Zhao et al., 2001) found that dantrolene reduces the effect of RyR1 activators (but interestingly, not in RyR2) including CaM, suggesting that CaM might augment dantrolene inhibition of RyR1. During the process of RyR2 isolation from the heart and their incorporation into artificial lipid bilayers, the RyR macromolecular complex stays mostly intact (Marks et al., 2002) except for CaM, which is reported to dissociate from the RyR complex with a time constant of less than 1 min (Guo et al., 2011). Hence, bilayer-based channel studies will generally have been made devoid of this important regulatory molecule in the RyR complex, whereas CaM is abundant in intact cell and cell homogenates. Therefore, we hypothesize that CaM is the missing protein and that its absence in bilayer experiments provides explanation as to why dantrolene inhibition has not been observed in single channel RyR recording experiments. We test this hypothesis by examining the effects of dantrolene, in the absence and presence of CaM, on the gating of RyR1 and RyR2 Ca^{2+} release channels incorporated into artificial lipid bilayers and on the frequency and amplitude of Ca^{2+} waves in permeablized cardiomyocytes.

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MATERIALS AND METHODS

Chemicals

SR vesicles containing RyR1 were isolated from rabbit skeletal muscle and RyR2 were isolated from sheep hearts (Laver et al., 1995) and incorporated in artificial bilayer membranes composed of a lipid mixture of phosphatidylethanolamine and phosphatidylcholine (8:2 wt/wt, Avanti Polar Lipids) in n-decane (50 mg/ml, ICN Biomedicals). Experimental solutions contained (in mM) 150 Cs^+ (130 $CsCH_3O_3S$ + 20 CsCl). All solutions were pH buffered using TES (N-tris[hydroxymethyl] methyl-2-aminoethanesulfonic acid; ICN Biomedicals), and titrated to pH 7.4 using CsOH (ICN Biomedicals). Cytoplasmic solutions were buffered to a redox potential of -232 mV with reduced glutathione disulfide (GSSG (0.2 mM) and GSH (4 mM), and luminal solutions were buffered to a redox potential of -180 mV with GSSG (3 mM) and GSH (2 mM). A Ca^{2+} electrode (Radiometer) was used in our experiments to determine the purity of Ca^{2+} buffers and Ca^{2+} stock solutions as well as free $[Ca^{2+}] > 100$ nM. The cesium salts were obtained from Aldrich chemical Company. $CaCl_2$ was obtained from BDH Chemicals. Calmodulin was obtained from two sources, Sigma (St Louise, USA; prepared from bovine testes) and Enzo Life Sciences (New York, USA; prepared from pig brain). Dantrolene (powder) were obtained from Sigma (St Louise, USA). Dantrolene was prepared as stock solutions in DMSO and calmodulin was prepared in milliQ. During experiments the concentrations of calmodulin, dantrolene and Ca^{2+} in the cytoplasmic solution were altered by a local perfusion system (O'Neill et al., 2003), which allowed exposure of a single channel to multiple bathing conditions applied in any chosen sequence with an exchange time of ~ 3s.

Data Acquisition and Analysis

Experiments were carried out at room temperature (23 ± 2^o C). Electric potentials are expressed using standard physiological convention (*i.e*. cytoplasm relative to SR lumen at virtual ground). Control of the bilayer potential and recording of unitary currents was done using an Axopatch 200B

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amplifier (Axon Instruments Pty, Ltd). The current signal was digitized at 5 kHz and low pass-filtered at 1 kHz. Single channel dwell-time histograms of open and closed time, open probability, mean open time and mean closed time, were measured using a threshold discriminator at 50% of channel amplitude (Channel3 software by N.W Laver). Individual readings were derived from 45-120 seconds of RyR2 recording. Hill equations were fitted to the dose-response data by the method of least squares. Average data are given as mean ± SEM. The statistical significance of differences was tested using Student's t-test.

Ca^{2+} wave experiments in ventricular myocytes

Single ventricular myocytes from 12- to 16-week-old C57Bl/6 mice were isolated using an enzymatic digestion method as previously described (Knollmann et al., 2006). Myocytes were first exposed to a Ca^{2+} free relaxing solution and then permeabilized with saponin (40 μ g/mL) for 60 s and placed in internal solution composed (in mM) of 120 K-aspartate, 15 KCl, 5 KH_2PO_4, 0.75 $MgCl_2$, 4% dextran (40,000), 10 HEPES, 5 Mg_2ATP, 10 glutathione (reduced), 0.025 Fluo-4 and 10 phosphocreatine (di-Na). These solutions also contained 10 U/ml creatine phosphokinase (Hwang et al., 2014) and had free [Ca^{2+}] = 120 nM. To allow complete removal of CaM binding to RyR2 in permeabilized myocytes (Yang et al., 2014), all Ca^{2+} wave recordings were done after 30-minute incubation with either dantrolene alone or dantrolene + CaM. Free [CaM] was kept at the physiological concentration of 100 nM. Ca^{2+} waves in myocytes were imaged with a confocal microscope (LSM 510 Zeiss) in line scan mode. Ca^{2+} wave analysis was performed as described (Hwang et al., 2014). Given the variability between different experimental days, the Ca^{2+} wave frequency and amplitude data were normalized to the mean of vehicle group obtained on the same day.

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RESULTS

Essential role of CaM on RyR1 and RyR2 inhibition by dantrolene

To investigate if CaM binding to RyR1 and RyR2 is a prerequisite for their inhibition by dantrolene, RyR activity was measured in the presence of 100 nM cytoplasmic Ca^{2+} (+ 2 mM ATP) for periods of 1 min (vehicle) and then during 1 min exposure to added dantrolene and then again after dantrolene washout. This sequence was repeated in the absence and presence of exogenous 100 nM CaM as shown in figure 1A for rabbit RyR1 and figure 1B for sheep RyR2. In the absence of CaM, dantrolene had no observable effect on the channel open probability (P_o) of either RyR1 or RyR2. However, when CaM was present in the experimental solutions, dantrolene reduced the P_o of both RyR isoforms. This effect of dantrolene on RyR1 and RyR2 was reversible on washout and inhibition could be seen in multiple applications (Figure 1C). The data summary from application-washout experiments in figure 1D shows that dantrolene at both 10 μM and 50 μM significantly reduced the open probability of RyR1 to 50% and RyR2 to 45% of control (*i.e.* vehicle alone), respectively. When CaM was subsequently washed out by perfusion with CaM free solutions for 1 min, dantrolene inhibition was abolished (RyR2 open probability 95 ± 9 % of vehicle, $p = 0.24$). Therefore, dantrolene inhibition of RyR1 and RyR2 requires the presence of CaM. The concentration-dependence of dantrolene inhibition of RyR2 is shown in figure 1E. In the presence of 100 nM CaM (●), inhibition exhibited a sigmoidal dependence on log-concentration with an IC_{50} of 0.16 ± 0.03 μM, a Hill coefficient of ~1 and with a saturating RyR2 open probability (E_{max}) of 52 ± 4 % compared with the absence of dantrolene. Reducing the CaM concentration to 10 nM approximately halved the magnitude of dantrolene inhibition (Figure 1E, ○, E_{max} = 80 ± 5 %).

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Effect of dantrolene on RyR dwell-times.

In order to gain more insight into the mechanism of dantrolene inhibition, we compiled dwell-time histograms of channel open and closed events of sheep RyR2 at four cytoplasmic [Ca^{2+}] ranging from 0.1 μM (end-diastolic) to 100 μM (systolic) (Figure 2A, B and Supplementary Figure 1). Histograms are displayed using the log-bin method of Sigworth and Sine (1987), where individual exponential components appear as peaks centred on their time constant value. In the absence of dantrolene, open and closed dwell times in 1 μM cytoplasmic Ca^{2+} exhibited peaked distributions that were fitted by two exponential components (see Supplementary Table 1). Addition of dantrolene (10 μM) shifted the peak of the open distributions to shorter times and closed distribution to longer times. Dantrolene had a similar effect in 0.1 μM cytoplasmic Ca^{2+} but had no effect at 100 μM cytoplasmic Ca^{2+} (Supplementary Figure 1). It was not possible to resolve significant differences in the parameters of the exponential fits except for the slow time-constant of the closed times at 0.1 μM cytoplasmic Ca^{2+} (T2, Supplementary Table 1). However, it was possible to resolve relative changes in the RyR2 mean open and closed durations (Figure 2C). In 0.1 μM cytoplasmic Ca^{2+}, dantrolene reduced RyR2 P_o via a decrease in mean channel open duration and an increase in mean closed duration. At 1 μM cytoplasmic Ca^{2+}, the effect of dantrolene was diminished and there was no significant inhibition occurring at higher [Ca^{2+}]. The effect of dantrolene was to shift the Ca^{2+}-activation response of RyR2 to higher [Ca^{2+}].

Effect of Dantrolene on Ca^{2+} waves in mouse cardiomyocytes.

The amplitude and frequency of spontaneous Ca^{2+} waves, two parameters that have been implicated as independent predictors of arrhythmogenicity (Galimberti and Knollmann, 2011), were measured in mouse ventricular myocytes. Examples of the effect of 30 min exposure to dantrolene (3, 10, 50 μM) on Ca^{2+} waves recorded in the presence or absence of CaM are presented in figure 3A.

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Dantrolene reduced Ca^{2+} wave amplitude and frequency in the presence of CaM but had no effect in the absence of CaM (Figure 3A). This finding is consistent with the single channel experiments with the concentration-dependence of these effects (Figure 3B, C) exhibiting remarkably similar IC_{50}'s and E_{max} values to that measured in the single channel experiments (Figure 1).

Dantrolene inhibition can be mediated by CaM mutants.

Since both dantrolene and CaM are RyR2 inhibitors we investigated the possibility that dantrolene acts by amplifying CaM inhibitory action on RyR2. To test this possibility we measured dantrolene inhibition of sheep RyR2 in the presence of 100 nM N54I-CaM. This mutation, as shown in Figure 4A and in our previous work, increases RyR2 P_o, the opposite effect to wt-CaM (Hwang et al., 2014). If dantrolene merely amplifies the action of CaM, then one would expect dantrolene to be an activator in the presence of N54I-CaM. This was not the case. Dantrolene had the same inhibitory action in the presence of wt- and N54I-CaM (Figure 4B). We also show in figure 4A that addition of wt-CaM to RyR1 caused channel activation in accord with previous findings (Tripathy et al., 1995).

We also investigated dantrolene inhibition in the presence of N98S-CaM that is a CaM mutant that has no inhibitory effect on RyR2 in the absence of dantrolene (Figure 4C, open circle). The advantage of this CaM mutant is that we can examine the effect of varying its concentration of on facilitating dantrolene inhibition without the confounding effect of CaM inhibition. In the absence of CaM, dantrolene (10 μM) had no effect on RyR P_o. Figure 4C shows that addition of only 6 nM N98S-CaM was sufficient to facilitate significant dantrolene inhibition of RyR2. The N98S-CaM facilitation of dantrolene inhibition had a sigmoidal dependence on log-concentration with an IC_{50} of 5.9 ± 0.3 nM, a Hill coefficient of 5 ± 2.6 and an E_{max} of 53 ± 4 %.

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DISCUSSION

Our study presents the first demonstration of dantrolene inhibition of mammalian RyR1 and RyR2 from recordings of single RyR and permeabilized cardiomyocytes. The finding that a physiological concentration of CaM is required for dantrolene inhibition of these RyRs provides an answer to the long-standing question of why dantrolene, an inhibitor of SR Ca^{2+} release, had no effect on the activity of mammalian RyR1 and RyR2 in previous single channel studies (Diaz-Sylvester et al., 2008; Szentesi et al., 2001; Wagner et al., 2014). Since CaM readily dissociates from the RyR complex (Guo et al., 2011), CaM would have been absent during those experiments. IC_{50} for CaM facilitation of dantrolene inhibition appears to be ~10 nM for wt-CaM (Figure 1E) and 5.9 nM for N98S-CaM (Figure 4C). These values are ~2-fold lower than the binding affinities for these CaMs on RyR2 (Guo et al., 2011; Hwang et al., 2014).

^{3}[H]-ryanodine binding assays have demonstrated a reduction of CaM activation of purified pig RyR1 by dantrolene (Fruen et al., 1997). However, that finding was contradicted by a single channel study (Cherednichenko et al., 2008) that, using similar experimental conditions (100 nM cytoplasmic Ca^{2+} and 35 ^{o}C), reported no inhibition by dantrolene (20µM) of purified rabbit RyR1 channels in bilayers in the presence of exogenous FKBP12 and CaM. Together with the findings reported here, these results suggest that the inhibitory effect of dantrolene on RyR not only requires CaM, but also other RyR-associated proteins that are present in native preparations but presumably absent in some purified RyR preparations.

The maximum RyR2 inhibition (E_{max}= 52%) and IC_{50} (0.16 ± 0.03 µM, Figure 1D) are in close agreement with dantrolene inhibition of Ca^{2+} wave frequency and amplitude in saponin permeablized cardiomyocytes (Figure 3) and inhibition of Ca^{2+} release in SR vesicles from failing dog heart (IC_{50} = 0.3 ± 0.07 µM, (Kobayashi et al., 2009)) and activity of purified RyR1 in ^{3}[H]-ryanodine binding assays (0.15 ± 0.02 µM (Fruen et al., 1997)). The dantrolene IC_{50} reported here coincides with the binding affinity of dantrolene to skeletal muscle SR vesicles (0.277 ± 0.025 µM

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(Parness and Palnitkar, 1995)) and its IC_{50} (0.3 ± 0.11 μM) for inhibiting the unzipping of the central and N-terminal domains of RyR2 (Kobayashi et al., 2009). The potency of dantrolene in our study is also consistent with the inhibitory action of 1 μM dantrolene on Ca^{2+} spark frequency in isoproterenol-stimulated cardiomyocytes from R2474S knock-in mice (Kobayashi et al., 2010). However, the therapeutic actions of dantrolene in skeletal and cardiac muscle occur at much higher concentrations than required for inhibition of Ca^{2+} release from the SR For example, 20 μM, or more, dantrolene was required to prevent exercise induced cardiac arrhythmias in R2474S knock-in mice (Kobayashi et al., 2010), increase survival after ventricular fibrillation (Zamiri et al., 2014) and prevent anesthetic induced MH in skeletal muscle (Podranski et al., 2005). This has lead others to consider alternative therapeutic mechanisms for dantrolene such as modulating store operated Ca^{2+} entry (Cherednichenko et al., 2008) or by acting as an antioxidant (Buyukokuroglu et al., 2001) or regulating antioxidant enzymes (Buyukokuroglu et al., 2002; Ucuncu et al., 2005).

Our finding that dantrolene inhibition is only seen at cytoplasmic [Ca^{2+}] =<1 μM (Figure 2C) is consistent with previous findings that dantrolene (1 μM) inhibits the frequency Ca^{2+} sparks (and hence SR leak) yet does not inhibit the amplitude of Ca^{2+} transients (Maxwell et al., 2012; Zamiri et al., 2014). Thus dantrolene is a diastolic inhibitor of Ca^{2+} release in failing heart, which has the beneficial actions of increasing diastolic Ca^{2+} loading of the SR (Maxwell et al., 2012) and reducing diastolic SR Ca^{2+} leak after ventricular fibrillation (Zamiri et al., 2014). Since dantrolene is not an effective RyR inhibitor at high cytoplasmic [Ca^{2+}] then it is not surprising that other dantrolene mechanisms may be more important for suppressing Ca^{2+} release during skeletal muscle twitches (Flewellen et al., 1983) or suppressing MH episodes.

Single channel recordings of dantrolene inhibition provide a unique opportunity to probe the mechanism of dantrolene inhibition. RyR2 dwell-time distributions (Figure 2A, B) indicate that dantrolene decreases the duration of channel openings and increases the duration of closures, characteristics typical of an allosteric inhibitor rather than a channel blocker like the local

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anesthetics that cause distinct blocking events in single channel recordings that introduce new exponential components in closed time distributions (Tinker and Williams, 1993; Tsushima et al., 2002; Xu et al., 1993). Like CaM, dantrolene inhibits RyR2 by destabilising their open state and stabilising their closed state. By using a CaM mutation that causes CaM to activate RyR2, we show that dantrolene does not merely increase the efficacy of CaM, but is an inhibitor in its own right (Figure 4). The RyR has a homotetrametic structure that includes four dantrolene binding sites and at least four CaM binding sites. The dantrolene dose-response (Figure 1D) exhibited a Hill coefficient ~1, consistent with values obtained from ^{3}[H]-ryanodine binding assays (Fruen et al., 1997). Such a value indicates that the binding of only one dantrolene molecule is sufficient to cause inhibition of RyR2 activity. Interestingly, the dose-response of N98S-CaM facilitation of dantrolene inhibition (Figure 4C) had a much higher Hill coefficient, consistent with a requirement for multiple CaM molecules on RyR2. The mechanism by which CaM facilitates dantrolene inhibition remains unclear. It is unlikely that dantrolene acts by binding to a site on CaM since that would not explain the different Hill-coefficients for the dantrolene and N98S-CaM dose responses. Also, given the redox buffering of our experimental solutions (4 mM GSH in bilayer experiments and 10 mM GSH in myocyte experiments) it is unlikely that the reducing properties of dantrolene underlie its inhibition. However, one possibility is that CaM puts the RyR into a conformation that gives dantrolene access to its binding site on the RyR. Two studies have demonstrated that dantrolene has restricted access to its binding site that is regulated by RyR conformation and on the presence of RyR ligands such as Ca^{2+} and ATP (Paul-Pletzer et al., 2001; Paul-Pletzer et al., 2005). An alternative possibility is that CaM is a part of the signaling pathway that transduces dantrolene binding into RyR inhibition. Several studies present evidence that dantrolene modulates inter-domain interactions in RyR1 (Kobayashi et al., 2005) and RyR2 (Kobayashi et al., 2009; Maxwell et al., 2012; Suetomi et al., 2011; Uchinoumi et al., 2010) between the N-terminal (1-619 aa),

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central (2,000-2500 aa) and C-terminal domains (3900-end). Our data are consistent with both these possibilities.

In conclusion, we show that CaM binding to the RyR is required in order to produce dantrolene inhibition in both RyR1 and RyR2. It is likely that other, as yet undefined factors play a similar role in facilitating dantrolene inhibition.

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AUTHOR CONTRIBUTIONS

Participated in research design: Oo, Laver, Knollmann, Imtiaz

Conducted experiments: Oo, Gomez-Hurtado, Walweel

Contributed to new reagents or analytic tools: Laver, Knollmann

Performed data analysis: Oo, Laver, Gomez-Hurtado, Knollmann

Wrote or contributed to the writing of the manuscript: Oo, vanHelden, Gomez-Hurtado, Knollmann, Laver

The authors declare that there is no conflict of interest in this report.

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LEGENDS FOR FIGURES

Figure 1. Dantrolene inhibits RyR1 and RyR2 only in the presence of CaM. (A) Representative, 10s segments of activity of RyR1 from rabbit skeletal muscle illustrating the inhibitory effect of 10 μM dantrolene in the absence (-CaM) and presence (+CaM) of 100 nM CaM. **(B)** Corresponding activity of RyR2 showing inhibition by 50 μM dantrolene. Open probabilities (P_o) for 60 s periods of activity are given at the end of each. Experiments were done at +40 mV and upward current jumps represent the channel openings. **(C)** 140 s recordings of RyR1 and RyR2 showing channel activity during dantrolene application (bars) and washout. Values of open probability (P_o) are given for each segment of recording **(D)** Relative inhibition of RyR1 by 10 μM dantrolene and RyR2 by 50 μM dantrolene. Each sample is RyR P_o in the presence of dantrolene relative to the mean P_o bracketing periods in the absence of dantrolene. Mean values are indicated by the horizontal bars and SEM by the vertical bars. *p*-values indicate significant difference of the mean from 100%. **(E)** Concentration-dependence of dantrolene inhibition of RyR2 in the presence of 10 nM (○, mean ± SEM, n= 3 to 4) and 100 nM CaM (●, mean ± SEM, n= 7 to 20). The luminal [Ca^{2+}] is 0.1 mM and cytoplasmic [Ca^{2+}] is 100 nM. The solid curve shows the Hill fit to the data using the equation:

$Po = \{1 + E_{max}\ ([dantrolene]/IC_{50})^{H}\}/\{1 + ([dantrolene]/IC_{50})^{H}\}$ where IC_{50} = 0.16 ± 0.03 μM, H = 1.3 ± 0.3 and E_{max} = 52 ± 4%. The dashed curve uses the same parameter values except E_{max} = 80 ± 5%.

Figure 2. Effect of dantrolene on open and closed dwell-times of RyR2. (A) Open and **(B)** closed dwell-time histograms compiled using the log-bin method of Sigworth and Sine (1987) as described in the text. Histograms are averages of three experiments obtained in 1 μM cytoplasmic [Ca^{2+}] in the absence (○) or presence (●) of 10 μM dantrolene. **(C)** Statistical analysis of open and closed times of dwell-time histograms showing relative changes in mean dwell-times induced by 10

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μM dantrolene over a range of cytoplasmic [Ca^{2+}]. Also shown is the relative inhibition of channel P_o. Asterisks indicate significantly different to 100% (* p<0.05, **p<0.01).

Figure 3. Dantrolene reduces spontaneous Ca^{2+} wave frequency and amplitude only in the presence of CaM. Presence of CaM is required for Dantrolene action on arrhythmogenic Ca^{2+} waves in cardiomycytes. (**A)** Representative confocal microscope line scans from permeabilized mouse ventricular myocytes after 30-minute incubation with either dantrolene alone or dantrolene + CaM (100 nM). Red arrows indicate the location of the line-scans plotted below each confocal image. **(B-C)** Concentration response curves for Ca^{2+} wave frequency (**B**) and amplitude relative to vehicle (**C**) in the absence (black ●) or presence (red ●) of CaM 100 nM. The solid curves show the Hill fit to the data using the equation in the caption to figure 1. (B) IC_{50} = 0.42 ± 0.18 μM and E_{max} = 47 ± 4% and (C) IC_{50} = 0.19 ± 0.04 μM and E_{max} = 66 ± 2%.

Figure 4. Dantrolene inhibition of RyR in the presence of wt-CaM and mutant CaM. (A) Relative effect of wt-CaM (100 nM) on the open probability of RyR1 and RyR2 and N54I-CaM on RyR2. **(B)** Relative effect of 10 μM dantrolene on RyR2 P_o in the presence of wt- and mutant-CaM. Mean values are indicated by the horizontal bars and SEM by the vertical bars. *p*-values indicate significant differences between wt- and mutant-CaM. **(C)** Facilitation of dantrolene (10 μM) inhibition by N98S-CaM (●). In the absence of dantrolene (○), N98S-CaM has no inhibiting action on RyR2. Asterisks indicate significantly different to 100% (* p<0.05, **p<0.01). The solid curve shows the fit of the Hill equation (see caption to Figure 1) to the data.

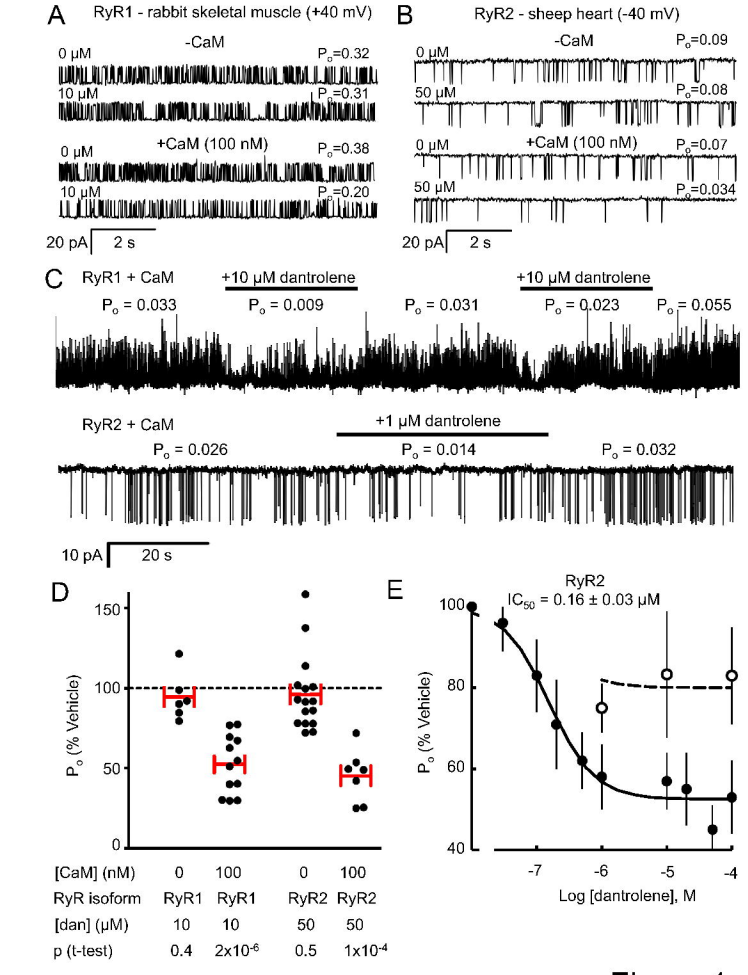
A
RyR1 - rabbit skeletal muscle (+40 mV)
-CaM
0 μM
P_o=0.32
10 μM
P_o=0.31
+CaM (100 nM)
0 μM
P_o=0.38
10 μM
P_o=0.20
20 pA
2 s
B
RyR2 - sheep heart (-40 mV)
-CaM
0 μM
P_o=0.09
50 μM
P_o=0.08
+CaM (100 nM)
0 μM
P_o=0.07
50 μM
P_o=0.034
20 pA
2 s
C
RyR1 + CaM
+10 μM dantrolene
P_o = 0.033
P_o = 0.009
P_o = 0.031
+10 μM dantrolene
P_o = 0.023
P_o = 0.055
RyR2 + CaM
+1 μM dantrolene
P_o = 0.026
P_o = 0.014
P_o = 0.032
10 pA
20 s
D
P_o (% Vehicle)
150
100
50
0
[CaM] (nM) 0 100 0 100
RyR isoform RyR1 RyR1 RyR2 RyR2
[dan] (μM) 10 10 50 50
p (t-test) 0.4 2x10-6 0.5 1x10-4
E
RyR2
IC50 = 0.16 ± 0.03 μM
P_o (% Vehicle)
100
80
60
40
-7
-6
-5
-4
Log [dantrolene], M

Figure 1

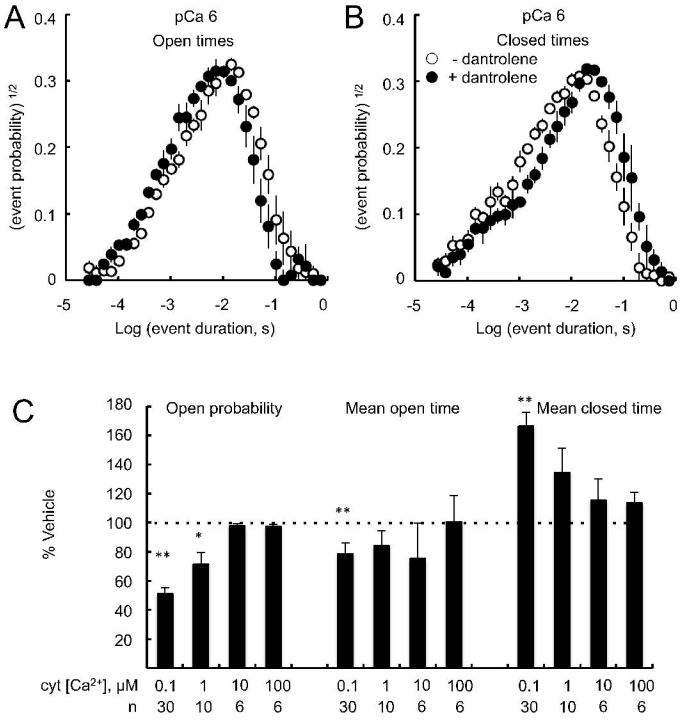
A
pCa 6
Open times
(event probability) 1/2
Log (event duration, s)
B
pCa 6
Closed times
- dantrolene
+ dantrolene
(event probability) 1/2
Log (event duration, s)
C
Open probability
Mean open time
Mean closed time
% Vehicle
cyt [Ca2+], µM 0.1 1 10 100 0.1 1 10 100 0.1 1 10 100
n 30 10 6 6 30 10 6 6 30 10 6 6

Figure 2

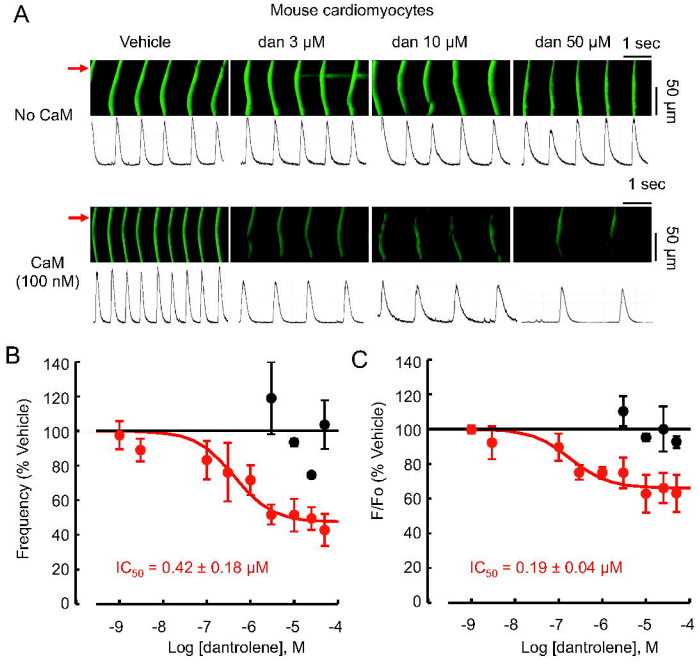
A
Mouse cardiomyocytes
Vehicle
dan 3 µM
dan 10 µM
dan 50 µM
1 sec
50 µm
No CaM
1 sec
50 µm
CaM
(100 nM)
B
Frequency (% Vehicle)
IC50 = 0.42 ± 0.18 µM
Log [dantrolene], M
C
F/Fo (% Vehicle)
IC50 = 0.19 ± 0.04 µM
Log [dantrolene], M

Figure 3

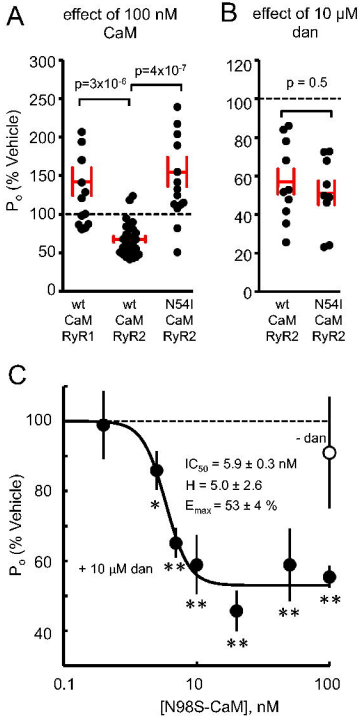
A
effect of 100 nM CaM
B
effect of 10 μM dan
p=3x10-6
p=4x10-7
p = 0.5
Po (% Vehicle)
wt CaM RyR1
wt CaM RyR2
N54I CaM RyR2
wt CaM RyR2
N54I CaM RyR2
C
IC50 = 5.9 ± 0.3 nM
H = 5.0 ± 2.6
Emax = 53 ± 4 %
- dan
+ 10 μM dan
[N98S-CaM], nM

Figure 4

Essential role of Calmodulin in RyR inhibition by dantrolene
Y.W. Oo, N. Gomez-Hurtado, K. Walweel, D.F. van Helden, M. S. Imtiaz, B.C. Knollmann and D.R. Laver
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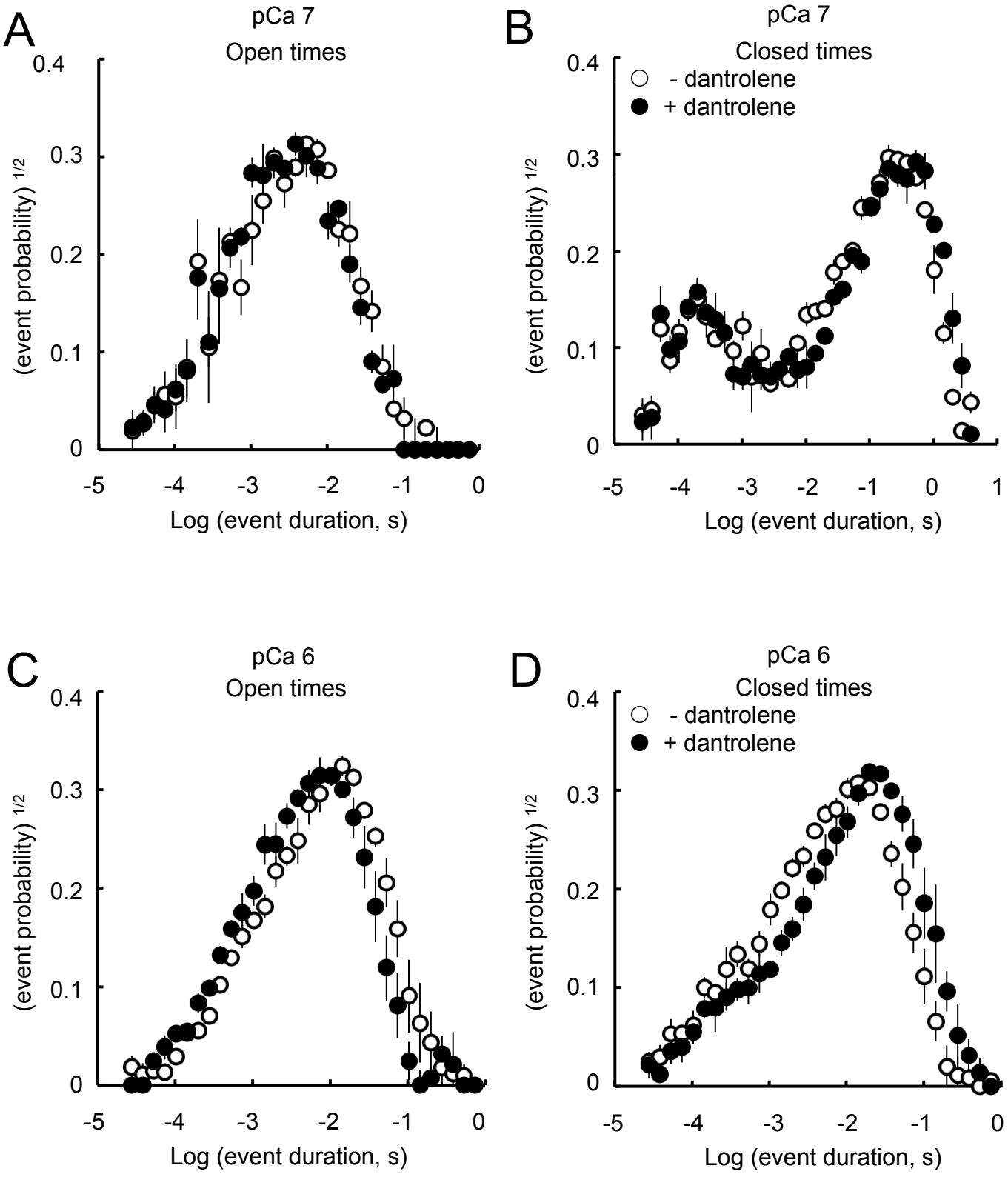
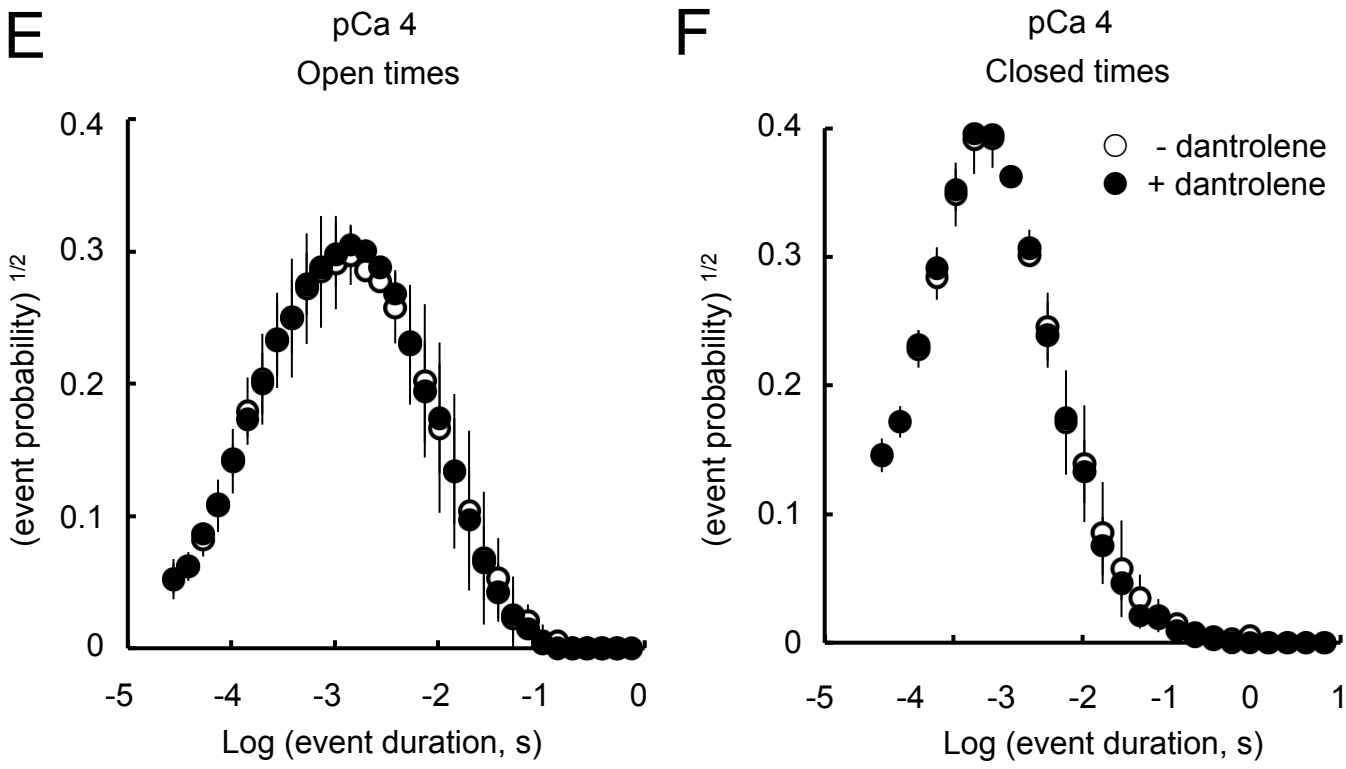


Supplementary 1 (part 1)



Supplementary figure 1. Effect of dantrolene on open and closed dwell-times of RyR2. (A,C,E) Open and **(B,D,F)** closed dwell-time histograms compiled using the log-bin method of Sigworth and Sine (1987). Histograms are averages of three experiments obtained in cytoplasmic [Ca^{2+}] (indicated by pCa in each pannel) in the absence (○) or presence (●) of 10 μM dantrolene. The constants of exponential constants fits to these dwell-time histograms are given in Supplementary Table 1. Figure 1C and D that the data from Figure 2A and B are re-plotted here for comparison purposes.

Supplementary 1 (part 2)

Essential role of Calmodulin in RyR inhibition by dantrolene
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	Fit to open dwell times				Fit to closed dwell times			
condition	A1 %	T1 ms	A2 %	T2 ms	A1 %	T1 ms	A2 %	T2 ms
pCa7	73 ± 16	3.8 ± 0.6	27 ± 16	22 ± 6	11 ± 3	0.77 ± 0.18	89 ± 3	500 ± 100
pCa7 + dan	81 ± 12	4.4 ± 1.0	19 ± 12	22 ± 5	8 ± 4	0.37 ± 0.04	92 ± 4	700 ± 150*
pCa6	37 ± 7	8 ± 2	63 ± 7	27 ± 9	31 ± 5	5 ± 2	69 ± 5	27 ± 6
pCa6 + dan	41 ± 17	5.2 ± 0.5	59 ± 18	16 ± 1	35 ± 10	15 ± 9	65 ± 10	50 ± 20
pCa4	68 ± 5	1.8 ± 0.4	32 ± 5	8 ± 2	93 ± 5	0.19 ± 0.01	7 ± 5	0.91 ± 0.09
pCa4 + dan	70 ± 10	1.7 ± 0.4	30 ± 10	7 ± 2	88 ± 3	0.18 ± 0.03	12 ± 3	0.61 ± 0.08

Table 1. Parameter values for multi exponential fits to RyR2 dwell-time histograms (***H(t)***). The conditions give the cytoplasmic [Ca^{2+}] in units of pCa in the absence and presence of 10 μM dantrolene. T1 and T2 are the exponential time constants and A1 and A2 give fraction of dwell times in each exponential where A1 + A2 = 100%. Asterisks indicate significant difference to absence of dantrolene in paired t-test (* $p<0.05$). The equation is: $H(t) = A1\ T1.exp(-t/T1) + A2\ T2.exp(-t/T2)$