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Divergent Effects of Disease-Associated Mutations on Type 2 Ryanodine Receptor Channel

Nagomi Kurebayashi[1], Takashi Murayama[1], Akira Uehara[2], Midori Yasukochi[2], Takashi Sakurai[1].
[1]Dept. of Pharmacology, Juntendo University School of Medicine, Tokyo, Japan, [2]Dept. of Cell Biology, Fukuoka University School of Medicine, Fukuoka, Japan.

Type 2 ryanodine receptor (RyR2) is a Ca^{2+} release channel in the sarcoplasmic reticulum and plays a pivotal role in excitation-contraction coupling in heart. RyR2 is the major target for arrhythmogenic diseases, e.g., catecholaminergic polymorphic ventricular tachycardia (CPVT) and arrhythmogenic right ventricular cardiomyopathy (ARVC). To date, over 150 mutations have been identified in the RyR2 gene of CPVT and ARVC patients. It is widely believed that CPVT and ARVC mutations cause hyperactivation of the Ca^{2+}-induced Ca^{2+} release (CICR), resulting in abnormal Ca^{2+} homeostasis in cardiac muscle. CICR shows biphasic Ca^{2+} dependence against cytoplasmic Ca^{2+}, thus the activity can be determined by three parameters: sensitivity to activating Ca^{2+}, sensitivity to inactivating Ca^{2+}, and the gain (i.e., peak activity). In addition, CICR is also regulated by luminal Ca^{2+}; high luminal Ca^{2+} activates CICR, and vise versa. However, it remains unclear how the disease-associated mutations affect these parameters. In this study, we expressed RyR2 channels carrying several CPVT/ARVC mutations in HEK cells and tested their CICR by live-cell Ca^{2+} imaging and [^{3}H]ryanodine binding. Our results suggest that the disease-associated mutations divergently affects the parameters of CICR depending on the sites for mutation. The underlying molecular mechanism will be discussed.

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Crystal Structure of Cardiac Ryanodine Receptor N-Terminal Region Contains Unique Anion Binding Site Targeted by Disease-Associated Mutations

Lynn Kimlicka[1], Ching-Chieh Tung[1], Anna-Carin C. Carlsson[2], Paolo A. Lobo[1], Zhiguang Yuchi[1], Filip Van Petegem[1].
[1]University of British Columbia, Vancouver, BC, Canada, [2]University of Gothenburg, Gothenburg, Sweden.

Ryanodine receptors (RyRs) are intracellular calcium-release channels of the endo/sarcoplasmic reticulum that are critical to the muscle excitation-contraction coupling. Mutations in the cardiac isoform (RyR2) are linked to life-threatening arrhythmias, such as catecholaminergic polymorphic ventricular tachycardia (CPVT), known for a sudden cardiac arrest. RyR2 is the target for over 150 disease-associated mutations. Here, we present the 2.0Å crystal structure of the N-terminal region of RyR2 (residues 1-547), an area containing 29 distinct disease-associated mutations. The protein folds up in three individual domains similarly to its counterpart in the skeletal muscle, RyR1, but contains a unique central chloride anion that holds together the three domains. The CPVT-associated mutant R420Q targets one of the Arginines coordinating the anion and ablates chloride binding. The crystal structure of the mutant shows reorientations in the first two domains relative to the third, likely destabilizing intersubunit interactions in the context of an intact channel. Chloride anion binding may represent one of the ways RyR2 uses to regulate channel opening.

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Arrhythmogenic Mechanisms in Catecholaminergic Polymorphic Ventricular Tachycardia Linked to RyR2 Loss-of-Function Mutation

Yan-Ting Zhao[1], Carmen R. Valdivia[1], Georgina B. Gurrola[1], Cong Li[1], Patricia P. Powers[2], Héctor H. Valdivia[1].
[1]Internal Medicine, University of Michigan, Ann Arbor, MI, USA, [2]University of Wisconsin-Madison, Madison, WI, USA.

To date, classical mechanisms of arrhythmogenesis in CPVT require spontaneous Ca^{2+} release via *hyperactive* RyR2 channels affected by gain-of-function mutation. Here, we report novel arrhythmia mechanisms in a CPVT-linked RyR2-A4860G mutation that *depresses* channel activity. Murine RyR2-A4860G cDNA was expressed in CHO cells to the same level than wild-type (WT), but recombinant RyR2-A4860G protein displayed dramatically reduced channel activity by [^{3}H]ryanodine binding assay and direct single channel activity recording. Mice heterozygous for RyR2-A4860G mutation (RyR2-A4860G$^{+/-}$) exhibited basal bradycardia without cardiac structural alterations; no homozygotes were detected at birth, indicating the mutation is phenotypically too strong to be harbored in two alleles. Anesthetized RyR2-A4860G$^{+/-}$ mice injected with arrhythmogenic cocktail (120mg/kg caffeine, 2mg/kg epinephrine) displayed ECG alterations of QRT alternans, premature ventricular complexes and bidirectional ventricular tachycardia, while ECGs of WT mice remained uneventful. Simultaneous recording of action potentials (APs) and $[Ca^{2+}]_i$ transients in isoproterenol-stimulated RyR2-A4860G$^{+/-}$ ventricular myocytes showed prolonged APs and interspaced bursts of altered Ca^{2+} release consisting of normal peak followed by a second, prolonged phase of release. Remarkably, early afterdepolarizations (EADs) were observed only during the prolonged phase of Ca^{2+} release, and were abolished by NCX inhibitor CB-DMB or intracellular Ca^{2+} chelator EGTA. SR Ca^{2+} load was higher in RyR2-A4860G$^{+/-}$ cells before EADs but decreased to levels comparable to WT after EADs. Simultaneous recording of $I_{Ca,L}$ and $[Ca^{2+}]_i$ transients showed decreased transient amplitude and prolonged Ca^{2+}-dependent-inactivation of $I_{Ca,L}$ in RyR2-A4860G$^{+/-}$ cells. In conclusion, RyR2-A4860G loss-of-function mutation decreases Ca^{2+} release amplitude and impairs $I_{Ca,L}$ inactivation, both of which gradually overload SR. The resultant SR overload then causes bursts of prolonged Ca^{2+} release, activating electrogenic NCX activity during APs and triggering EADs. The RyR2-A4860G mutation reveals novel pathways by which RyR2 channels engage membrane currents to produce life-threatening arrhythmias.

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Preventing RyR2-S2808 and RyR2-S2814 Phosphorylation does not Alter the β-Adrenergic Response of Mouse Hearts

Emmanuel Camors[1], Randall Loaiza[1], Francisco Alvarado[1], Yanting Zhao[1], Patricia Powers[2], Hector H. Valdivia[1].
[1]Center for Arrhythmia Research, University of Michigan at Ann Arbor, Ann Arbor, MI, USA, [2]Cell and Regenerative Biology, University of Wisconcin at Madison, Madison, WI, USA.

We previously showed that ablation of the cardiac Ca^{2+} release channel/ryanodine receptor (RyR2) phosphorylation at Ser2808 (RyR2-S2808A) has no functional impact on cardiac performance and/or heart failure progression. In this study, we investigated whether this lack of effect may be due to compensatory mechanisms involving altered phosphorylation of the nearby Ser2814 CaMKII phospho-site. We generated a new mouse model where Ser2808 and Ser2814 were genetically replaced by Alanine (RyR2-S2808A/S2814A). Anesthetized RyR2-S2808A/S2814A mice have normal ejection fraction at baseline compared to wild type (WT) mice ($55.1 \pm 2.6\%$ *vs.* $55.9 \pm 2.4\%$, respectively, n=7/group) but exhibit ~20% increase in heart rate (553 ± 33 *vs.* 460 ± 28 bpm, $p<0.05$, n=8/group). Langendorff-perfused hearts from both, RyR2-S2808A/S2814A and WT mice displayed an equally robust increase in the amplitude of isoprenaline (200nM)-stimulated LV contractions (60% and 63% above control in mutant and WT hearts, respectively, n=10/group, $p=0.7$). In agreement with the whole-heart results, isoproterenol (100nM)-stimulated RyR2-S2808A/S2814A myocytes displayed a comparable increase in Ca^{2+} transient amplitude and sarcoplasmic reticulum (SR) Ca^{2+} content than WT cardiomyocytes. Nevertheless, ablation of the S2808/S2814 phosphosites significantly decreased RyR2 refractoriness measured by a 2-pulse protocol (142 ± 7 *vs.* 194 ± 6 ms in the S2808A/S2814A and WT, respectively, n=7, $p<0.01$). This effect was associated with a ~27% increase in SR Ca^{2+} leak (3.22 ± 0.2 *vs.* 2.53 ± 0.2 $\Delta F/F_0.100\mu m^{-1}.s^{-1}$ in the RyR2-S2808A/S2814A and WT myocytes, respectively, n=17/group, $p<0.05$) causing earlier, faster and more frequent spontaneous Ca^{2+} waves. Interestingly, 60% of RyR2-S2808A/S2814A and 40% of the WT Langendorff-perfused hearts (n=10 each) exhibited arrhythmias upon isoprenaline stimulation. In conclusion, our data show that inhibition of S2808 and S2814 phosphorylation leaves intact the β-adrenergic response of the heart but accelerates RyR2 refractoriness, increasing SR Ca^{2+} leak and promoting arrhythmic events.

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Suppressed RyR2 Function Represents a Common Cause of Idiopathic Ventricular Fibrillation and Sudden Cardiac Death

Xiaowei Zhong[1], Yijun Tang[1], Ruiwu Wang[1], Vern Hsen Tan[2], Yingjie Liu[1], Lin Zhang[1], Carlo Napolitano[3], Silvia Priori[3], SuiRong Wayne Chen[1].
[1]University of Calgary, Calgary, AB, Canada, [2]Foothills Hospital, University of Calgary, Calgary, AB, Canada, [3]University of Pavia, Pavia, Italy.

Cardiac Ryanodine Receptor (RyR2) is the major intracellular calcium release channel located in the sarcoplasmic reticulum. A large number of mutations in RyR2 have been linked to inherited cardiac arrhythmias (CPVT, catecholaminergic polymorphic ventricular tachycardia and IVF, idiopathic ventricular fibrillation) and sudden cardiac death. We previously demonstrated that in contrast to CPVT RyR2 mutations, the IVF RyR2 mutation A4860G suppresses RyR2 function. However, A4860G represents the only IVF RyR2 mutation that has been functionally characterized to date. To determine how common suppression-of-function RyR2 mutations are, we systematically characterized 27 RyR2 mutations that are associated with IVF or sudden cardiac death. Each of these mutations was generated by site-directed mutagenesis and expressed in HEK293 cells. Ca release assays revealed that 7 of these mutations suppress or abolish caffeine-induced Ca release. Single cell Ca imaging studies also indicated that these suppression- or loss-of-function RyR2 mutations

reduce or abolish the propensity for store-overload induced Ca release (SOICR) in HEK293 cells. In addition, single channel analysis showed that these mutations significantly reduce the response of the channel to activation by cytosolic and luminal Ca. These observations demonstrate, for the first time, that suppressed or loss of RyR2 function may be a common mechanism underlying idiopathic ventricular fibrillation, which is opposite to the gain-of-function RyR2 mutations associated with CPVT. Thus, understanding the exact molecular defects of disease-causing RyR2 mutations will help to develop novel approaches to the diagnosis and personalized treatment of these lethal cardiac arrhythmias (Supported by CFI, CIHR, AIHS, and LCIA).

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Suppression of Spontaneous CA^{2+} Release by Cardioprotective Drugs

Jingqun Zhang[1], Chris D. Smith[2], Qiang Zhou[1], Jianmin Xiao[3], Guogen Wu[1], Alma Nani[1], Thomas G. Back[2], Michael Fill[1], S.R. Wayne Chen[1,3].
[1]Department of Molecular Biophysics and Physiology, Rush University Medical Center, Chicago, IL, USA, [2]Department of Chemistry, University of Calgary, Calgary, AB, Canada, [3]Department of Physiology and Pharmacology, University of Calgary, Calgary, AB, Canada.

Spontaneous sarcoplasmic reticulum (SR) Ca^{2+} release in the form of Ca^{2+} waves occurs in cardiac cells under conditions of SR Ca^{2+} overload. This store-overload induced Ca^{2+} release (SOICR) is a well-known cause of delayed afterdepolarization and triggered arrhythmias. Hence, inhibiting SOICR may represent a promising therapeutic strategy for Ca^{2+}-triggered arrhythmias. Indeed, we have recently shown that carvedilol, one of the most effective beta-blockers for preventing ventricular tachyarrhythmias and sudden death in heart failure, possesses a novel anti-SOICR activity. To identify more SOICR inhibitors, we searched the DrugBank database (http://www.drugbank.ca) for chemicals with structures similar to carvedilol. We selected a number of hit compounds and assessed their impact on SOICR in HEK293 cells expressing a SOICR-promoting cardiac ryanodine receptor (RyR2) mutation (R4496C). We found that curcumin and resveratrol (natural phenols) and bevantolol (a beta blocker and a Ca^{2+} channel blocker) suppress SOICR in HEK293 cells with IC50s of 6.7 ± 0.3, 48.4 ± 3.6, 64.7 ± 11.3μM, respectively. On the other hand, gliclazide, diphenhydramine, crocin, dexrazoxane, and trazodone displayed little or no SOICR inhibition. We also tested several other known cardioprotective drugs. We found that docosahexaenoic acid (DHA), eicosapentaenoic (EPA), arachidonic acid (AA), anandamide, and a novel derivative of anandamide (CS-X-95) inhibit SOICR in HEK293 cells with IC50s of 11.2 ± 4.2, 5.1 ± 0.5, 5.5 ± 0.9, 14.7 ± 5.1, and 11.5 ± 0.7 μM, respectively, whereas ranolazine and S107 have no significant impact on SOICR. These observations indicate that many cardioprotective drugs exhibit anti-SOICR activity, and that SOICR inhibition may contribute, in part, to their cardioprotective effect. (Supported by NIH)

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Cryo-EM Studies of RyR1 Channel in Detergent-Free Aqueous Environment

Olga B. Popova[1], Guizhen Fan[1], Mariah R. Baker[1], Joanita Jakana[2], Wah Chiu[2], Steven J. Ludtke[2], Irina I. Serysheva[1].
[1]Biochemistry and Molecular Biology, UT-Medical School at Houston, Houston, TX, USA, [2]National Center for Macromolecular Imaging, Department of Biochemistry and Molecular Biology, Baylor College of Medicine, Houston, TX, USA.

Skeletal muscle ryanodine receptor (RyR1) is a Ca^{2+} release channel in the sarcoplasmic reticulum membrane and plays a key role in excitation-contraction coupling. Obtaining high-resolution 3D structure of RyR1 is a formidable challenge due to its enormous size (~2.3 MDa), dynamic nature and location in the lipid membrane in native state. Detergents are traditionally used to make membrane proteins water soluble and suitable for single-particle cryo-EM. However, detergents tend to destabilize and inactivate membrane proteins. In addition, the presence of detergent in the protein sample reduces surface tension of water making it difficult to control the ice thickness and distribution of channel particles in cryospecimen, and leads to low-contrast in cryo-images. To overcome these difficulties we used amphipathic polymer, amphipol 8-35 (APol8-35), to substitute for detergent in RyR1 preparations. We tested functionality of RyR1/APol8-35 in a [^{3}H]ryanodine binding assay, which yielded K_d and B_{max} values similar to those of the purified RyR1 in the presence of CHAPS, indicating that the high-affinity binding site for ryanodine is retained in RyR1/APol8-35. The use of Apol8-35 allowed us to reproducibly obtain ice-embedded specimens of RyR1 for cryo-EM analysis and resulted in improved ice thickness with channel particles uniformly distributed across the holes in the grids. The protein contrast of ice-embedded RyR1/APol8-35 is substantially higher than in detergent preparations using a traditional CCD detector. Furthermore, low-dose images of vitrified RyR1/APol8-35 have been collected on the DE-12 back-thinned DDD camera using JEM3200FSC electron cryomicrosope operated at 300 keV at liquid nitrogen temperature. Preliminary analysis of these cryo-EM images demonstrates image signals that extend beyond ~6Å. Optimal conditions for both sample vitrification and data acquisition were reached in order to achieve a higher resolution RyR1 structure. Supported by NIH (R21AR063255, R01GM072804, R01GM080139, P41GM103832) and AHA (12GRNT10510002).

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Ligand-Induced Conformational Changes in Tetrameric IP_3R1 Revealed by Single-Particle Cryo-EM

Guizhen Fan[1], Olga B. Popova[1], Steven J. Ludtke[2], Irina I. Serysheva[1].
[1]Biochemistry and Molecular Biology, The University of Texas Medical School, Houston, TX, USA, [2]National Center for Macromolecular Imaging, Verna and Marrs McLean Department of Biochemistry and M, Baylor College of Medicine, Houston, TX, USA.

A functional hallmark of inositol 1,4,5-trisphosphate receptors (IP_3Rs), the main Ca^{2+} release channels in the endoplasmic reticulum of virtually all eukaryotic cells, is the coupled interplay between the binding of primary ligands, IP_3 and Ca^{2+}, and channel gating. IP_3Rs are exceptionally large integral membrane proteins, comprising four subunits of over 300 kDa each. A central mechanistic question of IP_3R function is how IP_3 binding in the *N*-terminal sequence of the channel protein is communicated to the ion-conduction pore, which is formed close to the *C*-terminus. Using single-particle cryo-EM, we have performed structural analysis of purified tetrameric IP_3R1 vitrified in the presence of micromolar Ca^{2+} and saturating concentration of adenophostin A (AdA), a structural mimetic of IP_3, that is a high-affinity, full agonist of IP_3Rs. Cryo-EM images of IP_3R1 were recorded on a Gatan 4k x 4k CCD camera in the JEM2010F cryomicroscope operated at low-dose conditions. Using EMAN2, ~40,000 particle images were merged to generate a preliminary 3D map of ligand-bound IP_3R1 at ~15 Å resolution. A comparison with our recent structure of IP_3R1, determined without the addition of any channel ligands (Ludtke *et al.*, 2010: Structure *19*, 1192-99), reveals structural rearrangements in the cytoplasmic domains of the ligand-bound IP_3R1 channel. Furthermore, we have performed docking studies of available crystal structures of the ligand-binding domains into reconstructed cryo-EM density maps of the entire IP_3R1. All together our studies suggest that AdA/Ca^{2+} binding induces conformational changes in the quaternary structure of tetrameric IP_3R1 that might underlie the long-range allosteric mechanism of agonist-mediated activation of the ion-conducting pore of IP_3R. Supported by grants from NIH (R21AR063255, R01GM072804, R01GM080139, P41GM103832) and by AHA (12GRNT10510002).

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Coupled Gating of Ryanodine Receptors: Evidence for a Role of Physical RyR-RyR Interactions

Paula L. Diaz-Sylvester[1], Jake T. Neumann[1], Sidney Fleischer[2], Julio A. Copello[1].
[1]Pharmacology, SIU-SOM, Springfield, IL, USA, [2]Biological Sciences, Vanderbilt University, Nashville, TN, USA.

We studied the synchronous function (coupled gating) of arrays of striated muscle ryanodine receptors (RyRs) reconstituted into planar bilayers. We think that understanding coupled gating would help explain local Ca^{2+} sparks and global Ca^{2+} transients which arise from the simultaneous activation of groups of RyRs in the sarcoplasmic reticulum. We have previously found that coupled gating of multiple cardiac or skeletal RyRs requires luminal Ca^{2+} as current carrier. In principle, this would suggest that Ca^{2+} flowing from the open pore of a RyR, could activate neighboring channels (local CICR). However, further analysis showed that coupled events are insensitive to changes in the magnitude of lumen-to-cytosol Ca^{2+} flux. This is not compatible with the idea of individual channels modulated by local CICR. As an alternative, we tested the involvement of physical RyR-RyR interactions in the process of coupled gating. We found that coupled gating of RyRs reconstituted in planar bilayers is not significantly affected by agents that disrupt cytoskeletal networks, FKBPs and/or kinases/phosphatases. However, the polycationic amine spermine interfered with coupled RyR gating (suggesting a role for electrostatic interactions). Additionally ryanodol, known to lock open channels in a sub-conductance state, also affected coupled RyRs. Nevertheless, event termination (synchronous channel closures) occurred even during ryanodol substates. This was unexpected because single channels do not transition from the ryanodol/ryanodine substate to the closed state. Overall, our results suggest that modulation of coupled RyRs is different from that expected for individual channels interacting by local CICR. We think that luminal Ca^{2+} promotes conformational changes in RyRs allowing for cytosolic RyR-RyR