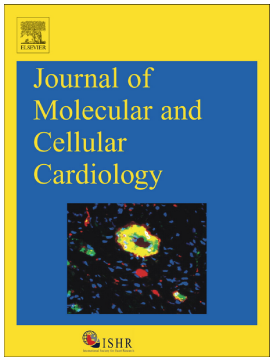

Ryanodine receptor modification and regulation by intracellular Ca2+ and Mg2+ in healthy and failing human hearts

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Ryanodine receptor modification and regulation by intracellular Ca^{2+} and Mg^{2+} in healthy and failing human hearts

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ABSTRACT

Rationale: Heart failure is a multimodal disorder, of which disrupted Ca^{2+} homeostasis is a hallmark. Central to Ca^{2+} homeostasis is the major cardiac Ca^{2+} release channel – the ryanodine receptor (RyR2) – whose activity is influenced by associated proteins, covalent modification and by Ca^{2+} and Mg^{2+}. That RyR2 is remodelled and its function disturbed in heart failure is well recognized, but poorly understood.

Objective: To assess Ca^{2+} and Mg^{2+} regulation of RyR2 from left ventricles of healthy, cystic fibrosis and failing hearts, and to correlate these functional changes with RyR2 modifications and remodelling.

Methods and Results: The function of RyR2 from left ventricular samples was assessed using lipid bilayer single-channel measurements, whilst RyR2 modification and protein:protein interactions were determined using Western Blots and co-immunoprecipitation. In all failing hearts there was an increase in RyR2 activity at end-diastolic cytoplasmic Ca^{2+} (100 nM), a decreased cytoplasmic $[Ca^{2+}]$ required for half maximal activation (K_a) and a decrease in inhibition by cytoplasmic Mg^{2+}. This was accompanied by significant hyperphosphorylation of RyR2 S^{2808} and S^{2814}, reduced free thiol content and a reduced interaction with FKBP12.0 and FKBP12.6. Either dephosphorylation of RyR2 using PP1 or thiol reduction using DTT eliminated any significant difference in the activity of RyR2 from healthy and failing hearts. We also report a subgroup of RyR2 in failing hearts that were not responsive to regulation by intracellular Ca^{2+} or Mg^{2+}.

Conclusion: Despite different aetiologies, disrupted RyR2 Ca^{2+} sensitivity and biochemical modification of the channel are common constituents of failing heart RyR2 and may underlie the pathological disturbances in intracellular Ca^{2+} signalling.

Keywords:

Ryanodine receptor, Mg^{2+} and Ca^{2+} signalling, heart failure, phosphorylation, lipid bilayer, regulatory proteins.

Abbreviations and Acronyms:

CaM	Calmodulin
CaMKII	Calmodulin kinase II
CF	Cystic fibrosis
CSQ2	Calsequestrin type 2
DADs	Delayed after-depolarizations
EDMD	Emery Dreifuss muscular dystrophy
FKBP	FK binding protein
ICM	Ischaemic cardiomyopathy
k_o	Opening rate
PKA	Protein kinase A
PP1	Protein phosphatase 1
PP2a	Protein phosphatase 2
P_o	Open probability
RyR2	Ryanodine Receptor Type 2
SERCA2A	Sarcoplasmic Reticulum/Endoplasmic Reticulum Ca^{2+}-dependent ATPase Type 2A
SR	Sarcoplasmic Reticulum
T_o	Mean open time
T_c	Mean closed time

1.0 INTRODUCTION

Cardiac muscle contraction (systole) is triggered by depolarization of the surface membrane. Subsequent activation of the L-type Ca^{2+} channel causes Ca^{2+} release from the internal sarcoplasmic reticulum (SR) Ca^{2+} store via the cardiac ryanodine receptor (RyR2) Ca^{2+} channel. During diastole (relaxation), Ca^{2+} is sequestered into the SR via the SERCA2A pump or extruded from the cell via the Na/Ca exchanger (NCX). Heart failure encompasses a complex and diverse set of disorders that involves changes in expression and post-translational modification of these Ca^{2+} handling proteins, along with altered Ca^{2+} dynamics and tissue remodelling [1-3]. In healthy hearts, minimal Ca^{2+} release during diastole serves to keep cytoplasmic [Ca^{2+}] low and to optimise SR refilling and end diastolic SR Ca^{2+} load [4, 5]. Under some pathological conditions, excessive RyR2 activity leads to diastolic Ca^{2+} leak and reduced end diastolic SR Ca^{2+} load resulting in reduced systolic Ca^{2+} release and contractile dysfunction [6, 7]. This leak can also raise diastolic cytoplasmic [Ca^{2+}] and induce delayed after depolarizations (DADs) [8] and arrhythmias.

RyR2 activity is influenced by the integrated effects of associated proteins, covalent modifications such as phosphorylation and oxidation (reviewed in [9]) and by levels of Ca^{2+} and Mg^{2+} in both the cytoplasm and SR lumen [10]. The cytoplasmic N-terminal domain of RyR2 is a scaffold for accessory proteins including FKBP12.6 (calstabin2), protein kinase A (PKA) and calmodulin kinase II (CaMKII) [11]. The RyR2 SR luminal domain is associated with the Ca^{2+}-binding protein calsequestrin (CSQ2) and its anchoring proteins triadin and junctin. Mutation, altered expression or chemical modification in many of these proteins has been implicated in a variety of pathological conditions (reviewed in [9, 12]). Importantly, hyperphosphorylated serine residues on RyR2 (S^{2808} and S^{2814}) are consistently associated with excess RyR activity [13-15] and reduced contractility. However, it is not yet known whether hyperphosphorylation is cause or consequence of heart failure (reviewed in [13, 16, 17]).

Heart failure is a complex clinical syndrome of heterogeneous aetiologies including atherosclerotic coronary artery disease resulting in ischemic damage [18], and a range of other cardiomyopathies [19]. In this study, the functional and structural remodelling of the RyR2 macromolecular complex is examined in heart tissue from patients with ischaemic cardiomyopathy (ICM), cystic fibrosis (CF), and for the first time, from Emery Dreifuss Muscular Dystrophy with cardiomyopathy (EDMD) [20]. ICM exhibits muscle weakness resulting from myocardial infarction secondary to atherosclerotic coronary artery disease, which reduces blood supply to heart muscle. EDMD is a rare skeletal muscle dystrophy arising from mutations in the genes encoding for lamin or emerin [21]. The autosomal dominant form of EDMD (lamin mutation) also leads to cardiac conduction defects and dilated cardiomyopathy [21, 22]. CF is an autosomal recessive genetic disorder that manifests as reduced function of several organs, primarily the lungs and causes increased after-load on the heart [23]. CF is not normally associated with heart failure, though secondary right ventricular enlargement [23-26] and reduced left ventricular filling [27-29] have been reported. Loss of the cystic fibrosis transmembrane conductance regulator (CFTR) Cl^- transporter causes cardiac dysfunction independent of lung disease [27]. This is possibly because of the contribution of CFTR to the maintenance of ventricular resting membrane potential, and action potential duration [30-32]. There are no reports of the structure and function of RyR2 in hearts from CF and EDMD patients. Therefore we investigated the possibility that RyR2 in these hearts show excess activity associated with hyperphosphorylation of S^{2808} and S^{2814}.

It is increasingly recognized that regulation of RyR2 by intracellular Ca^{2+} and Mg^{2+} is central to SR Ca^{2+} fluxes during diastole and systole, which may be altered under pathological conditions. Although we recently reported the Ca^{2+}/Mg^{2+} regulation characteristics of healthy human RyR2 [10], little is known about how these mechanisms are altered in heart failure. Published single

channel data on RyR2 from failing hearts are scant and conflicting. Studies of RyR2 isolated from ICM patients [33] and those with dilated cardiomyopathy and ICM [14] suggested normal activation by cytoplasmic Ca^{2+}. However, higher RyR2 activity in channels isolated from failing tissues has been observed in several other studies [15, 34, 35]. In our study, Ca^{2+} and Mg^{2+} regulation of RyR2 from healthy, CF, EDMD and ICM hearts were compared in single channel recordings of RyR2s in artificial lipid bilayers. Here, functional differences between heart failure groups were compared with remodelling of the RyR2 evaluated using a thiol probe assay to assess RyR2 thiol modification and Western Blot to assess RyR2 phosphorylation at S^{2808} and S^{2814}, and association of RyR2 with FKBP12.0, FKBP12.6 and phosphatases. We report a significant alteration in RyR2 regulation by intracellular Ca^{2+} in CF and failing heart, but only in failing hearts did we find this correlated with hyperphosphorylation of RyR2 S^{2808} and S^{2814}, enhanced RyR2 thiol modification, and reduced FKBP association with RyR2.

2.0 MATERIALS AND METHODS

The materials and methods section is found in the Online Data Supplement.

3.0 RESULTS

3.1 RyR2 channel characteristic from healthy and ICM hearts. RyR2s isolated from left ventricular tissue from four healthy hearts (H1-H4) and five failing hearts with ischemic cardiomyopathy, (ICM1-ICM5) were incorporated into lipid bilayers and activity measured with 0.1 μM cytoplasmic Ca^{2+}, 0.1 mM luminal Ca^{2+} and 2 mM cytoplasmic ATP (no Mg^{2+}) (Figs. 1A,B). We observed no differences in the mean open probability (P_o) of RyR2 among the four healthy hearts and among the ICM hearts (Fig. 1C). However, we found a significant difference ($p = 0.015$) between the means of the healthy and ICM hearts. The distribution of P_o from each healthy heart was skewed towards low P_o. Histograms of P_o pooled from all healthy hearts (Fig. 1D, blue line), showed a bimodal distribution with a low activity mode (P_o ~0.006) constituting 50% of channels with the remaining channels forming an intermediate activity mode (P_o ~0.1). In contrast, P_o from all ICM hearts showed a distribution with (i) 30% of channels in a low activity range (P_o <0.03), (ii) an intermediate activity group (P_o between 0.03-0.3) constituting 50% of channels, and (iii) the remaining channels in a high activity mode (P_o >0.3). Traces of RyR2 activity representative of the low, intermediate and high activity groups are shown in Figs. 1A,B.

In a second series of experiments, we investigated whether phosphorylative or oxidative modification of RyR2 could account for the higher P_o in failing heart. SR membranes from one healthy heart (H1) and one failing heart (ICM4) were incubated with the phosphatase PP1 (250 units per mg protein) prior to bilayer experiments. These hearts were selected because they showed highly significant differences in P_o and we wanted to measure how much of the differences in RyR2 activity from healthy and failing hearts were due to differences in their levels of phosphorylation and oxidation (Fig. 1E). Incubating healthy and failing hearts with PP1 to dephosphorylate the channels (whilst maintaining the thiol modifications in all samples) abolished the significant differences in P_o between RyR2 from the healthy and ICM groups. Similarly, treating with 5 mM DTT to reduce RyR2 thiol side chains (which did not alter RyR2 phosphorylation) yielded similar results, with channel P_o not significantly different between healthy and failing RyR2 after treatment. Our interpretation of this data is that both RyR2 phosphorylation and thiol modification make a contribution to the higher activity of RyR2 from ICM hearts.

3.2 Cytoplasmic Ca^{2+} regulation of RyR2 from healthy and ICM hearts. All RyR2s exhibited the characteristic Ca^{2+}-activation to a maximum P_o near 1 and half-activating [Ca^{2+}] (K_a) in the μM range (Fig. 2A, Supplementary Table 1). The average K_a for RyR2 from four healthy hearts (Fig. 2A, ○) was 3.05 ± 0.8 μM, and was significantly higher than the average of five ICM hearts (●) (1.3 ± 0.1 μM, $p<0.05$). These differences are reflected in the K_a values measured in RyR2 from individual hearts (Fig. 2B). In these experiments, all RyR2 from healthy hearts were in the low activity group. However, four RyR2 from ICM2-ICM4 hearts were in the intermediate activity group (group ii above) and their mean Ca^{2+} dose-response is shown separately in Fig. 2A (▲, also see Supplementary Fig. 1) where it can be seen that these channels were more sensitive to Ca^{2+} at diastolic concentrations.

3.3 RyR2 from hearts with Emery Dreifuss cardiomyopathy and cystic fibrosis. We obtained RyR2s from one heart donated from a patient with cystic fibrosis (CF) and from another with Emery Dreifuss muscular dystrophy (EDMD) (Supplementary Figs. 2A,B). The gating kinetics of cardiac RyR2s of CF and EDMD (Supplementary Figs. 2C-F) were compared with healthy and failing hearts. Single channel recordings of representative RyR2 from low, intermediate and high activity groups in CF and EDMD hearts are shown in Supplementary Figs. 2A,B. The mean P_o from CF and EDMD samples was 5-10 fold higher than RyR2 from healthy hearts, as was the ICM sample (Supplementary Figs. 2C). Interestingly, RyR2 from the CF heart had higher P_o than healthy channels, similar to the failing heart samples (*i.e.* EDMD, ICM) suggesting that RyR2 from

this CF heart had been altered as a consequence of pulmonary stress. P_o from the CF and EDMD hearts, like ICM hearts, was skewed to higher activity modes (Supplementary Fig. 2D) and a broad range of mean open times (T_o) and opening rates (k_o) (Supplementary Fig. 2E,F). The higher RyR2 activity seen in CF, failing, and EDMD hearts is due to an increase in both mean open time and opening rate.

RyR2s from CF and EDMD hearts showed activation with significantly higher sensitivity to cytoplasmic Ca^{2+} (EDMD $K_a \sim 1$ μM, Supplementary Table 1, Supplementary Fig. 3A). In these experiments one EDMD RyR2 had intermediate activity levels and four others had high activity levels and their Ca^{2+} concentration responses are shown separately in Supplementary Fig. 3A. The EDMD RyR2 with intermediate activity behaved similarly to the ICM RyR2 with intermediate activity (*c.f.* solid curve and ▲). However, the high activity EDMD RyR2 (△) lost their responsiveness to Ca^{2+} over the range of 1 nM to 100 μM [Ca^{2+}]. The K_a for RyR2 from CF hearts ($K_a = 4 \pm 2$ μM) was not significantly different to that seen for healthy hearts ($p = 0.2$).

3.4 RyR2 luminal Ca^{2+} dependence in the physiological range, in the absence and presence of luminal Mg^{2+}. The responsiveness of RyR2 to luminal [Ca^{2+}] was examined by comparing P_o in 0.1 μM cytoplasmic Ca^{2+} in the presence and absence of luminal Ca^{2+}. RyR2 from all hearts showed a 50 to 1000-fold activation ($p = 4.10^{-6}$) when luminal Ca^{2+} was raised from <1 nM Ca^{2+}_{free} to 1 mM (Fig. 3A; Supplementary Fig. 3B). Although the average luminal Ca^{2+} response within each of the RyR2s groups were not significantly different, three RyR2s from ICM and CF hearts exhibited no significant response to luminal Ca^{2+} (indicated by arrows, $p = 0.54$) and four others showed substantially muted luminal Ca^{2+} response compared to healthy.

Luminal Mg^{2+} plays a physiological role in shaping the luminal Ca^{2+} dependence of RyR2 activation in the cell [38] and in healthy human RyR2 it shifts the K_a for luminal Ca^{2+} activation from 35 μM to ~ 1 mM [10]. Therefore we measured luminal Ca^{2+}-activation of RyR2s in the presence of 1 mM free luminal Mg^{2+} (with cytoplasmic 2 mM ATP and 100 nM Ca^{2+}). In all groups with P_o<0.2, increasing luminal Ca^{2+} over the physiological range of 0.1 to 1 mM, increased P_o by ~2-fold (Fig. 3B; Supplementary Fig. 3C, $p<0.05$). However, P_o of RyR2 from CF and failing human hearts were higher at all luminal [Ca^{2+}] than seen in healthy hearts. P_o for RyR2 from ICM (n = 1-3) and EDMD (n = 1-4) hearts in high activity mode (P_o>0.2) were insensitive to luminal Ca^{2+} (Supplementary Fig. 3C, + and ×, respectively).

3.5 RyR2 regulation by Mg^{2+}. At diastolic cytoplasmic Ca^{2+}, cytoplasmic Mg^{2+} is mainly a competitive inhibitor that binds at the cytoplasmic Ca^{2+} activation site to prevent Ca^{2+} activation of the channel [38]. To determine whether Mg^{2+} inhibition is altered in heart failure, the inhibitory effects of 1 mM cytoplasmic $MgCl_2$ (0.24 mM free Mg^{2+} and 2 mM ATP) were measured at 0.1 μM cytoplasmic Ca^{2+} and 0.1 mM luminal Ca^{2+} (Fig. 4A; Supplementary Fig. 4A). This sub-physiological [Mg^{2+}] was chosen because, at physiological Mg^{2+} (1 mM), the RyR2 P_o in healthy hearts is too low to reliably measured [37]. Sensitivity to Mg^{2+} inhibition in the ICM hearts and the EDMD heart was significantly reduced compared to healthy hearts (Figs. 4A,B; Supplementary Figs. 4A,B). RyR2 from the CF heart showed similar sensitivity to cytoplasmic Mg^{2+} as RyR2 from healthy hearts.

The presence of Mg^{2+} on the luminal side of the RyR2s also inhibits channels by competing with Ca^{2+} for the luminal activation sites [38]. The average inhibitory action of luminal Mg^{2+} was not significantly different between any of the RyR2 groups (Fig. 4C; Supplementary Fig. 4C). However, we observed several RyR2 from ICM and EDMD hearts that were insensitive to either cytoplasmic or luminal Mg^{2+} (e.g. Fig. 4C, Supplementary Fig. 4C points indicated by arrows).

3.6 Protein expression changes in failing heart. We determined whether the expression levels of key Ca^{2+} handling proteins were altered in ICM hearts by subjecting SR vesicles from healthy, CF and failing hearts to quantitative Western Blot (Supplementary Fig. 5). Significant reductions in RyR2 protein levels were found in all ICM samples, as reported previously in human failing heart [39] and in the EDMD sample. In addition there was a significant reduction in SERCA2A expression in 2 of the 5 ICM hearts, but not in the EDMD heart. Curiously, there was a significant increase in triadin-1 expression in 1 ICM heart. Expression of other key SR proteins was similar to that observed in healthy heart. Protein expression in the CF heart was identical to all healthy hearts.

3.7 RyR2 phosphorylation. The level of RyR2 phosphorylation at both S^{2808} and S^{2814} was determined for all healthy and ICM samples using Western Blot (Figs. 5A,B). The phosphorylation at S^{2808} and S^{2814} (Fig. 5C,D) were determined relative to maximal phosphorylation inducible by exogenous PKA and CaMKII, respectively (see Methods, Figs. 5C,D and Supplementary Fig. 6). There was no significant difference in the level of RyR2 phosphorylation at both S^{2808} and S^{2814} between all healthy hearts, being sub-maximally phosphorylated at both sites. RyRs from all ICM samples were hyperphosphorylated, at both S^{2808} and S^{2814} (to ~80-90% of maximal phosphorylation). S^{2808} hyperphosphorylation in ICM hearts was ~1.65 to 2.0-fold higher, and S^{2814} ~1.5 to 1.8-fold higher than the phosphorylation levels found in healthy hearts (Figs. 5D,E and Supplementary Table 2). Phosphorylation levels of RyR2 from the CF heart were not significantly different to that in healthy hearts and levels in EDMD hearts mimicked the ICM samples (Supplementary Figs. 7A,B,E**)**. In our hands, the affinity of the commercially available S^{2030} antibody for human RyR2 was too low to reliably immunodecorate RyR2.

Increased RyR2 phosphorylation in failing heart has been associated with up-regulation of PKA/CaMKII, and/or removal of the RyR2 dephosphorylating enzymes PP1 and/or PP2a [15]. The relative levels of PP1 and PP2a bound to RyR2, determined in immunoprecipitates using Western Blot (Fig. 6A), were significantly less in all 5 ICM hearts than in healthy hearts (Fig. 6B). The CF heart displayed levels of PP1 and PP2a association with RyR2 that were not different to healthy heart (**S**upplementary Figs. 7C,F). Interestingly, only PP1 (and not PP2a) association with RyR2 was significantly reduced in the EDMD heart. Thus, reduced association of these phosphatases with the RyR2 complex could contribute to the hyperphosphorylation observed in failing tissue.

3.8 FKBP association with RyR2. Both FKBP12.6 and FKBP12.0 associate with RyR2 in healthy hearts [40]. The antibody used here recognizes both FKBP isoforms, with an 80-100 fold higher affinity for FKBP12.0 than FKBP12.6 [40]. The migration patterns of the FKBP isoforms (Fig. 6A) are consistent with previously published data [40]. We found a significant reduction in FKBP12.6 associated with RyR2 in 4 of the 5 ICM hearts (Figs. 6A,C); the 5^{th} ICM sample showed a trend to decline in FKBP12.6 association with RyR2 ($p<0.1$). There was a significant decrease in FKBP12.0 and 12.6 bound to RyR2 in the EDMD heart and no significant differences in FKBP content between healthy and CF hearts (Supplementary Figs. 7C,G).

3.9 RyR2 thiol modification. Modification of thiol side chains on cysteine residues within RyR2 has been reported to be a downstream consequence of chronic ROS production in damaged cardiac tissue in dog [35, 41]. Cysteine residues are subject to *S*-nitrosylation, *S*-glutathionylation and disulfide formation (*S*-oxidation). To determine the degree of thiol modification, RyR2 was coupled with maleimide, which forms a covalent bond with unmodified cysteines. RyR2 free thiol content was significantly lowered, indicating enhanced thiol modification by circulating ROS, in the EDMD and ICM hearts (Figs. 7A,B, Supplementary Figs. 7D,H), but not in the CF heart.

4.0 DISCUSSION

The present work extends our previous investigation of intracellular Ca^{2+} and Mg^{2+} regulation on RyR2 from healthy human hearts [10], by examining regulation of human RyR2 from a range of failing hearts. Our data illustrate significant dysfunction in RyR2 from failing hearts from very different aetiologies due to higher activity than healthy hearts at end-diastolic Ca^{2+} (100 nM). The fact that RyR2 from the failing hearts were not significantly altered at systolic Ca^{2+} (10 μM) (Fig. 2), reconciles the different findings by Marx and colleagues [15] using end-diastolic Ca^{2+} with those of Jiang and colleagues [14] who using systolic 5 μM Ca^{2+}. We have also identified a number of other modifications to the RyR2 macromolecular complex including changes in RyR2 phosphorylation, thiol modification and changes in the binding of accessory proteins. Our data suggest that RyR2 phosphorylation and thiol oxidation have the greatest bearing on the RyR2-mediated diastolic Ca^{2+} leak. Either dephosphorylation of RyR2 using PP1 (whist maintaining thiol oxidation) or thiol reduction using DTT (whilst maintaining phosphorylation) eliminated any significant difference in the mean activity of RyR2 from healthy and ICM hearts (Fig. 1E), illustrating that a significant proportion of dysfunction we observe in RyR2 channels from failing heart is a combined effect of excess phosphorylation and thiol oxidation.

4.1 *RyR2 phosphorylation in failing hearts.* Marx *et al*. [15] associated RyR2 hyperphosphorylation at S^{2808} and dissociation of FKBP12.6 with Ca^{2+} dysregulation and heart failure. That PKA hyperphosphorylates RyR2 S^{2808} and contributes to SR Ca^{2+} leak is controversial [13, 17, 42]. Another widely accepted hypothesis is that CaMKII-mediated S^{2814} hyperphosphorylation alone is the major contributor to SR Ca^{2+} leak and heart failure progression [13, 14]. An extension of these hypotheses is that RyR2 remodelling is initiated by a defective interdomain interaction in response to RyR2 oxidation and hyperphosphorylation [43, 44]. To our knowledge, our study is the only one that demonstrates that both S^{2808} and S^{2814} are hyperphosphorylated in failing human heart. This result implies that both PKA and CaMKII pathways play a role in RyR2 hyperphosphorylation. Our finding that S^{2814} is hyperphosphorylated in ICM does not agree with a report that S^{2814} phosphorylation is increased in patients with non-ischemic, but not with ischemic heart failure [7]. We cannot rule out that phosphorylation of S^{2030} or one of a number of other recently identified phosphorylatable residues [45] were altered in ICM, EDMD or CF hearts. The significant reduction in PP1 and PP2a association with RyR2 in failing heart tissue is consistent with the proposal that their reduced association [15, 46], contributes to RyR2 hyperphosphorylation and "leaky" channels, rather than being solely to due increased PKA activity.

4.2 *Thiol modificaiton of RyR2 from failing hearts.* In addition to the significant rise in phosphorylation, the EDMD and ICM hearts showed a significant increase in RyR2 thiol modification. Much of the research on RyR2 modification in heart failure has focused on phosphorylation, with only recent work implicating RyR2 oxidative modification. Evidence in tachypacing heart failure animal models indicates that oxidative modification occurs during later stages, with RyR2 phosphorylation an early “hallmark” of the disease [4]. Another study has reported that the underlying thiol modification state of RyR2 increases the degree of FKBP12.6 disposition from RyR2 induced by PKA phosphorylation, using the constitutively phosphorylated S2808D mouse model [47]. This suggests that both modifications are codependent in causing the significant FKBP 12.6 dissociation from RyR2 reported by Marks *et al* [15]. This is consistent with our measurements of RyR2 activity (Fig. 1E) that suggest that both excess phosphorylation and oxidation, are required to elicit significantly higher P_o under diastolic Ca^{2+}conditions. This is the first study to illustrate significant ROS and phosphorylation-dependent modification of RyR2 in pathogensis of human heart failure.

4.3 ***RyR2 accessory proteins in human heart***. We report altered levels of key RyR2-accessory proteins in failing heart. Reductions in PP1, PP2a and FKBP12.6 confirm previous reports [48], and we present the novel finding that levels of FKBP12.0 are reduced in ICM and EDMD failing hearts. At present, there is no consensus on how the FKBP isoforms regulate RyR2. One proposal is that the loss of FKBP12.6 from RyR2 increases channel activity and diastolic Ca^{2+} leak [15], while FKBP12.6 binding to defective channels restores normal gating [49]. Alternatively, FKBP12.6 can competitively antagonize FKBP12.0 inhibition [50]. We find reduced association of both FKBPs with RyR2 in failing hearts. FKBP12.0 was reduced to a greater extent than FKBP12.6, which may reflect differences in affinities of the isoforms for RyR2 [51]. Given the controversy over actions of these isoforms on RyR2 activity it is difficult to predict precisely how the changes FKBP association with RyR2 that we observe relate to the increase in channel activity. We found no significant change in expression of luminal SR proteins CSQ2 and junctin and a small increase in traidin-1 expression in 1 of 5 failing hearts, but a significant reduction in RyR2 expression in all ICM samples. This illustrates that stoichiometry of CSQ2 tethered to RyR2 via traidin-1 and junctin is altered in failing heart, and we cannot discount that this may have influenced RyR2 responses (to luminal Ca^{2+} in particular) from this sample group.

4.4 ***RyR2 heterogeneity in Ca^{2+} and Mg^{2+} regulation.*** There was no significant difference between individual healthy hearts in average RyR2 Ca^{2+} regulation (Fig. 1C & 2B), suggesting that RyR2 from these hearts are representative of healthy hearts in general. Among the ICM hearts there was an increased heterogeneity between hearts in the mean activity with end-distolic [Ca^{2+}] Fig. 1C) even though all ICM RyR2s were more sensitive to Ca^{2+} activation than those from healthy hearts (Fig. 2B). The increased heterogeneity may be due to heart to heart variations in the progression of the myopathy.

We found that even within a given heart sample, individual RyR2 activity was heterogeneous (Figs.1C, 3D-F), consistent with other studies of RyR2 from human [14] and sheep [52, 53]. The P_o distributions are consistent with three populations of RyR2, a low and intermediate P_o group that predominates in healthy hearts and aberrant high P_o RyR2 that are more strongly represented in failing hearts. RyRs from the aberrant group were less regulated by luminal and cytoplasmic Ca^{2+}, showing substantial activity at <10 nM Ca^{2+} (Fig. 3). They were also less inhibited by cytoplasmic Mg^{2+} and tended to be less inhibited by luminal Mg^{2+} (Fig. 4). The proportions of RyR2s associated with normal and aberrant groups are similar to that reported previously in failing human and dog hearts [15] where, under diastolic conditions, 95% of RyR2 from healthy hearts were in the low activity group compared to only 30% of RyR2 from failing hearts.

The reason for the heterogeneity may be due to different degrees of modification of individual channels by phosphorylation [15, 54, 55], *S*-oxidation [56], *S*-nitrosylation and *S*-glutathionylation [57], all of which alter RyR2 activity. Moreover, individual RyR2 macromolecular complexes may differ in their complement of co-proteins that would also contribute to functional heterogeneity (*e.g.* the sub-stoichiometric FKBP: RyR2 ratio in various species [40, 51]). Alternatively, heterogeneity may reflect localised structural remodelling of the SR and t-tubules. Confocal images of failing cardiomyocytes identify localised remodelling of the SR and t-tubules and loss of dyad junctions in heart failure [58]. These regions probably correspond to regions where increased Ca^{2+} activation of proteases (*e.g.* calpain) has degraded dyadic proteins including junctophilin-2 [59] and RyR2 [60]. It is possible that the aberrant group of RyR2 seen in our study came from these regions.

4.5 ***RyR2 from the cystic fibrosis heart*** had higher end diastolic activity than RyR2 from healthy hearts (Supplementary Fig. 2) but were regulated normally by Ca^{2+} and Mg^{2+}. It is curious that cystic fibrosis elicited some changes in RyR2 function which mimicked some changes in the failing hearts, even though the levels of RyR2 phosphorylation, thiol modification and association of

FKBPs, PP1 and PP2a with RyR2 were not different from healthy heart. This might be because RyR2 in CF have undergone alterations that increase their end diastolic activity as a consequence of pulmonary or electrophysiological stress, but these changes are at an early stage of pathological progression, where the nature of the RyR2 remodelling is not yet defined, and where Ca^{2+} regulation is unchanged. Such changes in the levels of RyR2 phosphorylation, thiol modification and association of FKBPs, PP1 and PP2a with RyR2 we observe in heart failure are down-stream from changes in RyR2 end diastolic activity but not necessarily downstream from changes in Ca^{2+} regulation. However, we cannot rule out other contributing factors that would cause activation of RyR2 in the CF heart.

5.0 CONCLUSION

Human heart failure is associated with increased RyR2 activity at end-diastolic Ca^{2+}, increased sensitivity to activation by cytoplasmic Ca^{2+} and decreased inhibition by cytoplasmic Mg^{2+} that correlates strongly with hyperphosphorylation at S^{2808} and S^{2814} and to excess thiol modification. In failing hearts ~20% of RyR2 were found to be unregulated by intracellular Ca^{2+} and Mg^{2+} such that they would be nearly maximally active under diastolic conditions. These changes, along with RyR2 thiol modification and reduction in binding of both FKBP isoforms to the channel are common to the various aetiologies of heart failure seen here (ICM and EDMD) and elsewhere (Idiopathic Dilated Cardiomyopathy [15]) and would contribute to the diastolic leak phenotype leading to reduced systolic contraction and arrhythmia in heart failure.

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DISCLOSURES

None

FIGURE CAPTIONS

Figure 1. Single channel properties of RyR2s from healthy human heart and hearts with ischemic cardiomyopathy (ICM). Representative traces of RyR2 from healthy hearts **(A)** and hearts with ischemic cardiomyopathy (ICM) **(B)** illustrating individual RyR2 with a range of observed P_o. Channel recordings were at -40 mV, with 0.1 μM cytoplasmic Ca^{2+}, 0.1 mM luminal Ca^{2+} and 2 mM cytoplasmic ATP. Channel openings are downward transitions from the baseline (solid arrowhead). (**C)** The open probabilities *(P_o)* recorded from individual RyR2 channels from four healthy hearts and 5 hearts with ICM shown along with their means ± SEM (red). **(D)** Histograms of individual RyR2 P_o, values grouped from all healthy (blue) and ICM hearts (red). **(E)** Mean P_o of RyR2 recorded from a healthy and an ICM heart (as controls) are compared with those obtained in a separate group of RyR2 that were dephosphorylated by incubating with PP1 (PP1) or reduced by incubating with 5 mM DTT (DTT) prior to single channel recording. Numbers within bears indicate the number of RyR2 in each group. Asterisks denote a significant difference compared with the healthy heart (*** $p<0.001$, ns = not significant).

Figure 2. Regulation of RyR2 P_o by cytoplasmic Ca^{2+}. (A) [Ca^{2+}]-dependencies of RyR2 P_o grouped from four healthy hearts (H) and five hearts with ischemic cardiomyopathy (ICM) under conditions in Figure 1.The number of experiments and Hill parameters are listed in Supplementary Table 1. The [Ca^{2+}] for half-maximal activation (K_a) determined from Hill fits to the data are 3.05 ± 0.8 μM for healthy (○) and 1.3 ± 0.1 μM for ICM (●, $p<0.05$ for difference by t-test). ▲ denotes an ICM subgroup of four intermediate and high activity RyR2 that had P_o between 0.2 and 0.6 at 100 nM cytoplasmic Ca^{2+}. (** $p<0.01$ different to healthy). **(B)** Values of K_a for RyR2 grouped according to individual heart samples. The individual [Ca^{2+}]-dependencies of P_o, number of experiments and Hill parameters are listed in Supplementary Table 1 (* $p<0.05$ difference in the means of each group).

Figure 3. Regulation of RyR2 by luminal Ca^{2+}. (A) P_o in presence of 1 mM luminal Ca^{2+} relative to that in the absence of Ca^{2+}. Red symbols indicate geometric mean ± SEM. The arrow indicates RyR2 with no significant response to luminal Ca^{2+}. **(B)** Luminal [Ca^{2+}]-dependencies of P_o in the presence of 1 mM Mg^{2+} under conditions given in Figure 1 (mean ± SEM n=3-6 or ± standard deviation for n=2). Data from RyR2 with P_o in the high activity group are shown separately (ICM high P_o, n=1-3). Asterisks denote a significant difference compared with the healthy heart using a two-sided Wilcoxon rank sum test (* $p<0.05$, ** $p<0.01$).

Figure 4. Inhibition of RyR2s cytoplasmic and luminal Mg^{2+}. (A) Single RyR2 channel recordings in the presence/ absence of 0.24 mM cytoplasmic Mg^{2+}(1 mM $MgCl_2$ and 2 mM ATP, conditions given in Figure 1). Channel openings are downward from the baseline (arrows). **(B)** Average P_o in presence of 0.24 mM cytoplasmic Mg^{2+} relative to that in the absence of Mg^{2+}. **(C)** Average P_o in presence of 1mM luminal Mg^{2+} relative to that in the absence of Mg^{2+}. Red symbols indicate geometric mean ± SEM. Arrow in C indicate RyRs with no significant response to Mg^{2+}. Asterisks denote a significant difference compared with the healthy heart (** $p<0.01$).

Figure 5. RyR2s are hyperphosphorylated at residues S^{2808} and S^{2814} in failing human heart. (A-B) Samples (0.5 - 4 μg) of ventricular SR were separated via SDS PAGE and subject to Western Blot. Blots were probed with antibodies to phosphorylated (P) S^{2808} or S^{2814}, stripped and re-probed with anti-RyR2 as a loading control. SR samples four healthy (H1-H4; lanes 1-4), 5 failing (ICM1-ICM5) donor hearts (lanes 5-9). **(C-D)** Average % maximal levels of phosphorylation at S^{2808} (**C**) and S^{2814} (**D)** for all heart samples. % maximal phosphorylation was calculated relative to the maximal phosphorylation achieved by treatment with PKA (**C**) or

CaM+ATP (**D**). Data are mean ± SEM for n= 5-11 blots of each heart; n is listed on each bar (* $p<0.05$; different to healthy heart).

Figure 6. Reduced PP1, PP2a and FKBP association with RyR2 from failing heart. (A) RyR2 Co-IP of samples as in Figure 5. Blots were probed with antibodies to RyR2, PP1, PP2a and to an antibody that detects FKBP12.6 and 12.0, but with different affinities. **(B-C)** Relative association of PP1 and PP2a **(B)** and FKBP12.6 and FKBP12.0 **(C)** with RyR2. Band densities of target proteins were normalized to RyR2 in each lane and expressed relative to normalized band densities of healthy heart sample H1. Data are mean ± SEM for n= 6-8 blots of each heart; n is listed on each bar (* $p<0.05$; different to healthy heart).

Figure 7. RyR2 thiol content is reduced in failing heart. (A) Identical SR samples as in Figure 5 were coupled with IRDye 680 maleimide and subject to SDS Page. IRDye 680 maleimide staining (top panel) and Sypro Orange dye for total RyR2 protein staining (bottom) of all human heart samples. **(B)** Relative amount of IRdye maleimide 680, indicating the relative level of free thiols within RyR2. Band densities of protein maleimide content were normalized to RyR2 total protein in each lane and expressed relative to normalized band densities of healthy heart sample H1. Data are mean ± SEM for n= 5-11 gels of each heart; n is listed on each bar (* $p<0.05$; different to healthy heart).

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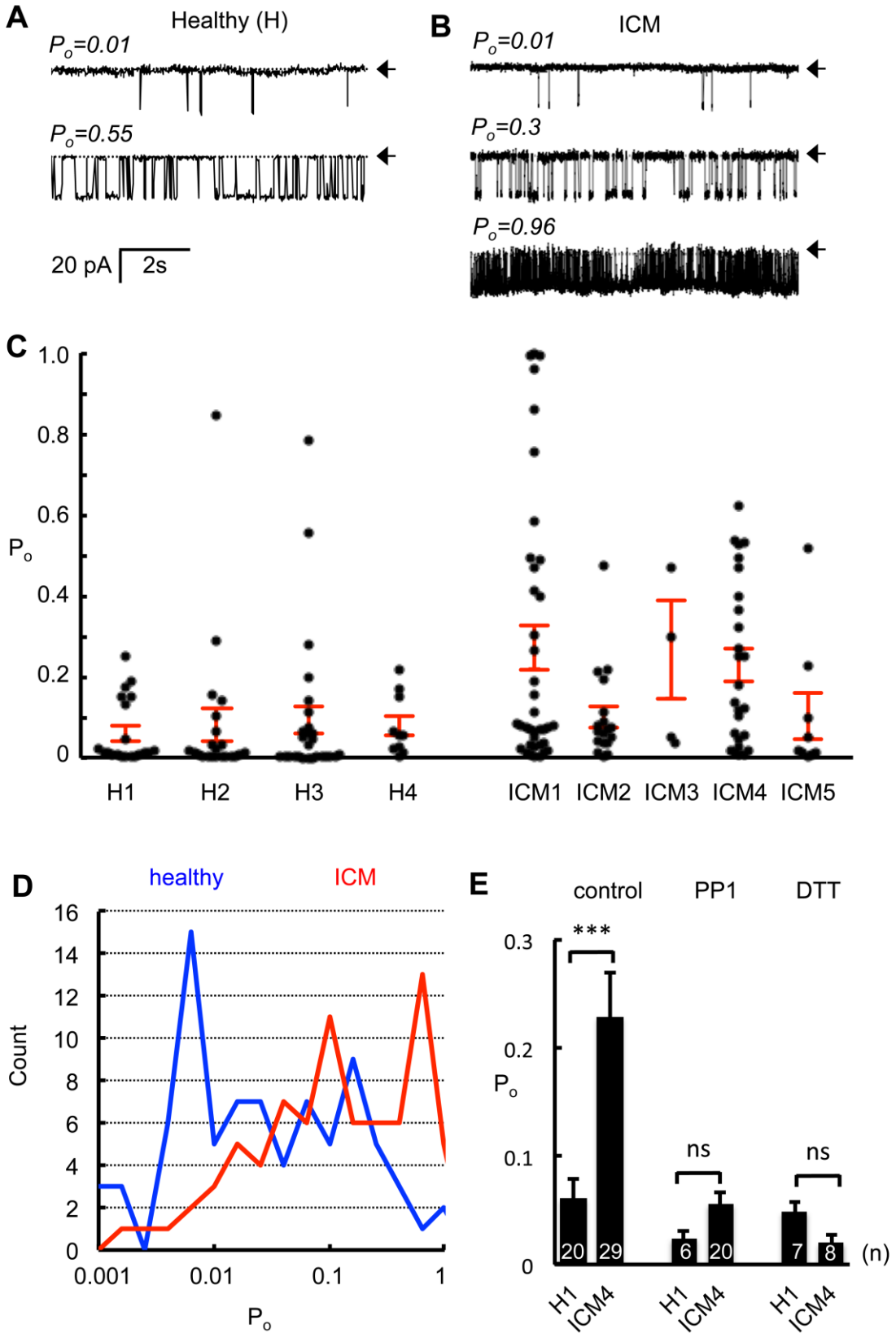

Figure 1

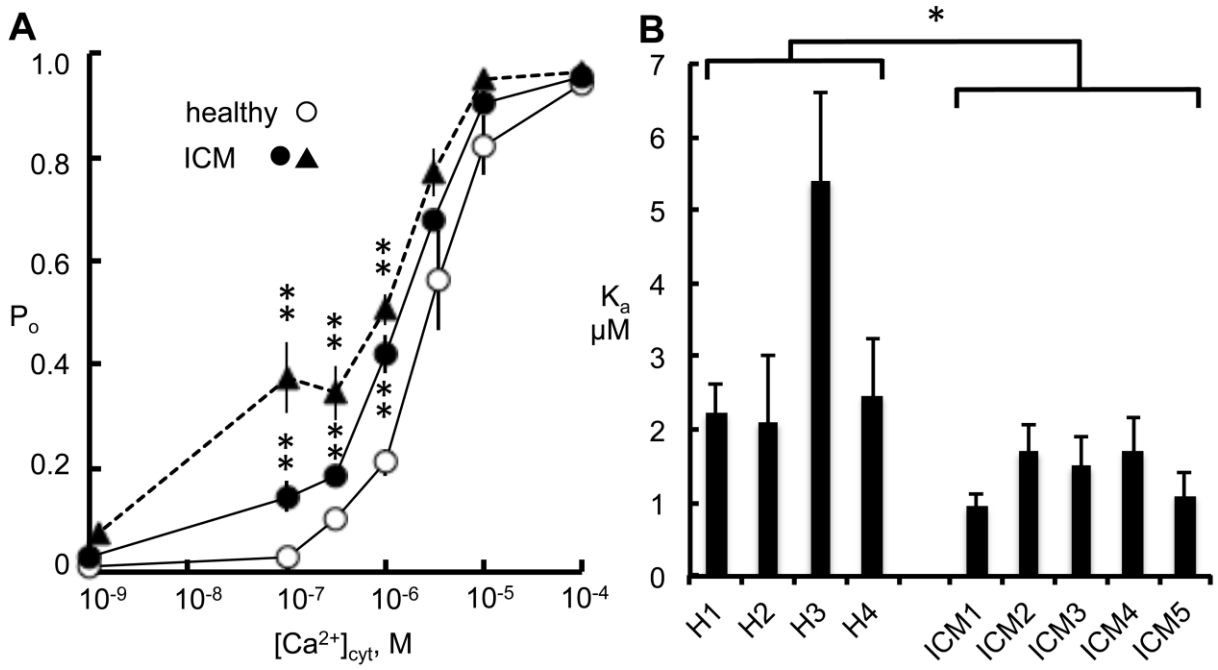

Figure 2

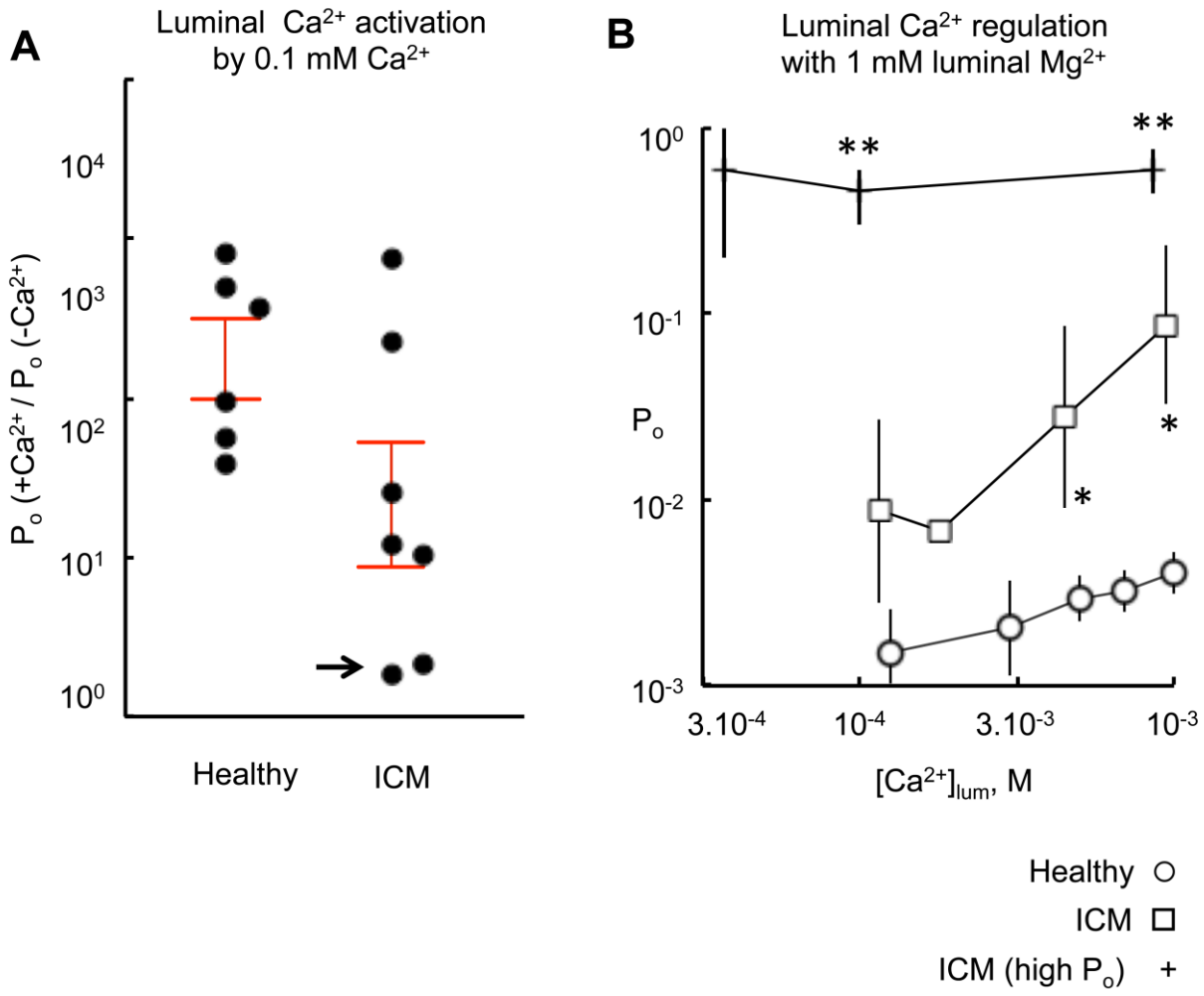

Figure 3

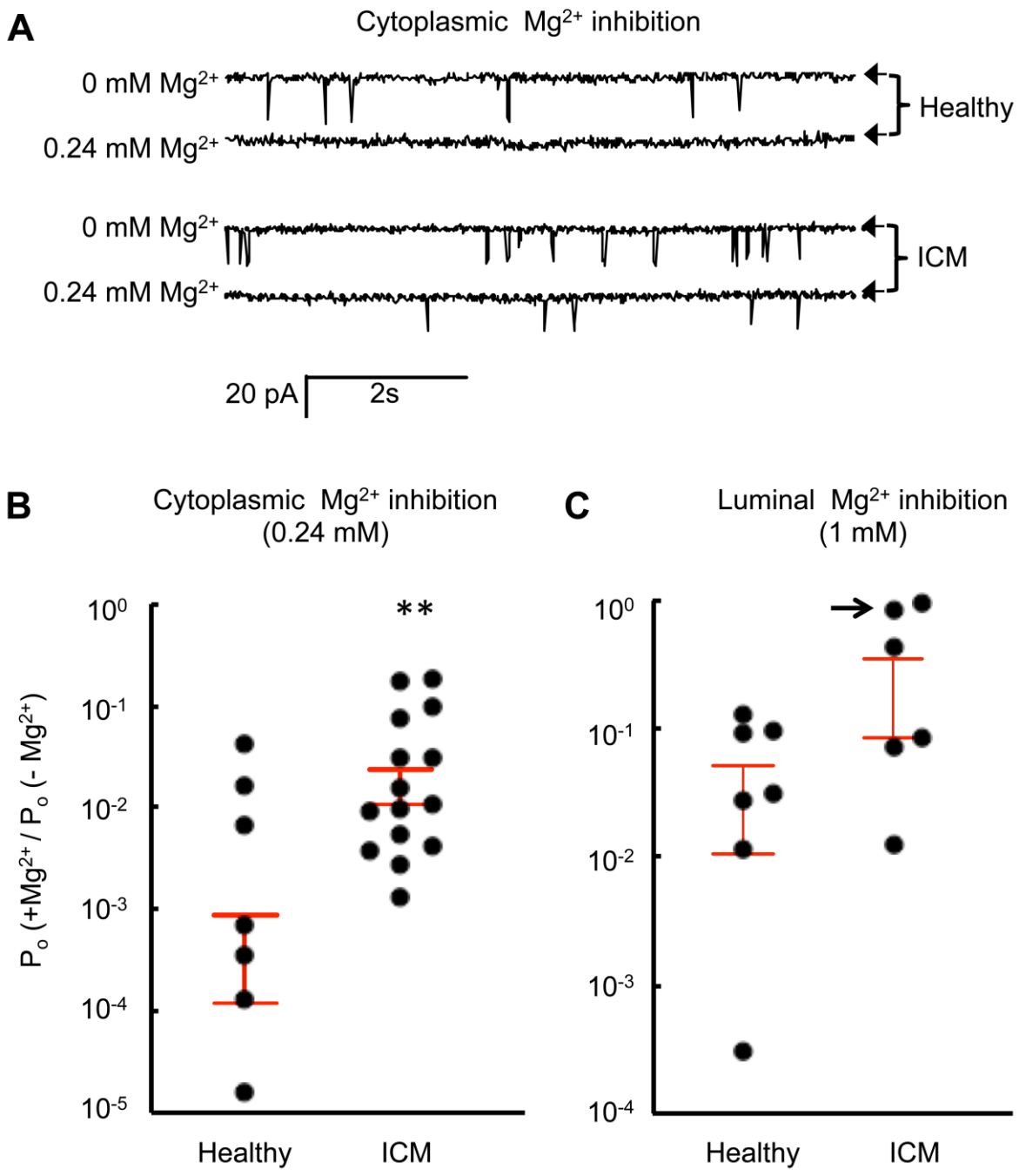

Figure 4

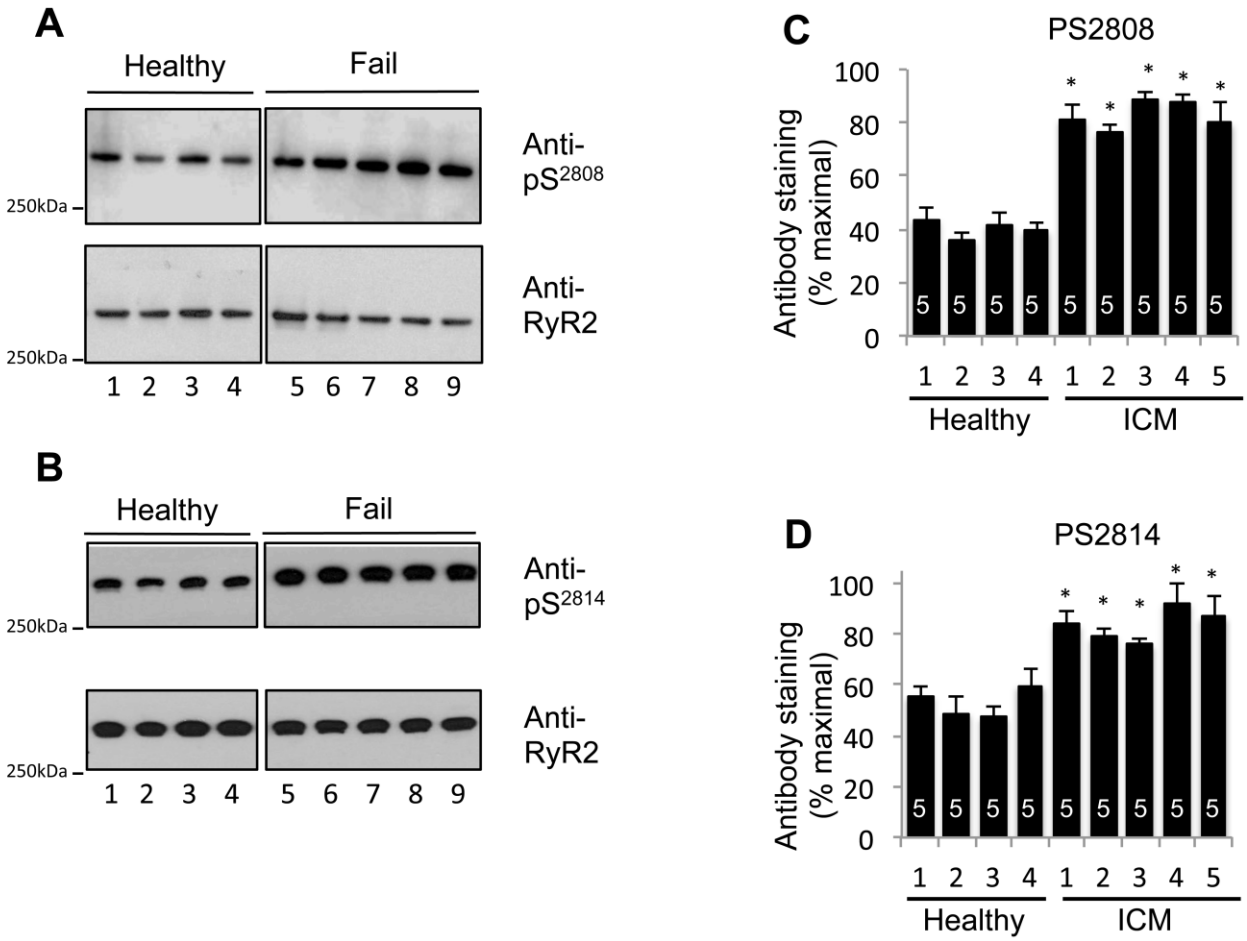

Figure 5

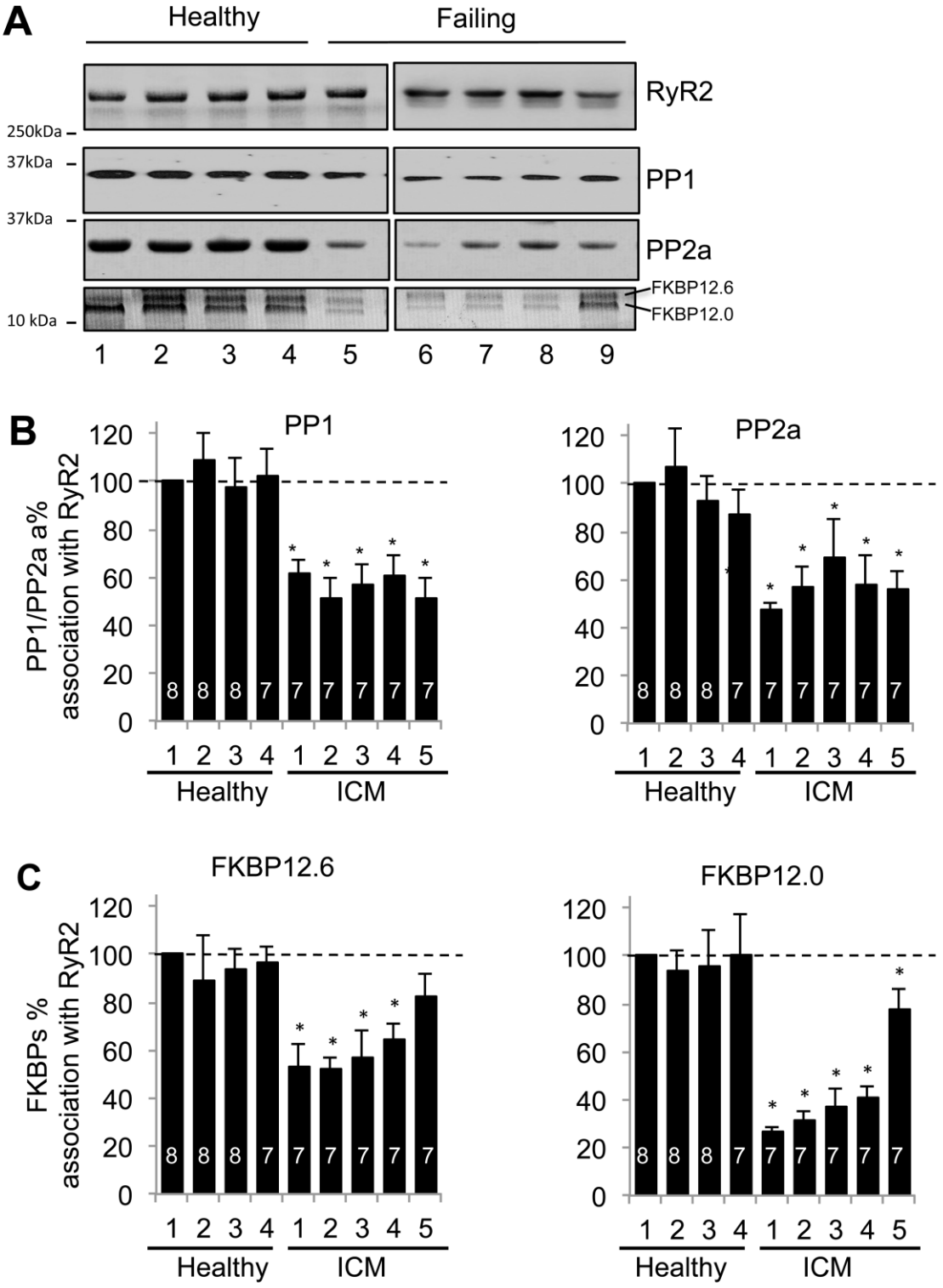

Figure 6

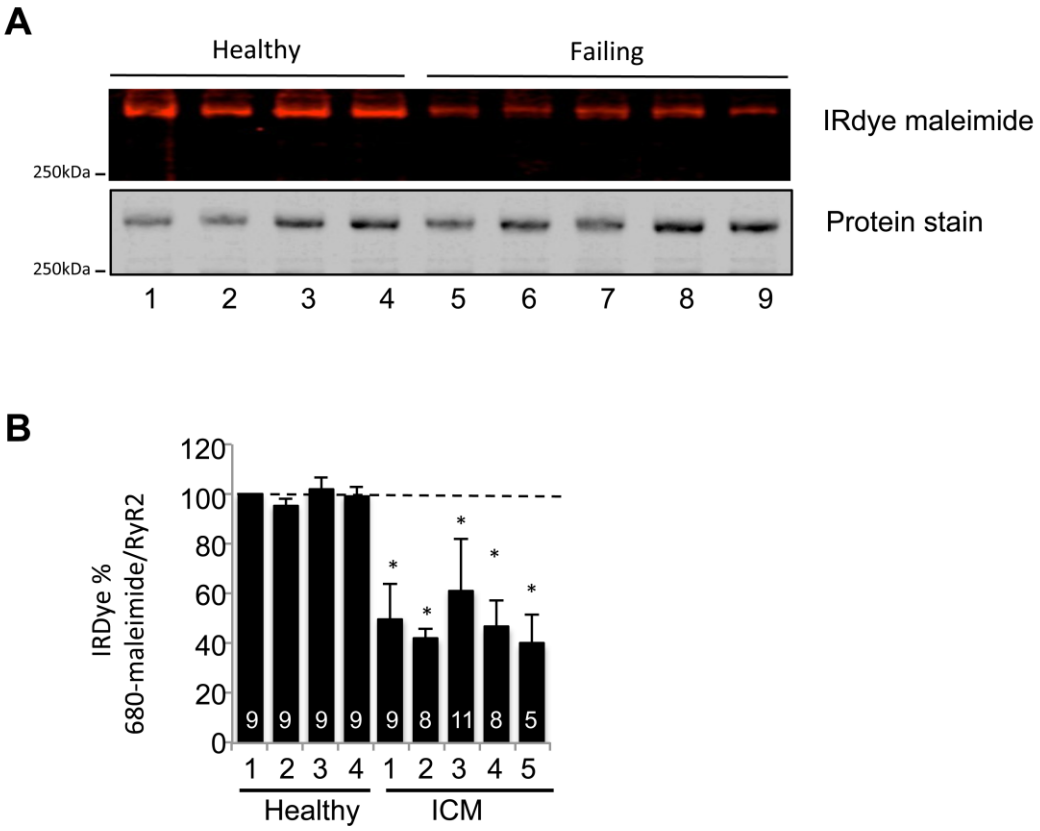

Figure 7

Highlights

- Human failing heart ryanodine receptor 2 Ca^{2+} release channels display an altered regulation to cytoplasmic Ca^{2+} and an increase in end diastolic channel activity.
- Alterations in ryanodine receptor 2 function in failing heart are correlated with channel hyperphosphorylation, enhanced ryanodine receptor 2 thiol modification, and reduced FKBP association.
- Observed changes in ryanodine receptor 2 function and protein modifications would contribute to the diastolic leak phenotype in heat failure.