Ryanodine Receptors Type II

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Relocation of Calmodulin in Hypertrophic Neonatal Cardiomyocytes

Jaya Gangopadhyay[1], Tomoyo Hamada[1], Noriaki Ikemoto[1,2].
[1]BBRI, Watertown, MA, USA, [2]Harvard Medical School, Boston, MA, USA.

An increasing body of evidence suggests that calmodulin (CaM) signaling is involved in the regulation of gene expression in cardiac hypertrophy (HT). According to recent report (Yamaguchi *et al*, 2007) mice expressing RyR2 mutant, that cannot bind CaM, develops cardiac HT. In an attempt to gain further insight into the role of RyR2-bound CaM in the process of development of HT, we have induced HT in neonatal rat cardiomyocytes by endothelin-1 (ET-1), and found distinct differences between the hypertrophied cells and control cells in several aspects. (1) The immuno-stain with anti-CaM antibody is chiefly localized in the cytoplasm of the control cells; whereas, in the HT cells, the stain is accumulated chiefly in the nucleus. (2) The cross linking/Western blot analysis shows that CaM is cross-linked with the RyR2 in control cells, but not in the HT cells. (3) We have prepared fluorescently labeled CaM by conjugating C^{34} mutant of CaM with Alexa 546, and introduced it into the myocytes using Bioporter. The exogenously introduced CaM-alexa is localized mainly in the cytoplasmic region in control cells, but within the nucleus in the HT cells. These findings suggest that stimulation of the neonatal cardiomyocytes with ET-1 dissociates RyR2-bound CaM from the receptor, then the dissociated CaM translocates to the nucleus to activate the HT gene program. In support of this conclusion, dantrolene (a specific blocker of RyR Ca^{2+} channel) inhibited HT in the ET-1-treated cells; at the same time prevented CaM translocation to the nucleus. Furthermore, an antibody raised against the CaM binding domain of RyR2, which was introduced into the cell via Bioporter, also prevented HT in the ET-1-treated cells. (Supported by HL072841)

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Endurance Training Abolish Arrhythmogenic Ca2+ Leak In Cardiomyocytes From Mice Over-expressing CaMKII

Tomas O. Stølen[1], Morten A. Høydal[1], Joan H. Brown[2], Lars S. Maier[3], Godfrey L. Smith[4], Ulrik Wisløff[1].
[1]Norwegian University of Science and Technology, Trondheim, Norway, [2]University of California, San Diego, CA, USA, [3]Georg-August-University Goettingen, Goettingen, Germany, [4]University of Glasgow, Glasgow, United Kingdom.

Background: Transgenic (TG) over-expression of calcium/calmodulin-dependent protein kinase IIδC (CaMKIIδC) causes depressed cardiac function, altered Ca2+ handling and increased diastolic sarcoplamic reticulum (SR) Ca2+ leak. The latter may trigger ventricular arrhythmias in heart failure. Aim of the study was to investigate the effects of endurance training on Ca2+ handling and diastolic SR Ca2+ leak in TG mice with over-expression of CaMKIIδC.

Methods: Four CaMKIIδC TG mice underwent high intensity endurance training 5 days per week over 12 weeks and were compared to TG sedentary and wild type (WT). Ca2+ handling, diastolic SR Ca2+ leak, t-tubule density and SR Ca2+ release synchronicity were measured in single cardiomyocytes.

Results: TG mice had depressed cardiomyocyte shortening ($3.3 \pm 1.8\%$ in TG vs. $6.2 \pm 1.2\%$ in WT, $P<0.01$). Ca2+ transient amplitude were lower (Fura-2AM ratio in TG was 0.09 ± 0.03 vs. 0.18 ± 0.02 in WT, $P<0.01$). SR Ca2+ leak over the RyR was significantly larger in TG mice ($19 \pm 3\%$ of total SR Ca2+ vs. $5 \pm 2\%$ in WT, $P<0.01$). Endurance training restored cardiomyocyte shortening ($5.9 \pm 1.3\%$ in TG trained) to WT level. Ca2+ amplitude was also significantly increased (Fura-2AM ratio 0.15 ± 0.02 in TG trained). Endurance training reduced diastolic SR Ca2+ leak to WT levels ($4 \pm 2\%$, $P<0.01$ vs. TG). CaMKIIδC inhibition and not PKA inhibition normalized SR Ca2+ leak in TG and was comparable to TG endurance trained and WT. TG had both reduced t-tubule density ($13 \pm 4\%$ in TG vs. $18 \pm 2\%$ in WT, $P<0.05$) and reduced SR Ca2+ release synchronicity ($P<0.05$), whereas t-tubule density and SR Ca2+ release synchronicity in endurance trained TG were comparable to WT.

Conclusion: Endurance training improved cardiomyocyte function and Ca2+ handling in mice with TG over-expression of CaMKIIδC. Increased diastolic SR Ca2+ leak, t-tubule density and SR Ca2+ release synchronicity was normalized after endurance training.

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Ca^{2+}-calmodulin Increases RyR2 Open Probability Yet Reduces Ryanoid Association With RyR2

Charalambos Sigalas, Belen Bayo-Martin, David Jane, **Rebecca Sitsapesan**.
University of Bristol, Bristol, United Kingdom.

We have previously demonstrated that low, physiological concentrations of Ca^{2+}-calmodulin (Ca^{2+}CaM; 50–100 nM) activate RyR2 channels incorporated into bilayers (Sigalas & Sitsapesan (2007) *Biophys.J.*, 88a). In contrast, we find that Ca^{2+}CaM dose-dependently inhibits [^{3}H]ryanodine binding to cardiac HSR. Since it appears that the [^{3}H]ryanodine binding assay is not accurately reporting the effects of Ca^{2+}CaM on RyR2 Po we have investigated, using the reversible ryanoid, ryanodol, whether Ca^{2+}CaM can directly influence the binding of ryanoid compounds to RyR2 independently of Po. Purified single sheep RyR2 channels were incorporated into artificial membranes under voltage-clamp conditions. In symmetrical solutions containing 250 mM K+, 100 μM free Ca^{2+}, pH 7.2, and 20 mM caffeine to clamp Po to high levels, 1 μM CaM had no significant effect on Po. This agrees with our previous work on native RyR2 channels demonstrating that high levels of CaM can reverse the activating effects of low [Ca^{2+}CaM] but do not significantly lower Po below control values. We find that Ca^{2+}CaM significantly reduces the rate of association of ryanodol to RyR2 but does not affect the rate of dissociation. Ca^{2+}CaM also significantly alters channel behaviour within the ryanodol-modified conductance state. Our results lead to two important conclusions: 1. A Ca^{2+}CaM-induced decrease in the rate of association of ryanoids to RyR2 is consistent with the reduction in [^{3}H]ryanodine binding caused by Ca^{2+}CaM, providing an explanation why the functional effects of Ca^{2+}CaM at the single-channel level are not faithfully mirrored in the [^{3}H]ryanodine binding studies. 2. The important, wider implications of this study are that [^{3}H]ryanodine binding assays, in the absence of other supporting data, may not provide reliable information about the Po of RyR channel populations.

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Phosphorylation Activates RyR2 By Uncoupling The Channel From The Influence Of Cytosolic Ca^{2+} But Also Inhibits RyR2 By A Distinct Mechanism

Simon Carter, Rebecca Sitsapesan.
University of Bristol, Bristol, United Kingdom.

Phosphorylation of RyR2 is generally thought to be associated with an increase in open probability (P_o), however, there is disagreement over which phosphorylation sites are responsible. To investigate the gating mechanisms underlying phosphorylation-induced changes in RyR2 P_o, we incorporated native sheep RyR2 into bilayers under voltage-clamp conditions with luminal Ca^{2+} as the permeant ion. Phosphorylation of RyR2 channels above basal levels, either by PKA or by activation of an endogenous kinase (by incubation with Mg^{2+}ATP) leads to massive increases in P_o that are associated with very long open states. The channels become effectively uncoupled from the influence of cytosolic Ca^{2+} because reductions in cytosolic [Ca^{2+}] to sub-activating levels do not reduce open lifetimes nor significantly reduce P_o. Treatment of channels with the phosphatase, PP1 reverses these gating changes returning the channels to a cytosolic Ca^{2+}-sensitive mode of gating. In contrast, to the gating changes described above which are specific for phosphorylation of RyR2 *above* basal levels, complete dephosphorylation of RyR2 by PP1 treatment causes significant increases in P_o. P_o increased from 0.074 ± 0.032 to 0.218 ± 0.028 (SEM;n=4) after incubation with PP1 (5 units). The channel gating associated with completely dephosphorylated channels, or channels phosphorylated to 75% of maximum at serine-2809, demonstrates that the channels are sensitive to cytosolic [Ca^{2+}]. We cannot yet assign specific phosphorylation sites to particular change in channel gating but it is clear that phosphorylation of RyR2 exerts effects which span the entire range of the RyR2 activity landscape. The huge scope for changing RyR2 channel activity suggests that phosphorylation of RyR2 is important for physiological regulation of the channel and that dysregulation of SR Ca^{2+}-release would be expected with prolonged hyperphosphorylation.

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FKBP12.6 Overexpression Blunts Cardiomyocyte Remodeling After Left-ventricular Pressure-overload

Liesbeth Biesmans[1], Virginie Bito[1], Laurent Vinet[2], Patricia Rouet-Benzineb[2], Jean-Jacques Mercadier[2], Karin Sipido[1].
[1]Catholic University of Leuven, Leuven, Belgium, [2]INSERM U698, Groupe Hospitalier Bichat-Claude Bernard, Paris, France.

Background. Increased open probability of the ryanodine receptor (RyR) has been implicated in the pathophysiology of heart failure. Since association of FK506 binding protein (FKBP12.6) with RyR enhances its closed state we investigated whether FKBP12.6 overexpression could improve cellular remodeling and Ca^{2+} handling in the setting of pressure-overload. **Methods**. We used a mouse model with heart-specific and conditional overexpression of FKBP12.6. Wild-type (WT) mice and mice overexpressing FKBP12.6 (TG) were submitted to 10 weeks transverse aortic constriction (TAC) and compared

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.