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Original article

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.

1. 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].

Abbreviations: 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 2a; 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.

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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). 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. Materials and methods

The materials and methods section is found in the Online data supplement.

3. 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+}) (Fig. 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 ($P_o \sim 0.006$) constituting 50% of channels with the remaining channels forming an intermediate activity mode ($P_o \sim 0.1$). 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 and 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 Fig. 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.