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Calmodulin inhibition of human RyR2 channels requires phosphorylation of RyR2-S2808 or RyR2-S2814

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Abstract

Calmodulin (CaM) is a Ca-binding protein that binds to, and can directly inhibit cardiac ryanodine receptor calcium release channels (RyR2). Animal studies have shown that RyR2 hyperphosphorylation reduces CaM binding to RyR2 in failing hearts, but data are lacking on how CaM regulates human RyR2 and how this regulation is affected by RyR2 phosphorylation. Physiological concentrations of CaM (100 nM) inhibited the diastolic activity of RyR2 isolated from failing human hearts by ~50% but had no effect on RyR2 from healthy human hearts. Using FRET between donor-FKBP12.6 and acceptor-CaM bound to RyR2, we determined that CaM binds to RyR2 from healthy human heart with a K_d = 121 ± 14 nM. *Ex-vivo* phosphorylation/ dephosphorylation experiments suggested that the divergent CaM regulation of healthy and failing

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Disclosures

The authors have declared that no conflict of interest exists.

human RyR2 was caused by differences in RyR2 phosphorylation by protein kinase A and Ca-CaM-dependent kinase II. Ca^{2+}-spark measurements in murine cardiomyocytes harbouring RyR2 phosphomimetic or phosphoablated mutants at S2814 and S2808 suggest that phosphorylation of residues corresponding to either human RyR2-S2808 or S2814 is both necessary and sufficient for RyR2 regulation by CaM. Our results challenge the current concept that CaM universally functions as a canonical inhibitor of RyR2 across species. Rather, CaM's biological action on human RyR2 appears to be more nuanced, with inhibitory activity only on phosphorylated RyR2 channels, which occurs during exercise or in patients with heart failure.

Keywords

Ryanodine receptor; calmodulin; phosphorylation; lipid bilayer; Calcium sparks; cardiomyopathy

Introduction

Calmodulin (CaM) is a ubiquitous calcium-binding protein that regulates target proteins either directly or via their regulatory kinases and phosphatases.[1, 2] In the heart, CaM regulates cardiac excitation contraction coupling via several key Ca^{2+} handling proteins. Targets include the ryanodine receptor (RyR2) Ca^{2+}-release channel in the sarcoplasmic reticulum (SR), voltage-dependent L-type Ca^{2+} channels in the sarcolemma membrane, and the Ca^{2+}-ATPase pump in the SR membrane (SERCA2a) via phosphorylation of its regulatory protein, phospholamban.[3][4] CaM regulates RyR2-mediated SR Ca^{2+} release either directly by binding to and inhibiting RyR2 or indirectly via Ca^{2+}-CaM kinase II (CaMKII)-mediated phosphorylation of RyR2 at S2814. [3] Single channel studies of RyR2 isolated from sheep, [5] dog [6] and mouse [7] show that saturating concentrations of CaM inhibit RyR2 activity by up to ~60%, leading to partial inhibition of SR Ca^{2+} release in cardiomyocytes. [6, 8, 9]

CaM binding to RyR2 is critical for normal SR Ca^{2+} release and cardiac function, and decrease in CaM binding has been observed in animal models of heart failure. [3, 9, 10] Moreover, loss of CaM binding by mutating RyR2 residues in the putative CaM binding domain causes cardiac hypertrophy and severe heart failure in mice. [7, 11] Furthermore, loss of RyR2 inhibition by mutant CaM (without loss of CaM binding) leads to pathological activation of RyR2 that triggers life-threatening arrhythmias in patients with catecholaminergic polymorphic ventricular tachycardia (CPVT). [5, 12] Increasing CaM binding to RyR2 restores normal Ca^{2+} handling and contractile function in cardiomyocytes from failing dog hearts. [13] In mice it appears to be modulated by RyR2 phosphorylation at two regulatory sites that are normally phosphorylated by acute adrenergic stress, residues corresponding to S2808 and S2814 in human. These are residues S2807 and S2813 in mouse and S2806 and S2812 in sheep, respectively. For clarity, we refer to these residues as S2808 and S2814 for all species. In mice, PKA phosphorylation of RyR2 S2808 results in a 50% reduction in CaM binding to the channel. [14] Similarly, CaM binding to RyR2 is reduced in mice homozygous for the phosphomimetic RyR2 S2814D mutation. [15] In animal models and humans, heart failure is also associated with hyper-phosphorylation of RyR2 S2808 [16] and S2814.[3, 17–20]

Despite the large number of experimental studies of RyR2 phosphorylation and CaM binding, data are lacking on the action of CaM on the activity of *human* RyR2 in healthy and failing hearts. To address this question, we investigated the action of CaM on the single-channel activity of RyR2 isolated from human donors with healthy hearts and from hearts from patients with ischemic cardiomyopathy (ICM). We found that CaM regulation of human RyR2 depends on the phosphorylation of RyR2 at both S2808 and S2814. CaM only exerts an inhibitory regulation of RyR2 channel activity when either RyR2-S2808 or S2814 are highly phosphorylated. We also examined the effects of CaM on RyR2 from sheep to see if RyR2 phosphorylation levels could explain differences seen in CaM regulation of RyR2 from sheep and human. These findings provide intriguing new insights into the function of CaM in healthy and failing human hearts.

Methods

Heart tissue

Human tissues were obtained with approval from the Human Research Ethics Committees of the University of Newcastle (approval number H-2009–0369), the Prince Charles Hospital in Brisbane (EC28114), the University of Canberra (2013/01), St Vincent's Hospital Sydney (H03/118), and the University of Sydney (#2012–2814 and #32016–923). Supplementary Table 1 summarizes the characteristics of donor hearts (four failing, ICM1–4, and four non-failing human hearts, H1–4) used in this study. Non-failing human left ventricle tissue was obtained from 'unused' donor hearts. These hearts were collected at the site of organ donation by the St Vincent's Hospital (Darlinghurst, Sydney, Australia) surgical team following the declaration of brain death by the transplant coordinator. These donor hearts were flushed with sterile, ice-cold cardioplegia, transported by the Australian Red Cross Blood Service within three hours to the Bosch Institute and immediately frozen in liquid nitrogen. These hearts were not required for orthotopic heart transplantation for a range of reasons, such as tissue incompatibility. Transmural sections of left ventricle (LV) free wall (~1 g) were snap frozen in liquid nitrogen (−196°C) within 15 min of coronary artery perfusion. Failing hearts were from patients with ischaemic cardiomyopathy (ICM) at the time of transplantation. LV was obtained from explanted hearts from patients with terminal heart failure undergoing heart transplantation (at The Prince Charles Hospital, Brisbane, Australia). Tissue was snap frozen in liquid N_2 within 15 min of the loss of coronary circulation. Sheep LV was obtained with approval from the Animal Care and Ethics Committee of the University of Newcastle (Australia; approval number #A-2009–153). Sheep hearts were obtained from ewes anesthetized with 5% pentobartitone [21] followed by oxygen/halothane. The use of mice in this study was approved by the Animal Care and Use Committee of Vanderbilt University, Nashville, TN, USA and performed in accordance with NIH guidelines.

Preparation of SR Vesicles

SR vesicles were isolated from human sheep and porcine left ventricle as previously described. [22] Details are given in Supplementary Methods.

In-vitro phosphorylation and dephosphorylation of RyR2 channels

In-vitro phosphorylation and dephosphorylation of RyR2 were conducted as previously described. [23] In RyR2 from healthy and failing human hearts and sheep heart, dephosphorylation was achieved by incubation of RyR2s in protein phosphatase 1 (PP1) reaction kit (containing $MnCl_2$, NEBuffer® and 2.5 unit/μl PP1, New England BioLabs) for 15 min at 30°C prior for single channel recordings. In-vitro phosphorylation of RyR2 by PKA enzyme was performed as follows: RyR2s were first maximally dephosphorylated by PP1 (15 min incubation at 30°C) and then incubated with exogenous PKA in PKA buffer containing (mM): 50 Tris-HCl (pH 7.4), 10 $MgCl_2$, 2 ATP, 1 cAMP, 10 NaF and 0.25 g/ml PKA (Sigma Aldrich) at 30°C for 5 min prior to incorporation into the bilayer. CaMKII phosphorylation of RyR2 was obtained by incubation of RyR2s in PP1 reaction kit first (15 min at 30°C, dephosphorylation) and then incubated in a CaMKII activating buffer containing (mM): 50 Tris-HCl (pH 7.4), 10 $MgCl_2$, 2 ATP, 25 NaF, 2.5 $CaCl_2$ and 62.5 μM CaM at 30°C for 5 min prior to incorporation into the bilayer. The CaMKII activation buffer contained no exogenous CaMKII because endogenous CaMKII is known to be associated with and phosphorylate the RyR2 *in-vitro*. [24–26] *In-vitro* phosphorylated and dephosphorylated RyR2 were used in bilayer experiments and SDS Page gels and Western Blots.

Single-Channel recording

RyRs from human and sheep hearts were incorporated into artificial lipid bilayers that were formed from phosphatidylethanolamine and phosphatidylcholine (8:2 wt/wt) in n-decane (50 mg/ml). For single channel recording, the *cis* (cytoplasmic) and *trans* (luminal) solutions contained 250 mM Cs^+ (230 mM cesium methane sulfonate, 20 mM cesium chloride). Channel gating was measured at −40 mV (cytoplasmic potential relative to the luminal potential at virtual ground) by single channel recording in the presence of cytoplasmic ATP (2 mM), Ca^{2+} (0.1 μM) and in the presence and absence of CaM (100 nM). CaM was added and removed from the RyR2 complex by using continuous local perfusion via a tube placed in close proximity to the bilayer, which could produce solution change within 1 s. All solutions were buffered using 10 mM TES (N-tris [hydroxymethyl] methyl-2-aminoethanesulfonic acid (ICN Biomedicals) and titrated to pH 7.4 using CsOH (ICN Biomedicals). A Ca^{2+} electrode (Radiometer) was used in our experiments to determine the purity of Ca^{2+} buffers and Ca^{2+} stock solutions. Free Ca^{2+} was adjusted with $CaCl_2$ and buffered using 4.5 mM BAPTA (1,2-bis(o-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid, obtained from Invitrogen). The cesium salts were obtained from Aldrich Chemical Company; $CaCl_2$ and $MgCl_2$, were from BDH Chemicals. Details of single channel recording and analysis are given in Supplementary methods.

Ca^{2+} sparks and SR Ca^{2+} content in ventricular myocytes

Single ventricular myocytes from 10–16 week-old C57Bl6, RyR2-S2814A, RyR2-S2814D and RyRS2808A knock-in mice [27, 28] were isolated by enzymatic digestion using collagenase as previously described [29]. Briefly, the ascending aorta was cannulated, and the heart perfused with collagenase (Worthington type II) and protease at 37°C. After perfusion, the hearts were chopped into small pieces, filtered through a 250 μm mesh and

washed twice in a standard Tyrode solution containing 0.2 mM of CaCl. Finally, the cells were resuspended in a solution containing 0.6 mM $CaCl_2$. For Ca^{2+} sparks measurements freshly isolated myocytes were permeabilized with saponin (40 μg/ml) for 60 seconds and placed in internal solution composed (in mM) of K-aspartate 120, KCl 15, KH_2PO_4 5, $MgCl_2$ 5.6, dextran 4%, HEPES 10, MgATP 5, phosphocreatine di-Na 10, creatine phosphokinase 10 U/ml, glutathione (reduced) 10, and Fluo-4 0.025. Free [Ca^{2+}] was 100 nM (calculated with MaxChelator). To dialyze CaM out or into the cell, measurements were done after 30-minutes wash, followed by 10-minute incubation with either the internal solution or the internal solution supplemented with CaM (100 nM). To prevent CaMKII activation, a group of cells were incubated with the internal solution containing AIP 1 μM plus or minus CaM. Finally, to activate PKA, a group of cells were incubated with the internal solution containing cAMP (10 μM) plus or minus CaM. Ca^{2+} sparks were imaged with a 510 Zeiss inverted confocal microscope in line-scan mode for 3 seconds. Image analysis was performed with ImageJ and Ca^{2+} spark master. SR Ca^{2+} load was assessed as the Ca^{2+} transient amplitude induced by rapid application of 10 mM caffeine.

SDS PAGE gels and Western blots

SDS polyacrylamide gel electrophoresis and Western Blots were performed on samples obtained from human and sheep hearts. Details given in Supplementary methods.

FRET assay of CaM binding to RyR2

CaM binding to RyR2 was inferred from the FRET signal between AF488-FKBP12.6 (D-FKBP) and AF568-CaM (A-CaM) in SR vesicles as described previously.[30] SR (4 mg/ml protein concentration) was stripped of endogenous CaM by incubation with 300 nM MLCK CaM-binding-domain peptide for 30 min at 37°C in the presence of 150 mM KCl, 20 mM PIPES (pH 7.0), 100 μM Ca^{2+}, 1 μg/ml aprotinin and leupeptin, and 0.1 mg/ml BSA. The donor probe was targeted to RyR2 by incubating this 0.4 mg/ml SR with 200 nM D-FKBP in the same buffer. Excess native CaM and D-FKBP were removed in two steps of centrifugation, supernatant removal and resuspension in TES buffer (containing 10 mM TES (pH 7.4), 1 μg/ml BSA, 250 mM cesium methane sulfonate, and 1 μg/ml aprotinin/leupeptin). FRET was measured by adding A-CaM (up to 1 μM) to 3 mg/ml SR prep in a solution containing 250 mM cesium methane sulfonate, 0.1 μg/ml BSA, 1 μg/ml aprotinin and leupeptin, 1 mM EGTA, Ca^{2+} for 100 nM free concentration and 10 mM TES, pH 7.4. Samples (21 μg) were loaded in triplicate wells on a low volume 384-well plate, and fluorescence decays were acquired following 473 nm excitation with 517/20 nm band-pass emission filter. To measure D-FKBP dissociation kinetics, we acquired a time course of FRET upon displacing 500 nM A-CaM by supplementing the bath with 25 μM wt-CaM.

Statistics

Gaussian distribution of the samples was assessed by D'Agostino-Pearson test. Then, data significances were tested using a student's t-test or ANOVA and post hoc analyses as appropriate. Unless otherwise stated, all other data are presented as means ± standard errors of the mean. $p<0.05$ was considered significant (*), and $p < 0.01$ was considered highly significant (**).

Results

CaM regulation of RyR2 isolated from healthy and failing human hearts.

Since CaM readily dissociates from RyR2, [6, 30] channels that are incorporated into artificial lipid bilayers are devoid of CaM unless exogenous CaM is included in the cytoplasmic bathing solution. In the absence of CaM, RyR2 from failing hearts showed a significantly higher mean open probability (P_o=0.15±0.04) than RyR2 from healthy heart (P_o=0.08±0.03, Supplementary Figure 1A), consistent with our previous findings. [31] The effect of CaM was measured by repeatedly applying CaM for periods of 1 min interleaved by 1 min periods of washout, all in the presence of end-diastolic levels of cytoplasmic [Ca^{2+}] (100 nM; Figure 1A). Previous single channel studies have shown that addition of exogenous CaM decreases the activity of RyRs isolated from sheep, [5] dog [6] and mouse. [7] This was confirmed in our experiments on RyR2 from sheep heart where addition of 100 nM CaM to the cytoplasmic bath caused a 40% reduction in RyR2 open probability at end-diastolic [Ca^{2+}] (P_o, Figure 1) but did not inhibit at systolic [Ca^{2+}] (see Figure 6B, •). Surprisingly, addition of CaM to RyR2 from healthy human hearts in end-diastolic [Ca^{2+}] produced no significant change to RyR2 activity, either at a sub-saturating 100 nM CaM (Figure 1) or at a nearly saturating 500 nM CaM (Supplementary Figure 2). However, addition of CaM to RyR2 isolated from human hearts with ischemic cardiomyopathy (ICM) did produce a 50 ± 4% decrease in open probability (Figure 1B). The CaM-induced reduction in ICM RyR2 activity occurred within the time taken for solution exchange by the perfusion system (2 s, Figure 1A) whereas upon CaM washout, inhibition reversed with a time constant of ~25 s (Supplementary Figure 3). CaM inhibition was indicated by a decrease in the channel open time and an increase in the channel mean close time (Figure 1C). Consistent with our previous findings [31], we found large channel-to-channel variations in P_o and this is reflected in the single channel traces in Figure 1A. Given this large variation we investigated whether high activity RyR2 from failing hearts responded differently to CaM than low activity channels and found no correlation between CaM inhibition and P_o prior to CaM addition (Supplementary Figure 1B). In the presence of CaM, RyR2 from failing hearts had the same mean open probability as those from healthy heart (P_o=0.10 ± 0.02 for both healthy and ICM, Supplementary Figure 1A).

CaM inhibition of RyR2 from failing human hearts depends on RyR2 phosphorylation status

Previous studies have shown that human RyR2 isolated from failing hearts are more highly phosphorylated at residues S2808 and S2814, than those isolated from healthy hearts. [17, 31] Our data (Supplementary Figure 4) documents that RyR2 of ICM hearts also exhibit higher phosphorylation levels relative to those from healthy heart controls. Phosphorylation of RyR2s from four healthy hearts was on average 40 ± 2% and 54 ± 4% of maximal for S2808 and S2814, respectively, whereas in four ICM hearts the corresponding levels were 83 ± 2% and 87 ± 3%. Interestingly, the S2808 phosphorylation of RyR2 from the sheep heart was similar to that seen in RyR2 from ICM human hearts (71%), yet S2814 phosphorylation was similar to that from healthy human heart (50%). These results suggest that the different levels of RyR2 phosphorylation may have influenced the responses of RyR2 from each heart group to CaM in Figure 1. To test this hypothesis, we modified the

degree of RyR2 phosphorylation by applying kinases and phosphatases and then examined the effect of CaM on RyR2 single channel activity. To dephosphorylate the serine residues, ICM heart SR vesicles containing RyR2 were incubated with PP1 for 15 minutes at 30 °C. Re-phosphorylation of RyR2 after PP1 incubation was done by incubating in phosphorylation buffers containing either PKA (in the presence of CaMKII inhibitor, KN93 (10 μM), this phosphorylates RyR2 at S2808) or CaM (in the presence of PKA inhibitor, H89 (2 μM), CaM activates endogenous CaMKII to phosphorylate RyR2 at S2814) for 5 min. The degree of RyR2 phosphorylation at S2808 and S2814 was quantified by Western blot (Figure 2A & B). As expected, PP1 decreased both S2808 and S2814 phosphorylation, whereas PKA increased S2808 phosphorylation and CaM increased PS2814 phosphorylation. Next, we obtained single-channel recordings of RyR2 isolated from ICM hearts subjected to the described incubations (Figure 2C). CaM inhibited RyR2 from ICM hearts (trace 1-none). Complete dephosphorylation of RyR2 with PP1 abolished the inhibitory action of CaM (trace 2- PP1). In contrast, re-phosphorylation of RyR2s after PP1 incubation with CaMKII buffers (trace 3-PP1-CaM) or PKA buffers (trace 4- PP1-PKA) restored the inhibitory action of CaM. Figure 2D summarizes the effect of CaM on RyR2 P_O. PP1 incubation completely abolished the inhibitory action of CaM on RyR2. The inhibitory CaM effect was restored by incubation with CaMKII or PKA phosphorylation buffers. Importantly, incubation of SR vesicles with buffers lacking either CaM or PKA (CaM-ctrl or PKA-ctrl) did not reinstate CaM inhibition (Figure 2D).

Ex vivo phosphorylation of RyR2 from healthy human heart restores inhibitory action of CaM.

We next tested the effect of CaM on RyR2 isolated from healthy human hearts. Healthy RyR2 were moderately (~40–60%) phosphorylated at both S2808 and S2814 (Supplementary Figure 4) and single-channel recordings did not detect CaM inhibition of RyR2 (Figure 3C, trace1-none). To test if the relatively low phosphorylation levels in healthy RyR2 were responsible for the lack of inhibitory action of CaM, we first incubated SR vesicles with PP1 to dephosphorylate the channel at both S2808 and S2814 and then rephosphorylated S2814 or S2808 by incubating with CaMKII or PKA buffers, respectively (Figure 3A & B). Dephosphorylation of RyR2s did not cause a significant change in the lack of CaM inhibition (Figures 3C, trace 2 -PP1; 3D). In contrast, increasing the level of RyR2 phosphorylation at S2814 or S2808 induced a substantial inhibitory response to CaM in RyR2 activity (Figures 3C, traces 3 & 4; 3D). Treating SR vesicles with control buffers lacking phosphorylating properties did not restore responsiveness to CaM inhibition (Figure 3D). Taken together, our experiments on RyR2 from healthy and failing human hearts suggest that CaM inhibition requires high levels of phosphorylation at RyR2 residues that are dependent on CaMKII or PKA (such as S2808 or S2814).

CaM binds to RyR2 from human healthy hearts

We then measured CaM binding to RyR2, to determine whether failure of CaM to inhibit RyR2 from healthy hearts was due to deficient CaM binding or a lack of inhibition by bound CaM. SR vesicles from one of the healthy hearts were stripped of endogenous CaM by incubation with MLCK CaM binding domain peptide, decorated with D-FKBP and then incubated with 0–1000 nM A-CaM. CaM binding specifically to RyR2 was inferred from

FRET measurements, using a previously reported method.[30] We observed A-CaM binding (Figure 4A) with K_d value of ~121 ± 14 nM. We also measured the dissociation of CaM in order to determine if failure of exogenous CaM to inhibit RyR2 was due to inability of endogenous CaM to dissociate from RyR2. We measured this from the decay of FRET after 500 nM A-CaM was displaced from RyR2 by adding 25 μM unlabeled WT-CaM to the medium (Figure 4B). Therefore, CaM binds to, and readily dissociates from RyR2 obtained from healthy human hearts.

Ex vivo dephosphorylation of RyR2 from sheep heart abolished CaM inhibition.

Channel experiments analogous to those in Figures 2 and 3 were also performed on RyR2 from sheep heart, to test if the differences in the CaM inhibitory response between human and sheep RyR2 can be attributed to differences in their phosphorylation levels. The degree of RyR2 phosphorylation was manipulated using the same protocols as for human RyR2. These experiments showed that CaM inhibition of RyR2 from sheep heart responds to RyR2 phosphorylation in the same way as those from human heart (Figure 5). We found that a higher level of endogenous PKA-induced phosphorylation in sheep RyR2 compared to healthy human RyR2 was associated with higher mean P_o (0.13±0.02, Supplementary Figure 1A) and the presence of CaM inhibition of RyR2 from sheep heart and its absence from healthy human heart. The latter suggests that the different CaM effects on RyR2 from healthy human and sheep hearts were due to differences in S2808 phosphorylation.

We next considered the alternative possibility that RyR2 after PP1 incubation failed to be inhibited by CaM because RyR2 dephosphorylation by PP1 significantly reduces RyR2 P_o, thereby masking any further inhibition by CaM. Hence, we repeated the PP1 experiment in the presence of increased concentrations of the cytoplasmic agonist, Ca^{2+} (EC_{50} ~ 1 μM; Figure 6A). CaM inhibition of RyR2 prior to PP1 incubation was ~ 50% at 0.1 and 0.3 μM Ca^{2+} and was abolished at $Ca^{2+} \geq 3$ μM (Figure 6B). However, after PP1 incubation, CaM did not cause any inhibition over the entire [Ca^{2+}] range. At 100 nM Ca^{2+}, PP1 incubation decreased RyR2 P_o by 65% from 0.13 to 0.057 (Figure 6A). Importantly, restoring P_o to 0.13 in the presence of PP1, by increasing cytoplasmic Ca^{2+} to 0.3 & 1 μM, did not restore CaM inhibition, showing that CaM inhibition of sheep RyR2 did not depend on P_o (like RyR2 from failing hearts in Supplementary Figure 1B).

CaMKII or PKA phosphorylation is necessary for CaM inhibition of RyR2-mediated Ca^{2+} sparks in mouse ventricular myocytes.

Spontaneous opening of native RyR2 channels in cardiomyocytes generate Ca^{2+} release from channel clusters that can be visualized as Ca^{2+} sparks. [32] Measuring the frequency of spontaneous Ca^{2+} sparks is widely used to investigate changes in RyR2 activity in intact and permeabilized cardiomyocytes. [15, 33, 34] Hence, we next tested the phosphorylation-dependence of CaM regulation of native RyR2 channel complexes by measuring spontaneous Ca^{2+} sparks (Figure 7A,B) and RyR2 phosphorylation using phospho-specific antibodies (Figure 7C,D) in C57Bl6 mouse ventricular cardiomyocytes. Since Ca^{2+} spark frequency is strongly dependent on SR Ca^{2+} load, [35] and RyR2 inhibition by CaM can cause a compensatory increase in SR Ca^{2+} load, [36, 37] all Ca^{2+} spark frequency measurements were normalised for SR Ca^{2+} store load, which was measured by rapid

caffeine application (Supplementary Tables 2 and 3). Adding CaM, at a physiological concentration of 100 nM, to the intracellular solution significantly reduced Ca^{2+} spark frequency (Figure 7B). CaMKII phosphorylation of RyR2 residue S2814 was significantly increased in presence of CaM whereas phosphorylation of S2808, the PKA consensus site, was unchanged (Figure 7C, D). Blocking CaMKII activation with AIP abolished both the inhibitory effect of CaM on Ca^{2+} sparks (Figure 7A, B) and phosphorylation of S2814 (Figure 7C, E). Next, we used cAMP to directly activate PKA and via Epac to activate CaMKII,[38] which significantly increased both S2808 and S2814 phosphorylation (Figure 7C–E). Under these highly-phosphorylated conditions, CaM inhibition of Ca^{2+} sparks was significantly enhanced (Figure 7A, B). Taken together, these results demonstrate that CaM inhibition of RyR2 is enhanced in the presence of significant levels of RyR2 phosphorylation.

Phosphorylation of RyR2 residues S2808 or S2014 is required for CaM inhibition of RyR2-mediated Ca^{2+} sparks.

Although three phosphorylation sites on RyR2 are confirmed to regulate RyR2 activity (S2030 and S2808 by PKA and S2814 by CaMKII), *in-vitro* phosphorylation studies using mouse RyR2 peptides (aa2699–2904) have identified five potential targets for PKA on RyR2 (S2797, S2808, S2810, S2811 and S2814) and four sites for CaMKII (T2876, S2808, S2811 and S2814). [39, 40] To identify which RyR2 residues are actually responsible for regulating the inhibitory action of CaM on RyR2 channels in response to PKA and CaMKII incubations, we used knock-in mice that are homozygous for phosphomimetic (S2814D) and phosphoablated (S2808A, S2814A) RyR2 mutations. We first examined the role of S2814 phosphorylation by CaMKII. A phosphomimetic aspartic acid residue in position 2814 was sufficient to produce an inhibitory action of CaM on Ca^{2+} sparks even in the absence of S2808 phosphorylation (Figure 8). Conversely, the phosphoablative alanine residue in position 2814 was sufficient to completely prevent an effect of CaM on Ca^{2+} sparks (Figure 8). Strikingly, addition of cAMP to maximally phosphorylate S2808 in 2814D cardiomyocytes did not produce any enhancement of the inhibitory activity of CaM on Ca^{2+} sparks, suggesting that one phosphorylated residue is sufficient to produce the CaM effect.

Next, we examined the role of S2808 phosphorylation by PKA. Cardiomyocytes homozygous for phosphoablated S2808A exhibited no regulation of Ca^{2+} sparks by CaM even in the presence of cAMP (Figure 8), as long as CaMKII activation and S2814 phosphorylation (Supplementary Figure 5) was prevented by including AIP in the solution. When S2808 was maximally phosphorylated in S2814A (*i.e.* cardiomyocytes lacking S2814 phosphorylation) the inhibitory effect of CaM was not significantly smaller compared to cardiomyocytes where both S2808 and S2814 were maximally phosphorylated (S2814D cardiomyocytes in presence of cAMP). These results strongly suggest there is no additive effect of S2808 and S2814 phosphorylation. Taken together, these experiments establish that RyR2 inhibition by CaM requires phosphorylation of RyR2 either at residues S2808 or S2814.

Discussion

The major new finding of this study is that the inhibitory CaM regulation of RyR2 channels requires RyR2 phosphorylation by PKA or CaMKII in human, mice and sheep left ventricle. Based on our results from gene-targeted mice, phosphorylation of either residue S2808 or S2814 is both necessary and sufficient for RyR2 regulation by CaM, whereas at low levels of RyR2 phosphorylation at these sites, CaM does not modulate channel activity. At intermediate phosphorylation levels such as seen in sheep RyR2, CaM still has an inhibitory action. As yet it is not clear what the phosphorylation threshold is for CaM inhibition. We also found that CaM inhibited Ca^{2+} sparks in wild-type mouse cardiomyocytes (Figure 7B-green) indicating that it had some inhibitory effect on mouse RyR2, consistent with other studies. [7, 11] In these experiments CaM inhibition could be abolished by the CaMKII inhibitor, AIP (Figure 7B-blue), again suggesting that RyR2 phosphorylation at S2814 is sufficient to promote CaM inhibition.

We find that in RyR2 from healthy human heart, the lack of a CaM effect is not due to lack of CaM binding. Using FRET assays, we show CaM binding (K_d = 120 ± 14 nM, Figure 4A) is similar to that seen in RyR2 from SR vesicles from pig (93 ± 3 nM [30]) and dog (~75 nM [10, 13]) but substantially higher than in reports of *in-situ* measurements from mouse cardiomyocytes (18.2 ± 2 nM). [9]

Our results have important implications for RyR2 regulation during β_2-adrenergic stimulation in response to exercise stress and in heart failure where phosphorylation at these sites is increased. [3, 17–20] Previous studies have focused on the effects of reduced CaM binding to RyR2 in animal models of cardiac pathologies, where it was shown that loss of CaM binding causes increased diastolic Ca^{2+} leak via RyR2. [9, 10, 13] Based on the finding that CaM binding to RyR2 was decreased by ~50% upon PKA phosphorylation of S2808 and introduction of a modified CaM (+Gly-Ser-His) with significantly higher binding affinity for RyR2 restored normal RyR2 function, [13, 14] it was assumed in these studies that loss of CaM binding would lead to pathological chronic activation of RyR2. Our results suggest that the current concept that CaM functions as a canonical inhibitor of RyR2 does not apply to channels isolated from healthy human hearts. Nevertheless, CaM does inhibit RyR2 from ICM human hearts, where CaM efficacy depends on the phosphorylation state of the channel. CaM inhibits only phosphorylated RyR2 channels, which are prone to spontaneous openings during diastole, such as might occur during exercise or in patients with heart failure. [28, 41] Since spontaneous RyR2 opening is an established molecular mechanism for triggering ventricular arrhythmias, [42] our findings suggest important implications for RyR2 regulation in health and heart disease.

The lack of effect of CaM on RyR2 isolated from healthy human hearts under diastolic conditions was not anticipated, since in sheep, dog and mouse models, [5–7] CaM reduces RyR2 activity by ~60% and partially inhibits SR calcium release in cardiomyocytes. [6, 8, 9] The lack of exogenous CaM effect in healthy human RyR2 is not due to interference by endogenous CaM on RyR2 because we established that CaM dissociates from these RyR2 samples within minutes (Figure 4B) and, therefore would have dissociated from RyR2 during washout in bilayer experiments. Interestingly, sheep RyR2s were significantly hyper-

phosphorylated at residue S2808 compared to healthy human RyR2, possibly due to higher catecholaminergic stress in sheep compared to humans prior to heart harvest. *Ex vivo* reduction in phosphorylation of S2808 in sheep by the phosphatase PP1 abolished CaM inhibition, reproducing the healthy human phenotype. Several studies have shown that the β_2-adrenergic receptor pathway activated by adrenaline, particularly during stress, is predominantly through a stimulatory $G_{s,}\alpha$-protein-cyclic AMP-PKA pathway in human heart [45, 46] as opposed to dual $G_{s,}\alpha$-inhibitory G_i -protein pathways in other animals, [47–49] which may explain differences in RyR2 phosphorylation and hence the difference in regulation by CaM between humans and other animals.

In heart failure, altered RyR2 *S*-oxidation *S*-nitrosylation and phosphorylation increase the SR Ca^{2+} leak through RyR2. [43, 44] Although we have demonstrated the importance of RyR2 phosphorylation in ICM hearts to CaM regulation, it is not yet clear how RyR2 *S*-nitrosylation and *S*-oxidation will affect CaM inhibition. Though single channel studies show that in the absence of CaM, RyR2 is more active in failing hearts than healthy, [16, 31] CaM inhibition of RyR2 in our ICM hearts ablated the difference in P_o between healthy and failing hearts (Supplementary Figure 1A). Hence the increased SR Ca^{2+} leak in heart failure may arise from other mechanism such as reduced inhibition of RyR2 by cytoplasmic Mg^{2+} which was not present in our experiments. [31]

Another case in which CaM inhibitory efficacy may be more important than CaM binding is in CPVT caused by RyR2 mutations. Our previous study of CPVT-linked CaM mutants (N54I and N98S) to modulate sheep and human RyR2, [5] resulted in the loss of RyR2 inhibition by CaM mutations without loss of CaM binding. Furthermore, the CPVT-linked mutant CaMs bind with even higher affinity to RyR2 than wild-type CaM. [5, 50] We also found that CPVT mutant CaMs failed to inhibit PKA phosphorylated human RyR2 channels whereas wild-type CaM did inhibit them. This suggests that the lack of inhibitory efficacy of CPVT mutant CaMs, rather than their decreased binding, is what facilitates pathological elevation of Ca^{2+} leak via RyR2 during stress and exercise, and this may explain their pathogenic role in CPVT. Similarly, CaM might have different efficacy (despite similar binding affinity) toward inhibition of RyR2 isolated from normal human hearts vs. ICM hearts.

A case in which CaM binding may be more important than CaM efficacy has been made based on RyR2 knock-in mice with partially ablated CaM binding sites. Homozygous $RyR2^{D/D}$ mice with impaired diastolic CaM binding and inhibition of RyR2 produced no significant cardiac hypertrophy, [51] consistent with the absence of cardiac remodeling associated with CaM mutations in humans that abolish CaM inhibition of RyR2 without loss of CaM binding. [12] Interestingly, homozygous knock-in mice with impaired diastolic and systolic CaM binding to RyR2 ($RyR2^{ADA/ADA}$) suffered from severe cardiac hypertrophy, poor LV contraction and death by postnatal day 16. The authors interpreted this data to indicate that systolic CaM inhibition of RyR2 is required for normal cardiac function. However, $RyR2^{ADA/ADA}$ mice manifest cardiac dysfunction already during fetal development when SR Ca^{2+} release does not contribute to excitation-contraction coupling. [52] Hence, it is likely that CaM binding to RyR2 is critical during cardiac development regardless of its inhibitory role. Since RyR2 binds approximately 20% of the total CaM

expressed, [9] and CaM is highly buffered in a cardiomyocyte (1 in 60 CaM molecules bound), [9] loss of CaM binding to RyR2 will significantly increase free CaM in the cell. The excess of CaM, unbound to RyR2, could activate other CaM regulated pathways such as Calcineurin/NFAT, which is known to cause cardiac hypertrophy. [53] In fact, mRNA levels of MCIP1 (myocyte-enriched calcineurin-interacting protein) are increased in $RyR^{ADA/ADA}$ mice, and loss of calcineurin mitigated the cardiac hypertrophy phenotype of $RyR^{ADA/ADA}$ mice. [7, 54]

Inspection of the structure of human RyR2 (PDB ID: 5GOA also see [55]) suggests that residues S2808 and S2814, the phosphorylation sites known to determine CaM action on RyR2, are 70–100 Å from one generally accepted component of the CaM binding site (K3583-F3603) [51] and at least this distance from the channel pore. Thus, both the CaM effect and the dependence on RyR2 S2808/S2814 phosphorylation would involve long-distance allosteric interactions. It has been observed that CaM can either stimulate or inhibit RyR1 while only inhibiting RyR2, with only one RyR binding site present. These observations have led to the proposals that isoform-specific inter-domain interactions mediate the transduction of CaM binding to the CaM effect on channel gating. [51, 56, 57] A structural analysis of RyR2 gating shows that channel opening and closing is associated with inter-domain conformational changes over the entire molecule. [55] Thus, it is quite plausible that changes in the RyR2 phosphorylation domain are important in CaM-dependent channel gating.

In summary, CaM inhibition of human RyR2 appears to depend on the phosphorylation of RyR2 at residues S2808 and/or S2814. Thus, the effect of CaM on RyR2 depends on two separate mechanisms; 1) the degree of CaM binding to RyR2 and 2) the effect of bound CaM on RyR2 activity. CaM protects against aberrant SR Ca^{2+} release in heart failure or periods of β_2-adrenergic stress where RyR2 is highly phosphorylated.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations:

AIP	Autocamptide-2 Related Inhibitor Peptide CaMKII inhibitor

cAMP	cyclic adenosine monophosphate
CaM	calmodulin
CaMKII	Ca^{2+}/calmodulin-dependent protein kinase II
CPVT	catecholaminergic polymorphic ventricular tachycardia
EPAC2	Exchange Protein Activated by cAMP 2
FKBP12.6	FK506 binding protein isoform 1B
ICM	ischemic cardiomyopathy
PKA	protein kinase A
PP1	protein phosphatase 1
RyR2	ryanodine receptor type 2
SERCA2a	sarco/endoplasmic reticulum Ca^{2+}-ATPase isoform 2a
SR	sarcoplasmic reticulum

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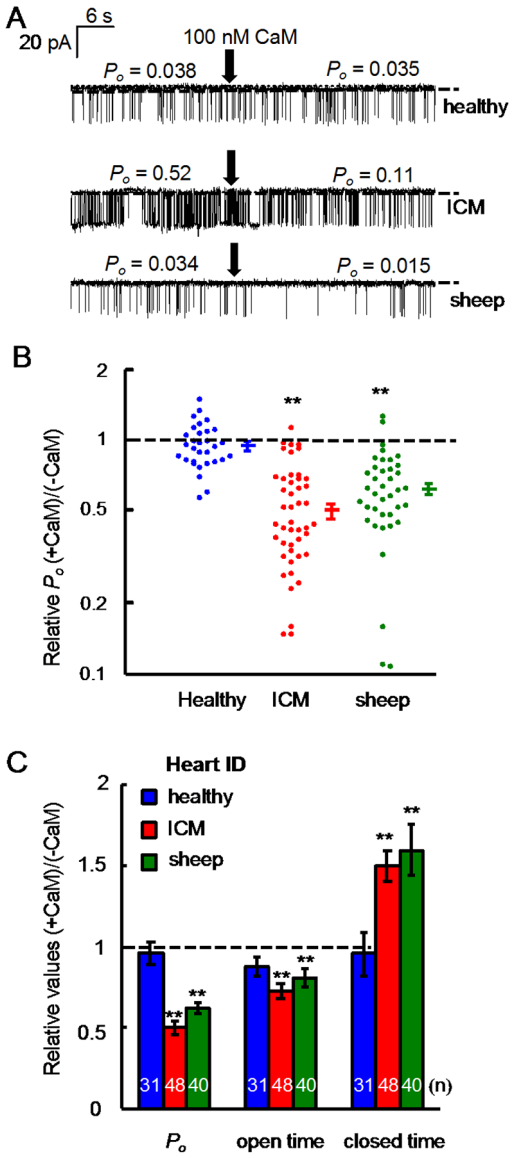

Figure 1: The effect of CaM on activity of RyR2 isolated from healthy and failing human hearts and from sheep heart.

Channel recordings were taken in the presence of 100 nM cytoplasmic Ca^{2+} and 2 mM ATP. Luminal [Ca^{2+}]= 0.1 mM and membrane potential= −40 mV. (A) Representative single channel recordings for RyR2 from each tissue, before and after addition of 100 nM CaM (arrows). Channel openings are downward current jumps from the baseline (dashed line). (B) Relative effect of CaM on the open probability (P_O) of RyR2 from healthy and failing human hearts and a sheep heart. Each sample measures the P_O in the presence of 100 nM CaM relative to mean of the bracketing intervals of CaM washout (excluding the first 15 seconds of each washout). The scatter in these values was due to inherent stochastic nature of RyR2 gating. Samples were pooled from RyR2s taken from four healthy and four failing human hearts with ischemic cardiomyopathy (ICM) and from one sheep heart. The means and SEM of each sample distribution are also shown. (C) Relative CaM effects on RyR2 P_O, mean open time and mean closed time. Data are shown as means ± SEMs with numbers of

samples shown in each bar. Asterisks denote a significant difference in relative values to 1 (** $p < 0.01$).

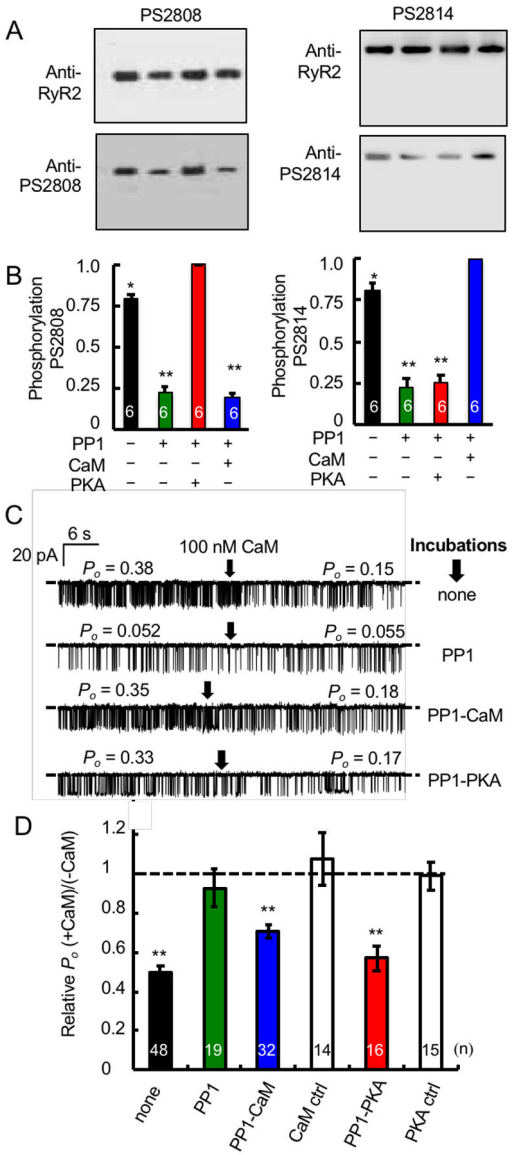

Figure 2: RyR2 phosphorylation modulates CaM inhibition of RyR2 activity in failing human heart with ICM.

(A-B) Representative Western Blots (A, complete Western Blots are shown in Supplementary Figure 6) and corresponding grouped mean data (B) of RyR2s isolated from human ICM heart that were subjected to various incubations to manipulate RyR2 phosphorylation prior to SDS Page and Western Blots. RyR2 were first maximally dephosphorylated by PP1, and then subjected to CaMKII phosphorylation (in the presence of PKA inhibitor H89 (2 μM)) or PKA phosphorylated (in the presence of CaMKII inhibitor KN93 (10 μM)). An untreated sample (neither dephosphorylated or subsequently phosphorylated) is included for comparison. Blots were probed with antibodies for PS2808 or PS2814 and then stripped and re-probed with antibodies for RyR2. In B, PS2808 and PS2814 signals were first normalized to total RyR2 detected by anti-RyR2. This data is then normalized to the maximum phosphorylation of S2808 by PKA (left) and to S2814 by CaM (right). (C) Representative single channel recordings of RyR2 from these ICM hearts that

were subjected to various incubations. Single channel recording conditions are the same as in Figure 1 and arrows indicate the addition of 100 nM CaM. Channel openings are downward current jumps from the baseline (dashed line). (D) RyR2 P_O in the presence of 100 nM CaM relative to that in the absence of CaM. Mean values are shown for the four incubation conditions described above plus for two control experiments where RyR2s were subjected to PP1 incubation followed by incubation with phosphorylation buffers lacking PKA (PKA-ctrl) or lacking CaM (CaM-ctrl) but including the PKA inhibitor H89 and CaMKII inhibitor KN93. Data in bar graphs are shown as means ± SEMs. Number of samples shown in each bar. In B, asterisks denote a significant difference in RyR2 phosphorylation from maximal PKA phosphorylation (left) or maximal CaMKII phosphorylation (right). In D, asterisks denote a significant difference in relative values to 1 (* $p<0.05$, ** $p<0.01$).

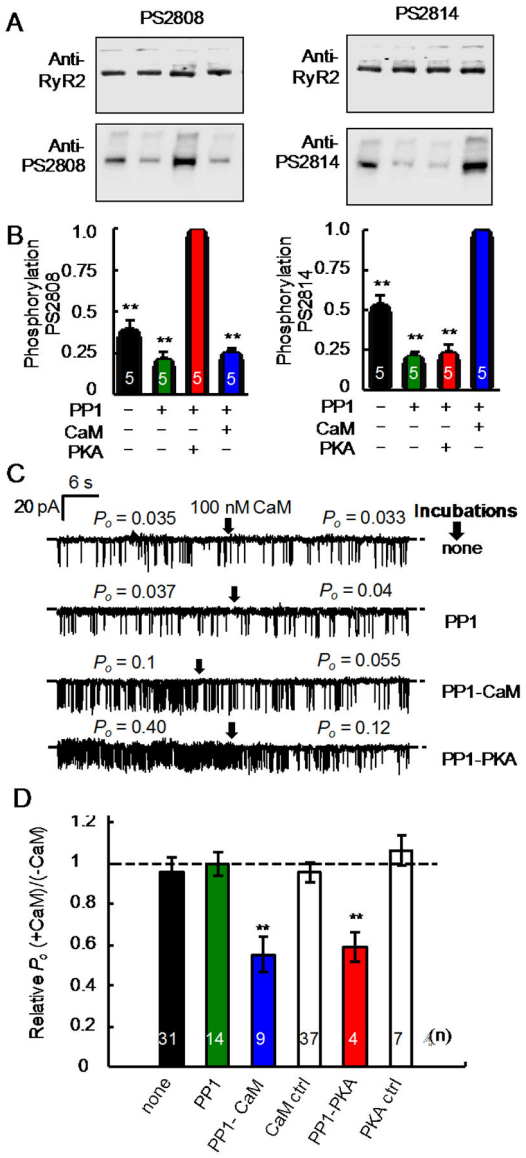

Figure 3: RyR2 phosphorylation modulates CaM inhibition of RyR2 activity in healthy human hearts.

(A-B) Representative Western Blots (A, complete Western Blots are shown in Supplementary Figure 7) and grouped mean data (B) of RyR2s isolated from healthy human heart that were subjected to various incubations as in Figure 2. In B, PS2808 and PS2814 signals were first normalized to total RyR2 detected by anti-RyR2. This data is then normalized to the maximum phosphorylation of S2808 by PKA (left) and to S2814 by CaM (right). (C) Representative single channel recordings of RyR2 that were subjected to various incubations. Single channel recording conditions are the same as in Figure 1 and arrows indicate the addition of 100 nM CaM. Channel openings are downward current jumps from the baseline (dashed line). (D) RyR2 P_O in the presence of 100 nM CaM relative to that in the absence of CaM. Mean values are shown for the four incubation conditions described above plus for two control experiments described in Figure 2 (PKA-ctrl and CaM-ctrl). Data in bar graphs are shown as means ± SEMs. Number of samples shown in each bar. In B,

asterisks denote significant differences in RyR2 phosphorylation from maximal PKA phosphorylation (left) or maximal CaMKII phosphorylation (right). In D, asterisks denote a significant difference in relative values to 1 (** $p<0.01$).

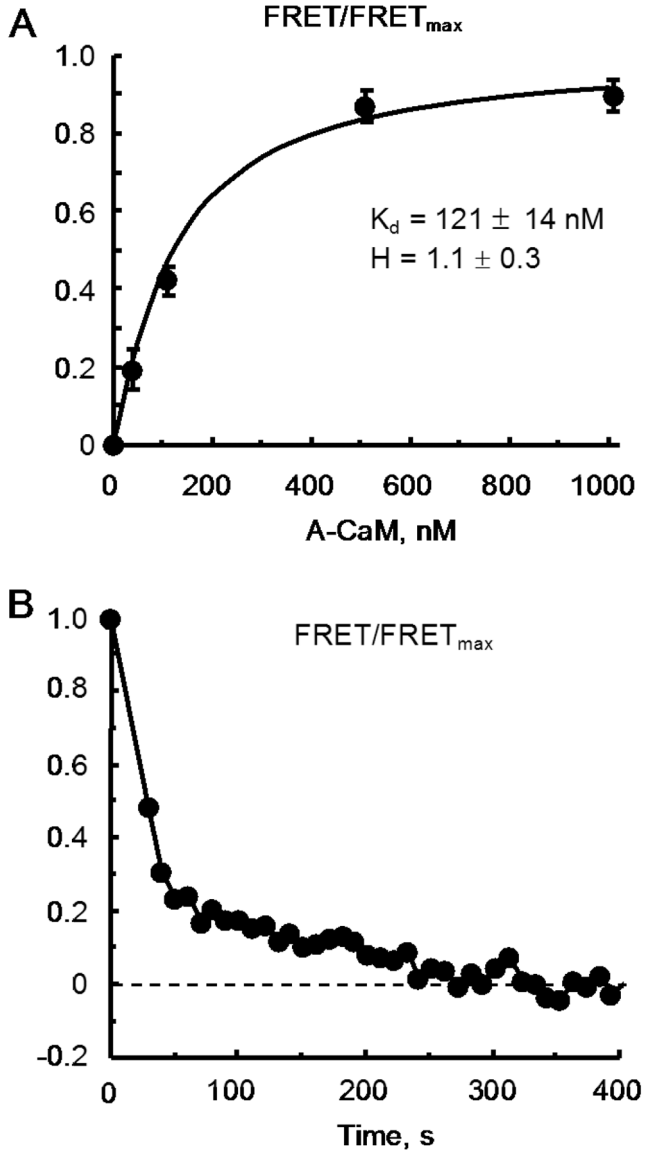

Figure 4: FRET determines that CaM binds to RyR2 from healthy human heart.

FRET between D-FKBP and A-CaM was measured from SR vesicles from one healthy human heart (H3) (details given in Supplementary Table 1). **(A)** FRET relative to the maximum FRET. The curves show the least-squares, Hill fit to the data. **(B)** Representative time course of FRET decay after 500 nM A-CaM was displaced from RyR2 by adding 25 μM WT-CaM to the medium. Measurements of FRET relative to the maximum FRET were made on 3 separate days and on each day, samples were measured in triplicate (means ± SEMs, n=3).

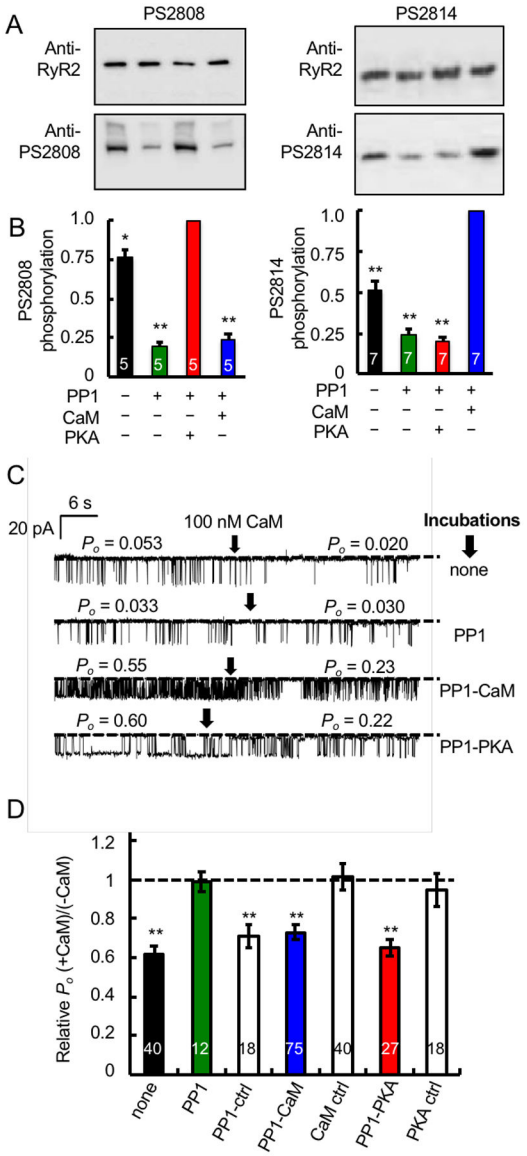

Figure 5: RyR2 Phosphorylation modulates CaM inhibition in sheep heart.

(A-B) Representative Western Blots (A, complete Western Blots are shown in Supplementary Figure 8) and grouped data (B) of RyR2s isolated from sheep heart that were subjected to various incubations as in Figure 2. **(B)** Corresponding average data for RyR2 phosphorylation at S2808 and S2814. PS2808 and PS2814 signals were first normalized to total RyR2 detected by anti-RyR2. Data is normalized to the maximum phosphorylation of S2808 by PKA (left) and to S2814 by CaM (right). **(C)** Representative single channel recordings of RyR2 that were subjected to various incubations. Single channel recording conditions are the same as in Figure 1 and arrows indicate the addition of 100 nM CaM. Channel openings are downward current jumps from the baseline (dashed line). **(D)** RyR2 P_O in the presence of 100 nM CaM relative to that in the absence of CaM. Mean values are shown for the four incubation conditions described above plus for three control experiments where RyR2s in one control experiment were incubated with dephosphorylating buffers

lacking PP1 (PP1-ctrl) and the other two control experiments are described in Figure 2 (PKA-ctrl & CaM-ctrl). Data in bar graphs are shown as means ± SEMs. Number of samples shown in each bar. In B, asterisks denote a significant difference in RyR2 phosphorylation from maximal PKA phosphorylation (left) or maximal CamKII phosphorylation (right) (* $p<0.05$, ** $p<0.01$). In D, asterisks denote a significant difference in relative values to 1 (** $p<0.01$).

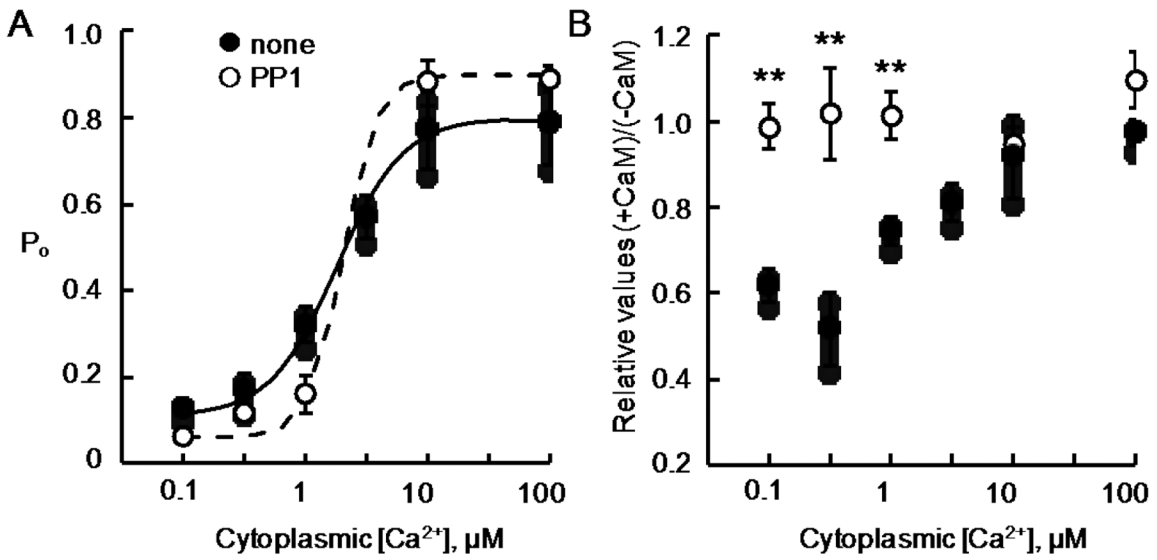

Figure 6: Dependence of CaM inhibition of sheep RyR2 on cytoplasmic Ca^{2+} and PP1 incubation.

(A) P_o of RyR2 before (none) and after (PP1) incubation (see methods for details). Curves show Hill fits to the data (IC_{50} values, none: 1.5 ± 0.3 µM, PP1: 2.0 ± 0.6 µM). **(B)** Relative inhibition by CaM. Asterisks denote a significant difference to no treatment 1 (** $p<0.01$).

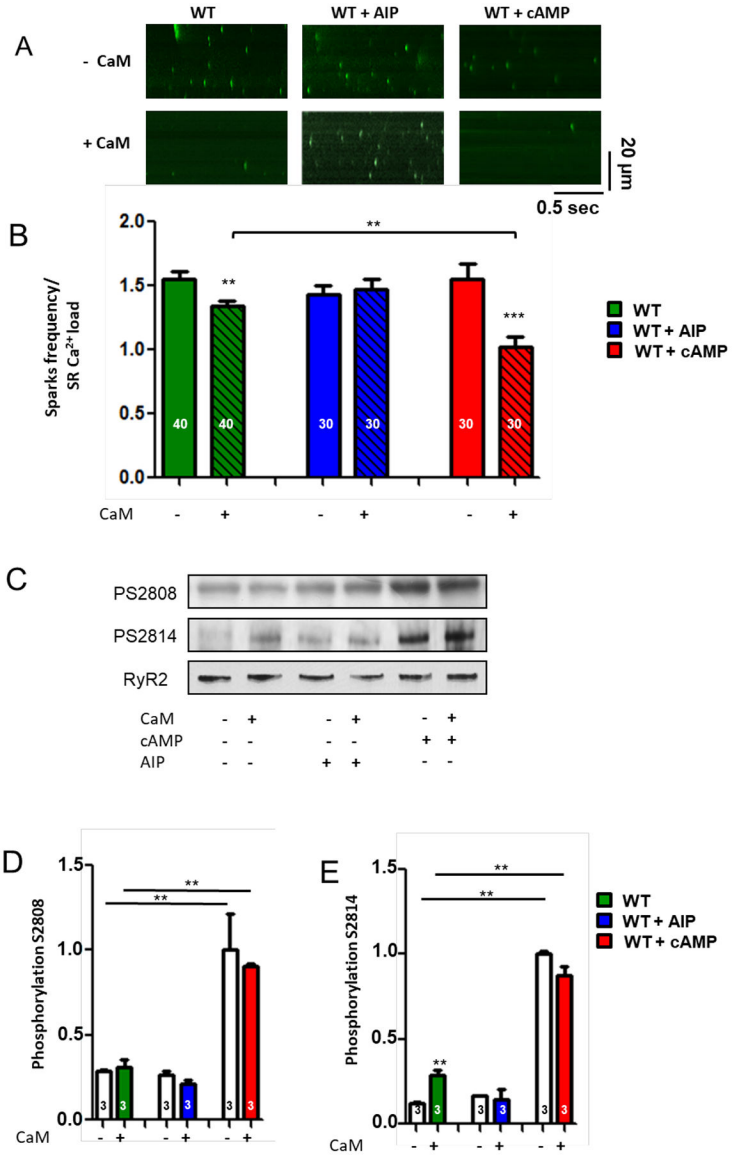

Figure 7: Phosphorylation of RyR2 is required for CaM inhibition of Ca^{2+} sparks in C57Bl6 mouse cardiomyocytes.

(A) Representative line-scan images of Ca^{2+} sparks obtained in wild-type (WT) permeabilized mouse ventricular myocytes in the absence or presence of CaM (100 nM), incubated with either vehicle, AIP (1 µM) or cAMP (10 µM). Representative line scans were chosen to illustrate the effect of CaM addition within each group rather than differences between WT, AIP and cAMP group averages. **(B)** Ca^{2+} spark frequency (sparks/100µm/sec) normalized by the SR Ca^{2+} load. **(C)** Representative Western Blots of permeabilized ventricular cardiomyocytes. Blots were probed with an antibody to S2808 (PS2808) and S2814 (PS2814). The membranes were then stripped and reprobed with anti-RyR2 as a loading control. **(D, E)** Grouped data showing the average phosphorylation levels relative to the maximal phosphorylation induced by cAMP exposure. Data in bar graphs are means ± SEMs with numbers of samples shown in each bar. Asterisks denote significant differences (** $p<0.01$).

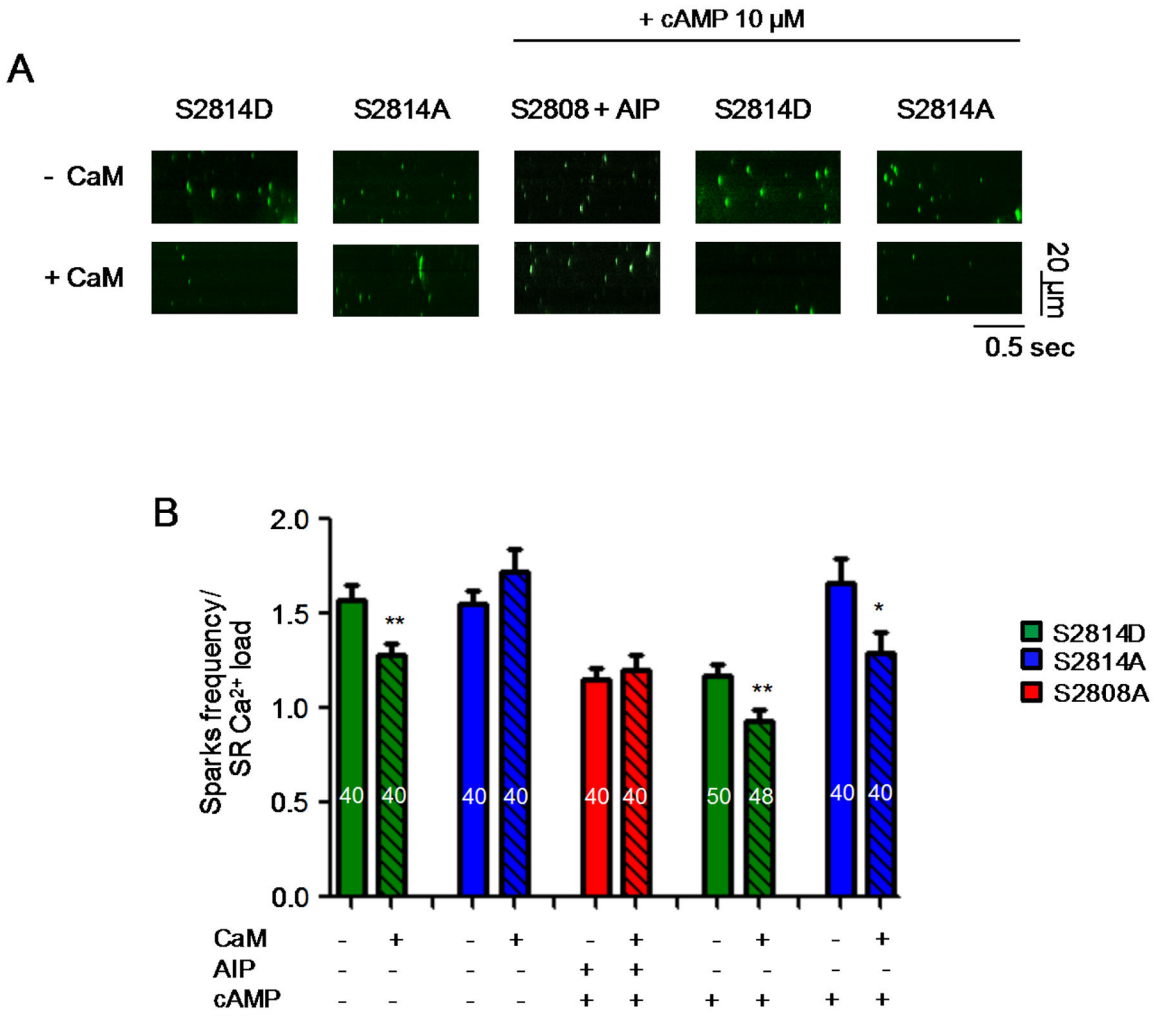

Figure 8. Phosphorylation of either residue S2808 or S2814 is sufficient for CaM inhibition of Ca^{2+} sparks in mouse cardiomyocytes.

(A) Representative line-scan images of Ca^{2+} sparks obtained in permeablized mouse ventricular myocytes from RyR2 S2814D, S2814A or S2808A mice in the absence or the presence of CaM (100 nM), incubated with vehicle, cAMP (10 μM) alone, or AIP (1μM) + cAMP (10 μM). Representative line scans were chosen to illustrate the effect of CaM addition within each group rather than differences between RyR2 genotypes. **(B)** Average Ca^{2+} spark frequency (sparks/100μm/sec) normalized by the SR Ca^{2+} load for every group. Data in bar graphs are means ± SEMs. Number of samples shown in each bar. Asterisks denote a significant difference to - CaM group for each experimental condition (* $p<0.05$, ** $p<0.01$).