Ryanodine and IDP Receptors

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The Cardiac Ryanodine Receptor (RyR2): Investigating Mechanisms of Gating at the Selectivity Filter

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Ryanodine receptors (RyR) provide the pathway for release of intracellular calcium ions (Ca^{2+}) that initiate muscle contraction. Mutations in cardiac RyRs, (RyR2) underlie arrhythmia and sudden cardiac death. RyRs are therefore an attractive therapeutic target, however more information regarding structure and function is required. The enormous size of RyRs precludes detailed structural analysis. Models, based on potassium channel templates, exist for the RyR pore-forming region (PFR) providing an invaluable framework in which predictions of specific processes of ion translocation and gating mechanisms can be tested.

Potassium channels have a conserved gating mechanism involving a distinct hydrogen-bonding network of residues at the selectivity filter that is responsible for holding the filter in an inactive, non-conducting conformation. This study examines interactions of equivalent residues in RyR2 to ascertain whether a similar gating mechanism exists.

Three alanine (D4829A, Y4813A, Y4839A) and one conserved tryptophan (Y4839W) RyR2 mutations were constructed to assess a proposed hydrogen-bonding network. Mutated channels formed functional homotetramers in vivo whereby they released Ca^{2+} upon caffeine addition. Differences in [3H]-ryanodine binding on isolated WT and mutant mix membrane populations revealed 1) altered ryanodine binding site and/or 2) altered calcium sensitivity for mutant channels. Preliminary single-channel experiments assessing ion handling and gating properties under steady state conditions suggest that the selectivity filer has a role in channel gating. Conductance and open probability (Po) for Y4839A was reduced by 15 % and 76 % respectively compared to WT RyR2. No single-channel experiments were performed for Y4813A due to inherent protein instability when purified. Four unique gating modes including subconductance states for D4829A were observed. Further experiments are required to assess the role of the selectivity filter in gating.

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Looking for the Calcium-Binding Site in the Ryanodine Receptor's Vestibule

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Ca^{2+} regulates ryanodine receptor's (RyR) activity through a high-affinity (activating) and a low-affinity (inhibiting) Ca^{2+}-binding site. Their altered sensitivity plays an important role in the pathology of malignant hyperthermia and heart failure. Although, the function of Ca^{2+}-binding sites has been extensively studied, their location within the channel protein remains elusive. We used $Eu3^{+}$ as a tool to learn more about the Ca^{2+}-regulation of RyR, because it has high affinity to Ca^{2+}-binding proteins and due to its high valency it is more susceptible to be moved across an electric field than Ca^{2+}.

$Eu3^{+}$ was tested on single RyR1 channels reconstituted into planar lipid bilayers. When the activating binding site was saturated by 50 μM Ca^{2+}, $Eu3^{+}$ potently inhibited RyR's open probability (kd=167 ± 5 nM nHill=2 ± 0.1), but in nominally 0 [Ca^{2+}], low [Eu3+] activated the channel. These results suggest that $Eu3^{+}$ acts as an agonist of both Ca^{2+}-binding sites. More interestingly, the action of $Eu3^{+}$ was voltage-dependent: at negative membrane potentials, when the electrical force tends to drive $Eu3^{+}$ into the pore, >1 μM $Eu3^{+}$ caused long closed states by occluding the pore, but unexpectedly, ≤1 μM $Eu3^{+}$ induced significantly weaker inhibition compared to positive potentials (which drives positively charged ions out from the outer vestibule). These findings led to the hypothesis that negative potentials prevent $Eu3^{+}$-inhibition by increasing relative [$Eu3^{+}$] in the vicinity of the activating site located within the vestibule. This idea was further tested with the peptide-toxin maurocalcine. We found that RyR open probability was insensitive to lowering [Ca^{2+}] to 100 nM as long as the toxin occluded the vestibule. These results suggest, that maurocalcine mechanically prevented Ca^{2+} from dissociating from its binding site, therefore the high-affinity binding site (but not the low-affinity) might be located in the RyRs vestibule.

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Ligand-Dependent Conformational Changes in the Cardiac Ryanodine Receptor

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Global conformational changes in the three-dimensional (3D) structure of the calcium release channel/ryanodine receptor (RyR) occur upon ligand activation. Of several ligands that activate RyR, Ca^{2+} is the primary activator. Although RyR Ca^{2+} activation is well characterized functionally, little is known about the conformational changes in RyR induced by Ca^{2+}. Here we generated three fluorescence resonance energy transfer (FRET)-based conformational probes. Each of these probes was constructed by inserting a CFP into one domain and a YFP into a neighboring domain in the cardiac RyR (RyR2) to yield a CFP- and YFP-dual labeled RyR2 (CFP/YFP FRET pair). These CFP/YFP FRET pairs were located in the "clamp" region ($RyR2_{S2367\text{-}CFP/Y2801\text{-}YFP}$), the calmodulin binding region ($RyR2_{R3595\text{-}CFP/K4269\text{-}YFP}$), and the "bridge" region ($RyR2_{S437\text{-}YFP/S2367\text{-}CFP}$), respectively. We monitored the conformational changes in these regions by recording the FRET signals, and the extent of Ca^{2+} release by measuring store Ca^{2+} depletion in HEK293 cells expressing each of the CFP/YFP FRET pairs upon activation by Ca^{2+}, caffeine, and ATP. Surprisingly, we found that different ligands induced different conformational changes in different regions of RyR2. For instance, we detected conformational changes in the clamp region for caffeine and ATP, but not for Ca^{2+}, although they all induced Ca^{2+} release. Considering Ca^{2+} as the primary activator of RyR2, we determined the impact of cytosolic Ca^{2+} sensing mutation E3987A on conformational changes. Interestingly, this single mutation abolishes caffeine-induced conformational changes, but not caffeine-induced Ca^{2+} release. These observations demonstrate that conformational changes in RyR2 are ligand-dependent, and that E3987, which is critical for cytosolic Ca^{2+} sensing, is essential for ligand-induced conformational changes (Supported by CFI, CIHR, HSFC, NIH, and LCIA).

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Structure-Function Relationship of Calcins, a Family of High-Affinity Peptide Ligands of Ryanodine Receptors

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Calcins is a novel family of scorpion peptides that bind with high affinity and specificity to ryanodine receptors (RyRs) and increase their open probability by inducing the appearance of a long-lasting subconductance state. Here we report two newly-identified calcins (Vejocalcin and Urocalcin) and provide a comprehensive analysis of the structure-function relationship of the eight calcins known to date, based on primary sequence examination, 3D structure modeling, and their effect on RyR1. [3H]Ryanodine binding assays, used as index of the open probability (Po) of RyRs, revealed that all eight calcins (Opicalcin1, Opicalcin2, Imperacalcin, Maurocalcin, Hadrucalcin, Hemicalcin, Vejocalcin, and Urocalcin) activate RyR1 dose-dependently, with EC50s (in nM) 0.35, 5, 8, 12, 37, 71, 91, and 523, respectively. At 1 μM, calcins significantly augmented the bell-shaped [Ca^{2+}]-activity curve of RyR1 with potency Opicalcin1>Opicalcin2>Vejocalcin>Urocalcin. In single channel recordings, the heretofore uncharacterized calcins Opicalcin1, Opicalcin2 and Vejocalcin increased the Po of RyR1 significantly and the fractional conductance was ~0.45, 0.30, and 0.65, respectively, of the full conductance state. Opicalcin1, Maurocalcin, Hadrucalcin and Vejocalcin induced Ca^{2+} release from rabbit skeletal SR with ED50s (in nM) 2.8 ± 0.02, 8.3 ± 0.25, 16 ± 0.4, 36 ± 0.5, respectively. A clear Ca^{2+} release-[3H]ryanodine binding activity correlation (r2=0.99) was obtained. Primary sequence alignment and evolutionary analysis (ClustalW and MEGA5.2) showed high homology among all calcins. An inhibitor cysteine-knot (ICK) motif with βαββ domains stabilized by three disulfide bridges characterizes this peptide family, although Vejocalcin lacks the β2 sheet. Positively-charged residue mutations in the high variable N-terminal region (G1-N14) and negatively-charged residue variations in the conserved C-terminal region (D15-R33) greatly decrease the effect of calcins on RyR1. In conclusion, natural variation in calcin peptides offers a diversified set of RyR ligands with capacity to modulate RyRs with high dynamic range and potency.

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Mapping the Interacting Sites Mediating Tetramerisation of Ryanodine Receptor Amino-Terminus

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The cardiac ryanodine receptor (RyR2) is an intracellular calcium release channel composed of four ~560kDa subunits. It mediates sarcoplasmic reticulum

calcium release playing a critical role in cardiac excitation-contraction coupling. Recently, we presented evidence that the RyR2 N-terminus self-associates into a tetrameric form, which stabilises the closed conformation of the channel. In order to identify the interacting domains mediating RyR2 N-terminus tetramerisation, we used truncated constructs in yeast two-hybrid and chemical cross-linking assays. RyR2 residues 1-418 remained in monomeric form, whereas residues 1-530 retained self-association ability although it was much weaker compared to longer fragments. Tetramerisation was not abolished by removal of the N-terminal 160 amino acids but further truncations were detrimental. A targeted approach for putative inter-subunit contact sites was also employed testing the effect of small internal deletions within RyR2 residues 1-906. We found that two small deletions (167-178 or 335-358 amino acids) severely compromised tetramerisation ability. These findings suggest that residues 167-178 and 335-358 are the primary interacting sites, whereas additional downstream determinants further strengthen the RyR2 N-terminus self-association.

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FRET-Based Trilateration of a Domain Peptide Bound within Functional Ryanodine Receptors in Cardiomyocytes

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We have used trilateration, the method of using distances to determine a location in space, simulated annealing calculations, and confocal FRET measurements in permeabilized rat myocytes to determine the topology of a modulatory interface within the ryanodine receptor (RyR) Ca^{2+} channel. A small peptide (~4kDa, termed DPc10), corresponding to a RyR central stretch, hypothetically destabilizes a key interaction between RyR N-terminal and central domains to promote the open channel. We used DPc10 labeled with a small fluorophore, HiLyte Fluor 647, as FRET acceptor. Five single-cysteine variants of the 12 kDa FK506-binding protein (FKBP) were labeled with FRET donor, Alexa Fluor 488 C5 maleimide, and targeted to RyR. Effective average positions of the donors were calculated from simulated annealing, constrained by the RyR cryo-EM map and by the FKBP atomic structure. FRET from the FKBP donors to the DPc10 acceptor was measured via confocal microscopy, and the calculated distances were used to trilaterate the acceptor location within the RyR 3D map. The trilateration method uses the distances determined by FRET from each donor's calculated average position, where the ranges of the distances were based on measurement precision for each donor labeling site. The DPc10-bound acceptor locus is found at a region in space matching four distance ranges from FRET, and was further constrained by FRET between donor-labeled calmodulin and F-DPc10. This locus is near the RyR N-terminal domain structure docked into the cryo-EM reconstructed map of RyR. Computational resources were provided by the Minnesota Supercomputing Institute. This work was supported by NIH grants R01HL092097 (to D.M.B. and R.L.C.), and R01GM27906 (to D.D.T.).

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Evolution, Structure and Function of Ryanodine Receptor Domains

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Ryanodine receptors (RyRs) control the Ca^{2+}-release from sarcoplamic reticulum (SR) and play an important role in excitation-contraction (EC) coupling. Misregulation of the channel by mutations or hyperphosphorylation can cause severe disease such as cardiac arrhythmias, heart failure and malignant hyperthermia. RyRs are the largest ion channels found in humans and consist of dozens of functional domains that may act as separate modules. Interestingly, a few domains are present multiple times in the channel due to the gene duplication. One of them, the so-called ''RYR domain'' is present four times in two tandem repeats, one near the N-terminus and the other in the central region. Previously we solved the crystal structure of the central repeat, which contains several major phosphorylation sites in RyR. By analyzing the sequences and structures of the different RYR domains from RyR and other proteins, we were able to gain interesting insights about the evolution of the domain. Some special structural features of the domain may be involved in the unique function of the channel. Some other repeating domains in RyRs were also studied and compared to their analogues from different protein families. Potential functions of these domains are proposed based on the evolutionary and structural relationships.

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Molecular Modeling and Structural Docking of a Ryanodine Receptor SPRY2 Domain

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Ryanodine receptors (RyRs) form a class of intracellular calcium release channels in various excitable tissues and cells such as muscles and neurons. SPRY domains are recognized as a protein interacting module and were so named because they were identified in a *Dictyostelium discoidueum*splA kinase and in the mammalian RyR. There are 3 SPRY domains present in all the RyR isoforms. A particular interest has been raised for the SPRY2 domain, as it has been identified as the binding partner for the II-III loop from the $\alpha 1_S$ subunit of the dihydropyridine receptor, an interaction that is thought to be crucial for skeletal excitation-contraction coupling. In the present study, we have generated pseudo-atomic structures for RyR1 fragment 1,071-1,208 and homologous RyR2 fragment 1,084-1,221, both of which contain a SPRY2 domain. Both modeled SPRY2 fragments contain multiple β-sheets. Our initial rigid-body docking model in the sub-nanometer resolution cryo-EM map of RyR has placed the modeled fragment into a cytoplasmic domain adjacent to the N-terminal and central mutation region of RyR. Based on the modeled SPRY2 structure, we have designed a RyR2-GFP construct, with GFP inserted after residue Arg-1084, the N-terminus of the SPRY2 domain, to test our docking position by a 3D cryo-EM study. Our preliminary 2D analysis result supported the docking position.

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A Regulatory Component of the Human Ryanodine Receptor 2 N-Terminus

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Human cardiac ryanodine receptor (hRyR2) is a channel mediating Ca^{2+} release from the sarcoplasmic reticulum during excitation-contraction coupling. The N-terminal (1-655) and central (2100-2500) regions of hRyR2 are thought to be involved in regulating channel gating. Mutations linked to several heart diseases are clustered within these two, as well as in the channel pore-containing C-terminal regions. High resolution structures of key regions involved in the regulation of RyR2 activity could further the understanding of the gating mechanism of hRyR2 and of its malfunction in disease. Here we present the structure of the $hRyR2^{1-606}$ N-terminal region; it shares a significant structural similarity with other solved N-terminal RyR structures (pdb code: 2XOA, 4J4K) but lacks the chloride binding site proposed for mouse RyR2 (4J4K). Structural data suggested high flexibility of the region starting at around residue 544 predicted to consist of helices forming an armadillo motif - a protein-protein interaction domain. Our SAXS data showed high flexibility and partial unfolding of the C-terminal part of $hRyR2^{1-606}$ beyond aa 544, suggestive of incompleteness of the C-terminus. Biochemical data showed a weak tendency of $hRyR2^{1-606}$ to form dimers. Prolonging the fragment to the end of the predicted RIH domain increased the propensity to dimerize. A mutation introduced to a specific spot of $hRyR2^{1-606}$ resulted in significant thermal destabilization and augmented dimerization. Docking of the $hRyR2^{1-606}$ structure into cryo-EM maps of RyR ruled out interactions of its C-terminus with neighbouring $hRyR2^{1-606}$ monomers. We interpret the formation of dimers as a surrogate for interaction of the N-terminal region with an armadillo motif of another part of RyR2, and propose the existence of a regulatory component at the secondary structure level controlling interaction power at inter-monomer interfaces within the RyR2 tetramer.

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