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Manipulating RyR2 Ca^{2+} Signalling in Cardiomyocyte Networks using Morpholino Oligonucleotides

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Pharmacologic and gene-based approaches to normalise excitation-contraction coupling (ECC) in heart disease often provoke compensatory adaptations. We proposed that such (mal)adaptation re-configures homeostatic Ca^{2+} signalling network to a new state that may directly contribute to disease pathogenesis (George et al., AJP 2012). We sought to investigate approaches that could modulate disease-linked Ca^{2+} release abnormalities without perturbing homeostatic Ca^{2+} signalling. In this proof-of-concept study we investigated the utility of a morpholino oligonucleotide targeted to exon1 of RyR2 (rMO) to modify Ca^{2+} release in HL-1 cardiomyocyte networks. Imaging of fluorescein-labelled MOs revealed a homogeneous time- and concentration-dependent accumulation of MO in the cytoplasm of >80% HL-1 cardiomyocytes. The efficacy of rMO-mediated protein knock-down was assessed using a construct encoding RyR2 exon 1 (bases 1-25) fused to the N-terminus of eGFP. The use of a control MO (cMO), that has no homology to known mammalian RNAs, and the expression of eGFP minus the RyR2 exon 1 confirmed the specificity of rMO effects. Incubation of HL-1 syncytia with rMO (24-72h, 1-10uM) did not alter RyR2 mRNA abundance and spontaneous Ca^{2+} oscillations and intercellular synchronisation of Ca^{2+} release remained unchanged. Despite near-identical basal Ca^{2+} oscillations in rMO- and cMO-treated HL-1 populations, caffeine-evoked Ca^{2+} transients were modulated by rMO in a concentration- and time-dependent manner. We expanded this study to determine the effects of rMO in human ES-derived cardiomyocytes over a longer time period (21 days). Our data support the development of MO-based methods to modulate discrete aspects of Ca^{2+} signalling in cardiac disease.

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Cardiac Hypertrophy Associated with Impaired Regulation of Type2 Ryanodine Receptor Calcium Channel by Calmodulin and S100A1

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The cardiac ryanodine receptor ion channel (RyR2) is inhibited *in vitro* by calmodulin (CaM). Simultaneous substitution of three amino acid residues in the CaM binding domain (W3587A/L3591D/F3603A, $RyR2^{ADA}$) leads to loss of CaM inhibition at submicromolar (diastolic) and micromolar (systolic) Ca^{2+} concentrations *in vitro* and cardiac hypertrophy and heart failure in mice. To address whether elimination of CaM inhibition at diastolic or systolic Ca^{2+} causes cardiac hypertrophy, a second mutant mouse was prepared with a single amino acid substitution (L3591D, $RyR2^{D}$) in the CaM binding domain. In single channel measurements, $RyR2^{D}$ lost CaM inhibition at diastolic but not systolic Ca^{2+}, and lost inhibition by the small Ca^{2+} binding protein S100A1 at both diastolic and systolic Ca^{2+}. In contrast to $Ryr2^{ADA/ADA}$ mice, $Ryr2^{D/D}$ mice had a normal lifespan and cardiac contractility. In 6-month old $Ryr2^{D/D}$ mice, heart-to-body weight ratio increased by 8% with a two-fold upregulation of atrial natriuretic peptide mRNA levels and a 40% decrease in RyR2 content. Differences between mutant and wild-type mice were more prominent in mice subjected to 4 weeks pressure overload using transverse aortic constriction. The results contrast those of $Ryr2^{ADA/ADA}$ mice that have an impaired cardiac contractile performance and die at ~2 weeks after birth, and suggest that CaM inhibition of RyR2 at systolic Ca^{2+} is important for maintaining normal cardiac function. Supported by NIH, AHA and NSF.

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The CPVT-Associated RyR2 Mutation G230C reduces the Threshold for Store Overload-Induced Ca Release (SOICR)

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Catecholaminergic polymorphic ventricular tachycardia (CPVT) is a life-threatening arrhythmia. Many congenital mutations in both the cardiac ryanodine receptor (RyR2) and calsequestrin (CASQ2) are known to be responsible for this disorder. It is now well established that CPVT is caused by delayed afterdepolarizations (DADs)-induced triggered activities, and that DADs are caused by spontaneous sarcoplasmic reticulum (SR) Ca release during Ca overload, a process also known as store overload induced Ca release (SOICR). A large body of evidence indicates that CPVT-causing RyR2 and CASQ2 mutations enhance the propensity for SOICR and DADs by increasing the response of RyR2 to SR luminal Ca. Recently, Marks and colleagues reported that a CPVT RyR2 mutation G230C increases the cytosolic Ca sensitivity (only after PKA phosphorylation) of single RyR2 channels in lipid bilayers, but has no effect on the luminal Ca sensitivity of the channel. These observations have led to the conclusion that SOICR is not involved in the disease mechanism of the RyR2-G230C mutation. However, the cellular impact of this mutation on SOICR has yet to be determined. To this end, we generated stable, inducible HEK293 cell lines expressing RyR2-WT and the RyR2-G230C mutant. We induced SOICR in these cells by elevating extracellular Ca, and found that the RyR2-G230C mutation markedly enhances the propensity for SOICR. Further, we employed single cell luminal Ca imaging to monitor the luminal Ca dynamics in RyR2-WT- and G230C-expressing cells during store Ca overload. We found that the G230C mutation significantly reduces the luminal Ca level at which spontaneous Ca release occurs (i.e. the SOICR threshold). Therefore, these results and those of previous studies demonstrate that reduced SOICR threshold is a common defect of CPVT-associated RyR2 mutations.

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The L433P Arrhythmia-Linked Mutation Disrupts Amino-Terminus Oligomerisation of the Human Cardiac Ryanodine Receptor

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The type 2 ryanodine receptor (RyR2) mediates calcium release from the sarcoplasmic reticulum of cardiomyocytes. RyR2 mutations found in three clusters including the amino-terminus are associated with arrhythmogenic cardiac disease. Arrhythmia-linked mutations are proposed to disrupt interactions between discrete functional domains within the RyR2 tetramer, resulting in abnormal channel gating. Recently, we presented evidence that the RyR2 N-terminus self-associates into a tetrameric form, which stabilises the closed channel conformation.

Here, we report that the arrhythmia-associated L433P mutation affects oligomerisation of the RyR2 N-terminus. Tetramerisation ability was tested by chemical cross-linking experiments of an RyR2 N-terminal fragment (residues 1-906) containing L433P, expressed in HEK293 cells. The mutant fragment displayed reduced ability for tetramerisation versus wild-type. Two additional techniques, the yeast two-hybrid system and co-immunoprecipitation assays, further indicated that the mutant fragment displays reduced self-interaction and reduced binding to wild-type N-terminus. Notably, dantrolene, a drug used to treat the clinical symptoms of malignant hyperthermia and whose target-binding site lies within the RyR N-terminus, was able to partially restore the tetramer in the L433P mutant.

The effect of the L433P mutation was also investigated in the context of the full-length channel. We performed sucrose density gradient centrifugation of CHAPS-solubilised RyR2 to assess the relative stability of tetrameric functional channels. We found that wild-type RyR2 remains a tetramer consistent with an intact functional channel, whereas a substantial proportion of RyR2/L433P channels is dissociated into monomers.

Our findings suggest that disruption of inter-subunit interactions within the N-terminus of mutant RyR2 might contribute to the mechanism by which some of the arrhythmia-associated mutations result in RyR2 channel dysfunction.

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ATP and Caffeine Binding to the Human RyR2 Central Domain Encompassing a CPVT Mutation Cluster

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The sarcoplasmic reticulum (SR) of cardiomyocytes contains the cardiac ryanodine receptor (RyR2) - a calcium release channel that plays a pivotal role in mediating the SR calcium release essential for cardiac excitation-contraction coupling. Discrete clusters of point mutations, that may comprise important regulatory regions within RyR2, have been associated with catecholaminergic polymorphic ventricular tachycardia (CPVT). In response to a physiological trigger, RyR2 bearing CPVT mutations are believed to cause diastolic calcium leak, which can result in a fatal arrhythmia. A RyR2 central domain, which encompasses both a CPVT-associated region and a predicted ATP binding motif, has been expressed as a soluble recombinant protein. The wild-type protein was compared with three constructs each containing a different CPVT mutation (P2328S, F2331S and N2368I). ATP is a physiological activator of RyR2 activity and caffeine has a similar effect. Fluorescence spectroscopy was used to record changes in the intrinsic fluorescence of the single tryptophan residue present within the expressed domain, and we found