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

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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

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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

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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

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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

interactions of electrostatic nature that induce coupled events (Supported by NIH R01 GM078665 and AHA-MWA 12180038).

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Phosphorylation of Maurocalcine Strongly Modifies its Effect on Type 1 Ryanodine Receptor

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Maurocalcine (MCa) is a 33-amino acid peptide isolated from the venom of *Scorpio maurus palmatus,* a Tunisian scorpion. It possesses efficient cell penetration properties and has been shown to strongly modify ryanodine receptor (RyR1) channel behavior by increasing open probability and inducing a long-lasting subconductance state. The amino acid residue threonine at position 26 belongs to a putative phosphorylation site within MCa sequence. We investigated the effect of a) T26 phosphorylation (MCa T26Phospho) by protein kinase A and b) replacement of T26 by glutamic acid residue to mimic phosphorylation on the effect of MCa on RyR1 properties. Using [^{3}H]ryanodine ([^{3}H]Ry) binding and single channel voltage-clamp measurements, we show that both MCa T26Phospho and MCa T26E analogs almost completely lose the ability to induce RyR1 activation. Neither MCa T26Phospho nor MCa T26E (up to 2μM) increase [^{3}H]rya binding. Small increase (~1.65-fold activation; $p<0.01$) was observed with high concentrations (10μM) of either MCa T26Phospho or MCa T26E. Single channel measurements revealed that neither MCa T26E nor MCa T26Phospho (up to 2μM) were able to induce the characteristic long-lasting subconductance state of RyR1 observed in presence of wild-type MCa. MCa T26Phospho altered RyR1 channel gating in a time-dependent biphasic manner with initial activation of Po followed by almost complete inactivation. These effects of MCa T26Phospho could be reversed by 200nM wt MCa. At concentrations $\geq$2μM, MCa T26E enhances Po~3 fold by shortening τ_c without altering τ_o. These results show that single phosphorylation of MCa modifies RyR1 activity.1P01 AR52354.

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Effects of Amino-Terminal Disease-Associated Mutations on the CICR Activity of RyR1 Channel

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Type 1 ryanodine receptor (RyR1) is a Ca^{2+} release channel in the sarcoplasmic reticulum and plays a pivotal role in excitation-contraction coupling in skeletal muscle. RyR1 is the major target for muscle diseases, e.g., malignant hyperthermia (MH) and central core disease (CCD). To date, over 200 mutations have been identified in the RyR1 gene of MH and CCD patients. It is widely believed that MH and CCD mutations cause hyperactivation of the Ca^{2+}-induced Ca^{2+} release (CICR), resulting in abnormal Ca^{2+} homeostasis in skeletal muscle. CICR shows biphasic Ca^{2+} dependence, 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). However, it remains unclear how the disease-associated mutations affects these parameters. In this study, we expressed several RyR1 channels carrying different MH/CCD mutations at amino-terminal region in HEK cells and tested their CICR by live-cell Ca^{2+} imaging and [^{3}H]ryanodine binding. Our results suggest that the amino-terminal 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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Functional Analysis of Ryanodine Receptor Carrying Malignant Hyperthermia Associated Mutations

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Ryanodine receptors, located in the sarcoplasmic/endoplasmic reticulum (SR/ER) membrane, are required for intracellular Ca^{2+} release that is involved in a wide range of cellular functions. Malignant hyperthermia (MH) is a pharmacogenetical complication of general anesthesia resulting from abnormal Ca^{2+}-induced Ca^{2+} release (CICR) via the type 1 ryanodine receptor (RyR1) in skeletal muscles. The typical symptoms include a rapid increase in body temperature and induction of a hypermetabolic state with skeletal muscle rigidity. More than 200 mutations in the RyR1 gene have been reported in MH patients. Most of those mutations have been found in three ''hot spots'' regions of RyR1. However, there were only a few experimental results confirming those mutations being responsible for the increment of the CICR sensitivities. We improved the method for making MH mutants in the cDNA of RyR1. We characterized the functional mutations on RyR1 in non-muscle cells, specifically HEK293 cells with tetracycline-regulated RyR1 expression. Rabbit RyR1 channels carrying corresponding mutations were expressed in HEK293 cells for functional assay. HEK293 cells were loaded at room temperature with fura-2 AM in physiological salt solution. Fluorescence images were acquired using an inverted microscope equipped with a objective, a cooled CCD camera and a polychromatic illumination system. We characterized the functional mutations on RyR1 in HEK293 cells. It was found that disease-associated mutations of the RyR1 resulted in enhanced Ca^{2+} release activity, therefore these mutations would be responsible for the MH incidence. These results suggest that exploration of the functional mutations of RyR1 is probably effective in preventive diagnosis of patients associated with MH disease.

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Malignant Hyperthermia Associated Mutations in S2-S3 Loop of Type 1 Ryanodine Receptor Calcium Channel Alter Calcium Dependent Inactivation

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Skeletal and cardiac ryanodine receptors (RyRs) are ~65% similar in their primary sequences, though differences in their regulation by physiological molecules have been observed. Skeletal RyR (RyR1) is inhibited by millimolar Ca^{2+} with ~10 fold higher affinity than cardiac isoform (RyR2). Using RyR1/RyR2 chimera channels and [^{3}H]ryanodine binding measurements, we found that two distinct regions are involved in isoform-specific Ca^{2+}-dependent inactivation. One region includes two EF hand Ca^{2+} binding motifs (RyR1 amino acids 4081-4092 and 4116-4127) and the other contains the second transmembrane segment (S2). The results suggest a possible cytoplasmic domain interaction between these two regions (or involving the flanking regions of S2). Human disease associated mutations have been identified in S2-S3 cytoplasmic loop of RyR1. We found that G4733E and R4736W malignant hyperthermia associated mutations reduced affinity for Ca^{2+} dependent inactivation of the channels by 5-6 fold (IC_{50}: 6.7 ± 0.6 mM (G4733E) and 5.5 ± 0.2 mM (R4736W) vs 1.1 ± 0.1 mM (wild type RyR1)), whereas mutations in S4-S5 cytoplasmic loop (T4825I and H4832Y) reduced affinity by 2-3 fold. We also found that the activities of G4733E- and R4736W-RyR1 mutants are suppressed at 10-100 μM Ca^{2+}, and the suppressions are relieved by 1 mM Mg^{2+}, which was observed in recombinant wild type RyR2 (Chugun et al., (2007) *Am. J. Physiol. Cell Physiol.*292, C535) but not in wild type RyR1. Taken together, G4733E and R4736W mutations in S2-S3 loop confer RyR2-type Ca^{2+} dependent inactivation and Mg^{2+} activation on RyR1. The S2-S3 cytoplasmic loop may play a key role for domain interaction involved in isoform-specific Ca^{2+}-dependent inactivation of RyRs. Supported by NIH (R03AR061030), AHA (10SDG3500001), and NSF (EPS0903795).

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Ryanodine Receptor Interaction with FKBP12 is Modulated by the RyR N-Terminus Repeat Region

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The ryanodine receptor (RyR) is an ion channel involved in the release of Ca^{2+} from intracellular stores (sarcoplasmic reticulum) in muscle cells. RyR plays a pivotal role in excitation-contraction coupling and is modulated by several accessory proteins in vivo, including the immunophilin FK506 binding protein 12 (FKBP12) which, our previous studies demonstrate, binds to RyR with extremely high affinity. The FKBP12 binding site within the RyR sequence has not been definitively identified and a number of candidate regions have been proposed. A central domain phosphorylation region of RyR (CDR) has been crystallised (Sharma, P. et al, FEBS (2013)288, 903-914; Yuchi, Z. et al., Structure (2012) 20, 1210-1211). This domain has considerable sequence and structural homology to its repeat region at the N-terminus (NTR) and a pseudo atomic model of the two repeat regions, docked onto a topology model of RyR, positions NTR adjacent to the previously proposed FKBP12 binding domain and suggests a potential interaction (Zhu, L. et al, JBC (2013) 288, 903-914). The RyR1 interaction with FKBP12 was tested using [3H]ryanodine binding to measure the Po of the channel of solubilised pig RyR preparations and microsomal preparations. A dose response to FKBP12 reduced activity