Using an animal model knocked in for the RYR1 Y522S mutation we observed that bleeding times were increased by more than two fold in heterozygous mice compared to their wild type littermates, with no differences in platelet numbers nor aggregation characteristics between heterozygous RYR1 Y522S carriers and control littermates. Bleeding abnormalities have also been seen in some patients with dominant RYR1 mutations; as part of a comprehensive study we investigated in detail RyR1 expression in smooth muscle cells isolated from different tissues and if there is a causal link between RYR1 mutations and prolonged bleeding times.

2274-Pos Board B293
Modeling a Ryanodine Receptor Amino-Terminal Domain Connecting the Central Vestibule and the Corner Clamp Region

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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. They are the major cellular mediators of the release of calcium ions from the sarcoplasmic reticulum, an essential step in muscle excitation-contraction coupling. Several crystal structures of skeletal muscle RyR1 peptide fragments have been solved, but these cover less than 15% of the full-length RyR1 sequence. In the present study, we obtain pseudo-atomic models for RyR fragments, consisting of residues 850-1056 in rabbit RyR1 or residues 861-1067 in mouse RyR2, by combining modeling techniques with sub-nanometer resolution cryo-electron microscopy (cryo-EM) maps. These fragments dock into a domain that connects the central vestibule and corner clamp region of RyR, with a good match between the secondary structure elements in the cryo-EM map and the pseudo-atomic models, and also consistent with our previous results of 3D cryo-EM RyR-GFP mapping and FRET measurement between RyR and FKBP. A combined model of the RyR fragment and FKBP docked into the cryo-EM map suggests that the fragment is positioned adjacent to the FKBP binding site. Its predicted binding interface with FKBP consists of primarily electrostatic contacts and contains several disease-associated mutations. A dynamic interaction between the fragment and a RyR phosphorylation domain, characterized using FRET data, also support the structural predictions of the pseudo-atomic models.

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Structural Insight into the Phosphorylation Domain in Ryanodine Receptors

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Ryanodine receptors (RyRs) are large Ca2+-release channels located in the SR membrane. They play a central role in the excitation-contraction coupling of skeletal muscle (RyR1) and cardiac muscle (RyR2). Over 500 disease mutations have been identified in RyRs that can cause several skeletal muscle disorders and cardiac arrhythmias. RyRs are also the target for phosphorylation, most notably by CaMKII and PKA. Although a lot of controversy surrounds these events, several studies indicate that phosphorylation can upregulate RyR activity. Here we present crystal structures of a domain in all three different RyR isoforms, containing the Ser2843 (RyR1) and Ser2808/Ser2814 (RyR2) phosphorylation sites. There are 11 disease mutations located in the RyR1 domain, several of which cluster near the phosphorylation site, suggesting that phosphorylation and disease mutations may affect the same interface. Crystal structures of the disease mutants show that the mutations affect either surface properties or intradomain salt bridges. One mutation, L2867G, causes a drastic reduction of thermal stability of the domain, and results in aggregation at room temperature. In vitro phosphorylation experiments identify several novel PKA and CaMKII phosphorylation sites in the same loop region of the RyR2 phosphorylation domain, also supported by another recent in vivo study. The observation of simultaneous phosphorylation events on multiple sites indicates that RyRs may be truly ‘hyperphosphorylated’. Docking into cryo-electron microscopy maps locates the domain in the clamp region, a region that has been shown to couple allosterically to channel opening. Disease mutations and phosphorylation may therefore cause conformational changes that affect the allosteric coupling and facilitate channel opening.

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Analysis of Fluorescence Microscopy Super-Resolution Data of Protein Assemblies

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In super-resolution microscopy applications, previously unknown fluorescent signal patterns are recorded. The interpretation of such nanoscale data is often unexpectedly complex, and can be performed by different analysis strategies: 1) empirical statistics of the spatial distribution of intensity values to identify local objects, 2) inverse problem approaches to convert signals into objects based on external data models or other external assumptions, 3) decomposition of spatial signal patterns into spatial modes. Here, we compare and relate two distinct approaches of analysing STED microscopy images of RyR2 clusters (cardiac Ryndine receptor type 2). RyR2 Ca2+-release channels are essential for heart muscle function (excitation-contraction coupling). Yet, the nature of lateral channel organization within super-structural clusters is unknown and important for models of local control mechanisms of RyR2 Ca2+-release activity. We established multi-scale analysis of RyR2 signal patterns employing wavelet analysis. This analysis decomposes the initial image with predefined wavelets into spatial modes identifying dominant scales of signal fluctuations. We tested the sensitivity of this approach for different wavelets with artificial and modified images. Distinct scales represent inter-cluster spacing and intra-cluster patterns, respectively. Furthermore, we compare the spatial mode analysis with object-based approaches. For object-based analysis, RyR2 cluster sub-structures were identified with a multi-step thresholding procedure. After increasing the threshold level step-by-step, the hierarchy of the segmentation outcome was analysed with logical operators. Accordingly, we identified cluster sub-structures as discrete objects of variable sizes with typical spacings ranging from 78 to 128 nm (IQR range) that we interpret as individual cluster building-blocks. We conclude that identification of common protein cluster building principles in highly variable signal structures as typical for RyR2 clusters benefits from combining object-based approaches with spatial mode analysis.

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Gating of the Pore of the Human Ryanodine Receptor Type 2 does not require Glycine Hinges

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Many potassium channels use a conserved glycine residue to allow flexibility of the inner helices and facilitate gating. A KcsA-based analogy model of the RyR2 pore-forming region (Welch et al Biophys J 87: 2335-2351) indicates that this region is composed of structural elements equivalent to those found in potassium channels and includes a potentially equivalent inner-helix hinge motif (GXXXA).

We studied the functional consequences of substitutions of the conserved glycine residue at position 4864 in recombinantly expressed human RyR2 GFP-tagged proteins. Whilst wild type (WT) RyR2 and the mutants G4864A and G4864V were expressed at equivalent levels in HEK293 cells, G4864P was less tolerated with a reduced expression. This was confirmed by western blot analysis in HEK293 membrane preparations. Caffeine-induced intracellular calcium release was observed in Fluor3-loaded HEK293 cells expressing WT, G4864A and G4864P RyR2 channels. In contrast cells expressing G4864V were not sensitive to caffeine. [3H]-ryanodine binding to HEK293 membrane preparations resulted in similar specific binding for WT and G4864A at 0.192 ± 0.005 and 0.190 ± 0.002 pmol/mg, while G4864V and G4864P showed comparable low values of 0.006 ± 0.001 and 0.002 ± 0.002 pmol/mg, respectively. Characteristic single channel current fluctuations were observed following the incorporation of purified WT and G4864A RyR2 proteins into planar phospholipid bilayers and G4864A channels displayed ion handling, and calcium-dependent gating properties comparable to WT channels. No equivalent activity was observed for G4864V and G4864P.

Our investigation indicates that whilst functionally sensitive to mutagenesis, a glycine residue at 4864 is not essential for RyR2 channel gating. Rather, there is a requirement for amino acids with small side chains that allow close packing of helices during transitions from closed to open states. The BHF supported this research.

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Structure of Glutaraldehyde Cross-Linked Ryanodine Receptor

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The ryndine receptor (RyR) and dihydropyridine receptor (DHPR) along with several other proteins form a large and dynamic complex, often referred to as the excitation-contraction coupling machinery. Complexes of