to sham operated animals of the same genotype (SH). Contraction of enzymatically isolated left ventricular myocytes was measured during electrical field stimulation; membrane currents and $[Ca^{2+}]_i$ were measured under whole-cell patch clamp, with Fluo-3 as Ca^{2+} indicator, all at 35°C. Data are shown as mean ± SEM. **Results.** In WT, TAC induced an increase in cell width (from 28 ± 0.7μm in SH to 30 ± 0.9μm in TAC, P<0.05), but not in cell length. This increase was prevented in TG mice (28 ± 0.7μμ ιν ΣΗ *νσ*. 28 ± 0.8μμ ιν TAX). At 1 Hz, unloaded cell shortening amplitude was not altered with TAC in both genotypes. However, the prolongation with TAC of time to peak (to 63 ± 3ms *vs*. 49 ± 2ms, P<0.05) and half-time relaxation (to 98 ± 8ms vs. 77 ± 3ms, P<0.05) in WT was less in TG (to 58 ± 4ms *vs*. 55 ± 4ms for time to peak and 91 ± 6ms *vs*. 84 ± 6ms for half-time relaxation). $[Ca^{2+}]_i$ transient amplitude, L-type Ca^{2+} current density and SR Ca^{2+} content were unchanged with TAC in both genotypes. RyR spontaneous activity (sparks) increased with frequency in SH animals. This frequency effect was reduced by TAC in WT but not in TG animals (P<0.05). **Conclusion**. FKBP12.6 overexpression reduces cellular hypertrophy and blunts functional remodeling in the setting of pressure-overload.

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FKBP12.6 Binding Characteristics of Ryanodine Receptor Mutations Associated with Arrhythmogenic Cardiac Disease

Spyros Zissimopoulos, N. Lowri Thomas, F. Anthony Lai.
Wales Heart Research Institute, Cardiff, United Kingdom.

The cardiac muscle ryanodine receptor-calcium release channel (RyR2) and its interaction with an accessory protein, FK506-binding protein (FKBP12.6), have been implicated in the molecular pathogenesis of acquired and inherited cardiac disease (e.g. catecholaminergic polymorphic ventricular tachycardia; CPVT). We have assessed the FKBP12.6 binding characteristics of recombinant wild-type and CPVT mutant RyR2 channels under basal and oxidising conditions. We find that the R176Q and S2246L mutations exhibit an increased FKBP12.6 binding by ~40% and ~20% respectively, whereas R4497C was similar to wild-type. The redox sensitivity of the FKBP12.6 interaction with the mutant RyR2 channels was very similar to wild-type. The oxidising reagents H2O2 and diamide resulted in reduced FKBP12.6 binding by ~10% and by ~50%, respectively, compared to the untreated state for all four RyR2 proteins. These results suggest that the FKBP12.6 regulation of RyR2 is unlikely to be defective in inherited arrhythmogenic cardiac disease.

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Impact of RyR2 Mutation Responsible for Cathecolaminergic Polymorphic Ventricular Tachycardia (CPTV) on the Short Term Interval-Force Relationship of Atrial and Ventricular Myocardium

Cecilia Ferrantini[1], Raffaele Coppini[1], Beatrice Scellini[1], Alexandra Belus[1], Chiara Tesi[1], Barbara Colombi[2], Elisabetta Cerbai[1], Carlo Napolitano[2], Silvia Priori[2], Corrado Poggesi[1].
[1]University of Florence, Florence, Italy, [2]University of Pavia, Pavia, Italy.

Mutant RyR2 responsible for CPVT can trigger on stress and exercise catecholaminergic induced-ventricular arrhythmias and sudden death. Defective RyR2, ubiquitously expressed in the heart, is responsible for sarcoplasmic reticulum dysfunction that may lead to atrial and ventricular contractile changes also detectable under basal conditions. Left atrial and right ventricular trabeculae were dissected from control (WT) and heterozygous mice (HE) carrying one of the most common CPVT-related RyR2 mutations (R4496C). Preparations underwent various stimulation protocols, including pause-delayed and premature stimuli while isometric tension was recorded. For intracellular Ca^{2+} and action potential recordings ventricular and atrial cardiomyocytes were isolated. In HE myocytes maximum post-rest increase in the amplitude of intracellular Ca^{2+}transients was reduced compared to WT. Maximum post-rest potentiation of isometric tension was lower in HE than in WT trabeculae (e.g.228 ± 37% vs.386 ± 71%, p<0.05, in atrial trabeculae) and was reached at shorter rest-intervals in HE preparations. Steady-state frequency and isoproterenol-induced positive inotropic responses were less pronounced in HE vs. WT trabeculae. These results seem consistent with the ''leaky phenotype'' of the mutant RyR2. Electrical refractoriness was the same in HE and WT myocytes but in HE trabeculae mechanical refractoriness was shorter and restitution of isometric tension was significantly faster than in WT. The rate constant of mechanical restitution at 1 Hz basal frequency was 10.07 ± 1.40 s^{-1} in HE atrial trabeculae vs. 5.28 ± 1.25 s^{-1} in WT and 3.68 ± 0.37 s^{-1} in HE ventricular trabeculae vs. 1.49 ± 0.99 s^{-1} in WT (p<0.05). Isoproterenol accelerated mechanical restitution of all types of trabeculae but did not abolish the difference between HE and WT preparations. A faster recovery from refractoriness of the mutant RyR2 may be responsible for these findings. Supported by Telethon-Italy GGP06007.

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An L433P Arrhythmia-linked Mutation In RyR2 Uncouples Agonist-evoked Ca^{2+} Release From Homeostatic Ca^{2+} Cycling

Aaron I. Clack, Debra L. Fry, N. Lowri Thomas, F. Anthony Lai, Christopher H. George.
Wales Heart Research Institute, Cardiff University, Cardiff, United Kingdom.

A common RyR2 polymorphism (G1885E) modulates the caffeine-sensitivity of an arrhythmogenic RyR2 mutation (L433P). However, despite the markedly different caffeine sensitivities that characterised homotetrameric channels formed from mutant subunits (L433P) or subunits in which the mutation was complemented *in cis* with the polymorphism (L433P/G1885E), cells expressing these channels exhibited equivalent reductions in the propensity for spontaneous intracellular Ca^{2+} oscillations under non-stimulated conditions. This finding suggested that a common mechanistic basis of perturbed basal Ca^{2+} handling may exist. We explored whether other factors, in addition to altered agonist sensitivity, contributed to the altered homeostatic Ca^{2+} signalling in these cells. We analysed the relationships between the amplitude and kinetic profiles of caffeine-evoked Ca^{2+} responses and multiple indices of basal Ca^{2+} homeostasis including mean Ca^{2+} levels, propensity for spontaneous oscillation and Ca^{2+} signal noise. Under non-stimulated conditions, intracellular Ca^{2+} signal noise was comparable in cells expressing WT RyR2, L433P, G1885E or L433P/G1885E. The amplitude and temporal characteristics of caffeine-evoked Ca^{2+} release was linked to basal Ca^{2+} cycling in cells expressing WT and G1885E channels. However, in cells expressing L433P the relationships between basal Ca^{2+} cycling and the amplitude and kinetic profiles of sub-maximal caffeine-triggered Ca^{2+} release was significantly altered. Under these conditions, the sequestration mechanisms that underlie the decay kinetics of the Ca^{2+} transients were uncoupled from basal Ca^{2+} handling events. *In cis* complementation with the G1885E polymorphism only partially reversed this phenomenon. Our data suggest that arrhythmogenic RyR2 mutations may perturb the regulatory link between homeostatic Ca^{2+} cycling events in non-stimulated cells and the cellular Ca^{2+} response following agonist stimulation. This functional uncoupling, that was a feature of channels exhibiting very different caffeine sensitivities, may be an important mechanistic defect that contributes to dysfunctional Ca^{2+} release in arrhythmogenesis.

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A G1885E RyR2 Polymorphism Modulates The Caffeine Sensitivity Of An Arrhythmia-linked Mutation

Aaron I. Clack, N. Lowri Thomas, Christopher H. George, F. Anthony Lai.
Wales Heart Research Institute, Cardiff University, Cardiff, United Kingdom.

More than 70 arrhythmia-linked mutations have been identified in the cardiac ryanodine receptor (RyR2). The observation that different mutations underlie similar clinical phenotypes supports the concept of a common mechanism of mutant channel dysfunction. However, RyR2 mutations are not exclusively 'gain-of-function' and some mutations may underpin different arrhythmogenic mechanisms. Additional mechanistic complexity may be conferred by the putative role of sequence polymorphisms in modulating the clinical severity of arrhythmias arising from ion channelopathies. A common RyR2 polymorphism (G1885E), that occurs at a frequency of approximately 6% in normal populations, is found on the same allele (in cis) as RyR2 mutations in symptomatic families (Tiso et al., (2001) Hum. Mol. Genet. 10 189–194). We investigated the modulatory effects of the G1885E polymorphism on human wild-type (WT) and L433P mutant channel function. eGFP-tagged WT RyR2, or RyR2 containing the L433P mutation, G1885E polymorphism or L433P and G1885E in cis (L433P/G1885E) were expressed in HEK293 cells. Consistent with previous findings (Thomas et al., (2005)), L433P channels exhibited decreased sensitivity to caffeine-evoked Ca^{2+} release although maximal responses were comparable to WT. Despite a trend toward increased caffeine sensitivity, G1885E channels were similar to WT. Unexpectedly, complementation of L433P in cis with G1885E reversed the right-shifted dose response characteristic of L433P channels and produced channels that exhibited significantly increased caffeine sensitivity when compared to WT RyR2. Despite their markedly different sensitivities to caffeine, cells expressing L433P or L433P/G1885E exhibited a reduced propensity of spontaneous Ca^{2+} oscillations under non-stimulated conditions. The mechanistic basis of these phenomena has been explored. We have extended the study to examine the functional consequences of L433P and G1885E complementation in trans. This data provides the first evidence that RyR2 polymorphisms may be a critical determinant of mutant channel dysfunction.

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Decoding The Molecular Basis Of Anti-Apoptotic Cardiac Ca^{2+} Signalling Via Human RyR2 Splice Variants

Wai Yin Yeung, Matthew Davies, F. Anthony Lai, Alan J. Williams, Christopher H. George.
Wales Heart Research Institute, Cardiff University, Cardiff, United Kingdom.

Apoptotic cell death is involved in normal and pathologic cardiac function. Apoptosis is pivotal in normal developmental cardiogenesis and yet unregulated cardiomyocyte loss via apoptosis contributes to cardiac degeneration in heart failure. There is emerging evidence that distinct populations of cardiomyocytes may exhibit differential susceptibility to apoptosis. We showed that alternative splicing of the cardiac ryanodine receptor (RyR2) modulated the susceptibility of cardiomyocytes to Ca^{2+}-linked apoptosis (George et al., (2007)). Specifically, a 24bp splice variant (encoding VTGSQRSK inserted distal to residue 3715 in human RyR2) suppressed homeostatic and agonist-evoked Ca^{2+} fluxes in cardiomyocytes. We proposed that alternative splicing of RyR2 may represent a mechanism for 'tuning' pro- and anti-apoptotic Ca^{2+} fluxes. Our data supports the concept that the 24bp splice insertion stabilises RyR2-mediated Ca^{2+} release, and we tested this hypothesis using single channel and cellular approaches. In single channel experiments using CHAPS-solubilised, sucrose gradient-enriched preparations incorporated into PE bilayers, we investigated the effects of synthetic peptides containing VTGSQRSK on the Ca^{2+} sensitivity of recombinant human RyR2 channels devoid of alternatively spliced insertions ($RyR2^{-/-}$). In a complementary approach we used confocal imaging to determine the effects of these peptides on Ca^{2+} handling in living cardiomyocytes. Peptides were injected into the nucleus and cytoplasm of synchronously-coupled HL-1 cells (that exclusively express $RyR2^{-/-}$) and cellular Ca^{2+} fluxes were analysed using the multi-parametric **S**ynchronicity-**A**mplitude-**L**ength and **V**ariability of **O**scillation (SALVO) program that describes the 'contractile' and 'non-contractile' aspects of cellular Ca^{2+} handling. In both approaches, peptides encoding a splice insertion that did not protect cells from Ca^{2+}-linked apoptosis (FAIDSLCGFG), or scrambled sequences were used as controls. Our data shows the utility of these complementary approaches in determining the mechanistic basis of altered cellular Ca^{2+} handling mediated by alternative splicing of RyR2.

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Use Of *Shaker* B K^+ Channel NH_2-inactivation Peptides To Probe The Ryanodine Receptor Ca^{2+} Release Channel Pore

Cedric Viero[1], Jo Carney[1], Sammy Mason[1], Mark Bannister[2], S R. Chen[3], Alan J. Williams[1].
[1]Wales Heart Research Institute, Cardiff, United Kingdom, [2]Boston Biomedical Research Institute, Watertown, MA, USA, [3]Department of Physiology and Biophysics, Libin Cardiovascular Institute of Alberta, University of Calgary, Calgary, AB, Canada.

Inactivation of K^+ channels occurs by the interaction of the pore with NH_2-terminal sequences. Synthetic peptides corresponding to these sequences were applied to the purified mouse cardiac ryanodine receptor type 2 (RyR2) and single channel activity was recorded in planar lipid bilayers. The wild type (WT) *Shaker* B NH_2-peptide MAAVAGLYGLGEDRQHRKKQ induced a block of the open RyR2 channel in a concentration and voltage-dependent manner, but also when the channel displayed a ryanodine-modified state [Mead *et al.*, J. Membrane Biol., 1998]. In the latter condition, in a 200 mM KCl buffer when the holding potential was at +50 mV, the open probability decreased by a factor of 2 ($p=0.001$, $n=10$ channels for control and $n=5$ for WT peptide) mainly due to a 4-fold reduction ($p<0.01$, $n=10$ channels for control and $n=5$ for WT peptide) of the mean open time in the presence of 20 μM WT peptide. It is noteworthy that the peptide at 20 μM had no effect in a 600 mM KCl buffer, suggesting the importance of charged residues in the blocking effect on RyR2. Furthermore we designed a mutant peptide where the amino acid Alanine in positions 3 and 5 was replaced by Glutamine residues, giving a less hydrophobic feature to the peptide. The mutant peptide at 20 μM in a 200 mM KCl buffer was less effective than the WT peptide, while showing a different mechanism of action. These findings are consistent with the idea that RyR2 contains well organised charged and hydrophobic residues in its conduction pathway and that they may play a general role in the ion translocation mechanisms of the sarcoplasmic reticulum Ca^{2+} release channel.

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Imperatoxin A, A Calcin Toxin From *Pandinus Imperator* Scorpions, Ablates Calcium Sparks In Permeabilized Cells

Erin M. Capes, Hector H. Valdivia.
University of Wisconsin-Madison, Madison, WI, USA.

Imperatoxin A (IpTxa) is a 33 amino acid peptide toxin from the African scorpion *Pandinus imperator*. Its definitive structural and functional characteristics designate it as a member of the calcin family, a unique group of peptide toxins, which to date includes Hemicalcin (HCa), Hadrucalcin (HdCa), IpTxa, Maurocalcin (MCa), and Opicalcins 1 & 2 (Opi 1 & 2). Calcins are unified by 1) a compact, globular structure containing an inhibitor cystine knot, 2) positive net charge, 3) the ability to activate ryanodine receptors (RyR) with high affinity and selectivity, and 4) presumed or confirmed cell-penetrating capabilities. Previous studies have demonstrated the ability of MCa to carry a fluorescent cargo to the interior of intact cells, and we have used a similar method to confirm that IpTxa is also a cell-penetrator. In addition, we have demonstrated IpTxa's ability to transiently enhance calcium release from the sarcoplasmic reticulum (SR) of intact cells. In the current study, we used confocal microscopy to explore the effects of IpTxa on saponin-permeabilized cells. Perfusion of 10nM IpTxa on cells resulted in a dramatic reduction of sparks to as low as 1% of control within two minutes. This effect was even more pronounced and rapid in the presence of 50nM toxin, with complete ablation of sparks in less than 1 minute. At the same time, mean fluorescence is often increased in our scans relative to control, although rapid application of caffeine reveals that SR calcium content is retained. Our results suggest that IpTxa depletes calcium from the SR, a hypothesis that would explain not only the ablation of sparks, but also the transient nature of enhanced calcium release in intact cells observed upon perfusion with IpTxa.

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Block Of Mouse Cardiac Ryanodine Receptor (mRyR2) By hERG Blocking Agents

Sammy A. Mason[1], Wayne S.R. Chen[2], Alan J. Williams[1].
[1]Wales Heart Research Institute, Cardiff, United Kingdom, [2]Libin Cardiovascular Institutes of Alberta, Calgary, AB, Canada.

This study investigates the novel block from the cytosolic face of mRyR2 by three hERG blocking agents. For this purpose, astemizole (A), terfenadine (T) and chloroquine (C) were investigated based on their different affinities of hERG block (IC_{50} = 0.9 nM, 56–204 nM and 2500 nM) and differences in their proposed mechanisms of action at the cytosolic face of ion channels. As well as exhibiting high affinity block of hERG, these blockers exhibit half maximal block of multiple ion channel types at concentrations exceeding 1×10^{-6} M. Whilst mRyR2 lacks the key residues (Y652 and F656) involved in high affinity block of hERG it does possess a wide pore containing a high density of hydrophobic residues. Therefore the potential exists for mRyR2, like hERG, to be blocked by a diverse range of compounds with high affinity. The aim of this study is to examine the properties/mechanisms of mRyR2 block to identify areas within mRyR2 that may be involved in drug binding. Similar to their action in non-hERG ion channels, these compounds block mRyR2 within the micromolar range (IC_{50}; (A) - 16.12 ± 2.12 μM; (T) - 30.04 ± 4.81 μM and (C) - 17.73 ± 5.14 μM; n = 5) at + 50 mV. Whilst all blockers reduce the single channel current amplitude to ~ 95 % of control levels, astemizole and terfenadine induce additional blocking states, whilst also allowing channel closure during periods of block. Chloroquine exhibits briefer blocking events, indicating a faster off rate and no additional reduced conductance states observed. This work was funded by the British Heart Foundation.

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Cardiac Ca2+ Release Channel/RyR2 -Molecular Mechanism Of Green Tea Extract epigallocatechin-3-gallate

George G. Rodney[1], Elaine Cabrales[2], Isaac N. Pessah[2], **Wei Feng**[2].
[1]University of Maryland School of Nursing, Baltimore, MD, USA, [2]University of California, Davis, Davis, CA, USA.

Consumption of green tea has been found to closely correlate with a lower risk of heart disease and reduced cardiovascular mortality. Extracted from green leaves, a group of catechin polyphenols appears to be responsible for these beneficial cardiovascular effects. Studies have shown that epigallocatechin-3-gallate (EGCG), one of the most abundant and potent catechins, can modulate myocardial contractility through some unknown mechanisms. Recent evidence suggests a role for Na^+/H^+ and Na^+/Ca^{2+} exchangers in the ionotropic effects of EGCG in rat heart. Here we report that cardiac Ca^{2+} release channel - RyR2 serves as a possible molecular target of EGCG that may be responsible for amplified Ca^{2+} transients, enhanced ventricular contractility and increased cell shortening in cardiac myocytes. In single isolated mouse ventricular myocytes EGCG (5μM) increased peak systolic Ca^{2+} transient amplitude, increased decay of the Ca^{2+} transient, increased fractional shortening and increased contraction and relaxation velocities. To assess the direct interaction of EGCG with RyR2, both purified and membrane-bound RyR2 single channels were reconstituted in planar lipid bilayers. In the presence of 0.5μM EGCG, the open probability (Po) increased from Po=0.012 in the control state to Po=0.387. Using [^{3}H]ryanodine as a probe to assess channel conformational states, our data further indicate that (1) EGCG dose-dependently enhanced junctional SR membrane-bound RyR2 channel activity and (2) EGCG increased Ca^{2+}-induced Ca^{2+} activation of RyR2 (EC_{50} = 7.0 and 1.7 μM for control and EGCG, respectively). In summary, our data are consistent with previous reports for positive inotropic and lusitropic effects by EGCG on heart cells. In addition, our data clearly supports a role of EGCG in modulating cardiac E-C coupling through interacting with RyR2 channel complex. Supported by K01 AR51519 to GR and P01 AR52354.