(D) An even lower $K_{1/2}$ of 1 μM is observed for the S549C mutation.

Surprisingly, the candidate cysteine residue is not located at NBD2, since Cd^{2+} still activates G551D/NBD2-C-less channel, in which all cysteine residues in NBD2 are converted to serines. These results suggest a gating mechanism independent of NBD dimerization. Identifying the involved Cd^{2+} interacting cysteine residues will likely provide insight into understanding the coupling mechanism of CFTR gating.

2622-Plat Studies at CFTR's composite site 1

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CFTR, whose failure causes cystic fibrosis, is a chloride channel, which belongs to the ATP binding cassette (ABC) transporter family. ABC proteins are characterised by their ability to bind and hydrolyse ATP. Like other ABC transporters, CFTR consists of two halves, each containing a cytosolic nucleotide-binding domain (NBD1, NBD2) and a transmembrane spanning domain (TMD1, TMD2). The NBDs are highly conserved, containing 3 motifs, the Walker A and Walker B motifs, and the signature sequence. Gating in CFTR is thought to be driven by the dimerisation of the two NBDs, in a head-to-tail configuration. Two functionally distinct composite ATP-binding sites are formed by the Walker A and B motifs of one NBD and the signature sequence of the other NBD. It is thought that binding and hydrolysis of ATP at the NBD2-composite site trigger channel opening and channel closure respectively, but the function of the NBD1 composite site is less clear.

We investigated the role of two residues, T460 and L1353 on either side of the NBD1- composite binding site, which have been shown to interact in other ABC composite sites. Surprisingly, neither of the mutations T460S or L1353M, appear to have any effect on normal gating. Measurements from patches containing up to 10 channels, show that the mean burst duration for T460S (347 ± 170ms) and L1353M (367 ±183ms) is not significantly different from WT (409 ±107ms) and noise analysis indicates no effect on open probability, P_o. Furthermore there is no change in apparent affinity for ATP. However, preliminary results indicate that the ability of pyrophosphate to lock open channels is reduced in the T460S mutant, consistent with this residue being important for stabilising the NBD1-NBD2 dimer, at least in non-hydrolytic conditions.

2623-Plat Kiss-and-run Gating Of CFTR Chloride Channels

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There is convincing evidence that opening of the CFTR chloride channel is associated with dimerization of CFTR's two NBDs (NBD1 and NBD2). We have shown previously that ATP binding to NBD2, but not NBD1, is critical for channel opening by ATP. Contrary to wild-type (WT) CFTR, which usually opens for hundreds of milliseconds, hydrolysis-deficient mutants can open for minutes. Interestingly, we observed that WT-CFTR occasionally shows long-lasting openings similar to that of hydrolysis-deficient mutants. This observation can be explained if the bound ATP at NBD2 dissociates before it is hydrolyzed, rendering the channel closure through a non-hydrolytic mechanism. This hypothesis predicts that mutants with a lower binding affinity of ATP to NBD2 will exhibit more long-lasting openings. For WT-CFTR, removal of ATP in excised inside-out patches results in a fast current decay that can be fitted with a single exponential function (τ = 533 +/− 104ms n = 9). Similar results were seen with W401G and Y1219W mutants (τ = 553 +/− 110 ms for W401G and τ = 726 +/− 115 ms for Y1219W). However, mutations including Y1219G, Y1219I and Y1219F with lower ATP binding affinity at NBD2 show different kinetic behavior. Current decay upon removal of ATP can be better fitted with a double exponential function. The time constants of the slow phase are 31.2 +/− 3.3 s, 28.7 +/− 1.63 s and 13.45 +/− 1.46 s for Y1219G, Y1219I and Y1219F respectively. In addition, the fraction of the slow component follows the same order as the degree of changes in ATP affinity characterized previously (i.e, Y1219G > Y1219I > Y1219F). These data are consistent with the idea that once ATP binding at NBD2 leads to channel opening, this ATP molecule does not have to stay there to maintain the open state.

Platform BA: Ryanodine Receptors

2624-Plat The Open and Closed Conformations of the RyR by CryoEM

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The intracellular calcium release channel/ryanodine receptor isoform of skeletal muscle (RyR1) controls a key step in the process that links nerve stimulation with muscle contraction. Using cryo electron microscopy of frozen-hydrated solubilized RyR1s followed by single-particle image processing we previously determined the basic architecture of RyR1's transmembrane domain in closed state. Our results (Samso, Wagenknecht et al., 2005) indicated that it has an ion gate defined by a four-helix bundle, a selectivity filter, and an intervening central cavity similar to the canonic architecture determined for the K^+ channel in the closed state.

To understand how the ion pathway is modified upon RyR1's opening, we now determine the 3D structure of RyR1 in open state. A highly uniform open state was obtained by using FKBP12 and BZ95 (Wong, Brackney et al., 1997), to induce long-lasting open states with a P_o of 0.99. The open state 3D reconstruction, obtained by single-particle cryoEM, displays a resolution that is sufficient to distinguish secondary structure within the transmembrane assembly. The comparison of RyR1 in the closed and open states reveals a conformational change that involves most of RyR1's domains. The changes in the cytoplasmic domains appear coordinated with changes in the central fourfold axis along the putative ion pathway. In particular, the diameter defined by the alpha helices in the region that we attribute to the ion gate increases in the open state. This conformational change is of the same order of that seen with opening in K^+ channels and appears to be pertinent to account for ion gating.

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2625-Plat An 8-nm Map of the Calcium Release Unit From Cryo-Electron Tomography

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The ryanodine receptor (RyR) is a sarcoplasmic reticulum membrane protein that interacts with the dihydropyridine receptor of transverse tubules to form the main components of the calcium release unit (CRU) in striated muscle. Using cryo-electron tomography on isolated SR vesicles combined with template matching and multivariate statistical analysis we obtained a more accurate representation of CRU in a near-native environment. RyR's were identified in several local environments. Some were found within triad junctions but most came from surfaces of SR vesicles not adjacent to a t-tubule. RyR's tended to cluster and were found mainly in pairs but a few extended arrays of four or more were present. The detected RyR's were aligned and computationally divided into monomers. Principal component analysis of the SR membrane and K-means classification resulted in three distinct classes from which averaged images were computed: monomers with an opposing t-tubule, monomers adjacent to another RyR, and monomers with no significant neighboring densities. All three averages show calsequestrin organized into nets below the receptor. These nets appeared to have no direct attachment to the RyR but are attached to adjacent densities in the membrane, possibly involving the triadin/junctin complex. The SR membrane is highly curved in the vicinity of the RyR but flattens when a neighboring RyR is present which allows the calsequestrin net to lie ~2nm closer to the bottom of the RyR. This suggests that the RyR is more strongly buffered by calsequestrin in the array form than when isolated. The presence of the t-tubule in the first class average suggests the presence of an SR-bound protein that is associated with the t-tubule. Taken together we have created an 8-nm map of the CRU.

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2626-Plat FRET-Based Mapping of Calmodulin within the Macromolecular RyR1 Channel

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The type 1 ryanodine receptor (RyR1) is regulated by calmodulin (CaM), which activates the channel in nanomolar Ca^{2+} and inhibits the channel in micromolar Ca^{2+}. To investigate the structural basis of RyR1 regulation by CaM, we have used site-directed labeling of channel regulatory proteins and FRET. Sarcoplasmic reticulum membranes were preincubated with a fluorescent FKBP donor (AlexaFluor488-FKBP12.6) and washed to remove unbound donor. FRET, evident as a decrease in donor fluorescence, was then determined following incubations in the presence of fluorescent CaM acceptors (AlexaFluor568-CaMs). Strong FRET between FKBP and CaM was observed when acceptor fluorophore was attached within CaM's N-lobe ($E = 0.38 \pm 0.2$ in 30 nM Ca^{2+}). By comparison, substantially weaker FRET was observed when acceptor fluorophore was attached within CaM's C-lobe ($E = 0.08 \pm 0.01$ in 30 nM Ca^{2+}). Addition of Ca^{2+} evoked little change in FRET to either CaM's N-lobe or C-lobe ($E = 0.43 \pm 0.2$ and 0.13 ± 0.02, respectively, in 30 μM Ca^{2+}). We conclude that CaM binds to the RyR1 in an extended conformation, oriented such that its N-lobe is nearest to FKBP (R = 65–67 Å) and its C-lobe furthest from FKBP (R = 85–93 Å) on a single lateral face of the tetrameric channel. Surprisingly, distance estimates are similar in nanomolar and micromolar Ca^{2+}, suggesting that Ca^{2+} dependent rearrangements of CaM on the RyR1 may operate on a smaller scale than predicted in structural models based on cryo-EM.

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2627-Plat Different Regions Mediate Targeting Of Triadin To The Junctional Sarcoplasmic Reticulum Membranes

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Triadin is an integral membrane protein localized to the junctional sarcoplasmic reticulum (jSR) containing a short N-terminal cytoplasmic domain, a single transmembrane alpha helix and a luminal C-terminal region. Sites of interactions with the ryanodine receptor have been identified both in the N- and the C-terminal regions, the latter of which partially overlaps with the binding site for calsequestrin. Different studies have pointed out the importance of these interactions in regulating Ca2+ release from the SR, either through a direct role of triadin on RyR activity or supporting the crosstalk between CSQ and RyR. In order to identify specific triadic-targeting signals in triadin we created a series of GFP-tagged cDNAs, characterized by deletions of the wild type sequence. Expression of these mutants in primary myotubes led us to identify three putative regions responsible for triadic localization, one localized in the N-terminus and two in the luminal domain of triadin. Interestingly, we found that none of these regions alone is able to drive triadin to the triads, but that a combination of at least two of them is sufficient for the correct localization of the protein. Fluorescence Recovery After Photobleaching (FRAP) analysis was further used to verify the association of wild type and mutated triadins to the jSR. These experiments revealed that deletion of the C-terminal tail led to a significant increase in protein mobility, suggesting that this region may be important in stably retaining triadin at the triads.

2628-Plat Calsequestrin Regulation of the Ryanodine Receptor is Isoform Specific

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Calsequestrin (CSQ) is the major Ca^{2+} binding protein in the sarcoplasmic reticulum (SR) of both cardiac and skeletal muscle. CSQ stores Ca^{2+}, buffers luminal free Ca^{2+} to 1mM and is a major luminal Ca^{2+} sensor for the skeletal muscle RyR1 calcium release channel and regulates RyR1 activity in a manner that would conserve store Ca^{2+}. CSQ1 (skeletal) and CSQ2 (cardiac) are determinants of the size of the SR Ca^{2+} pool. The SR Ca^{2+} store is smaller in cardiac muscle and is depleted after a single contraction, in contrast to the skeletal stores which is minimally reduced by a single contraction. The CSQ isoforms differ in their intrinsic properties and we have investigated the possibility that they might differentially regulate cardiac RyR1 and skeletal RyR2. Native RyR channels were incorporated into lipid bilayers and treated with low ($\leq$100μM) luminal Ca^{2+} to depolymerize CSQ and to dissociate all but the residual monomer of CSQ that remains bound to the triadin/junctin/RyR. This residual CSQ does not regulate the RyR or its response to changes in luminal [Ca^{2+}]. The RyRs were either maintained in $\leq$100μM luminal Ca^{2+}, or Ca^{2+} restored to 1mM Ca^{2+} and then the effects of adding CSQ examined. CSQ1 was found to inhibit RyR1 with 1mM luminal Ca^{2+}, but activated the channel with $\leq$100 μM Ca^{2+}. In contrast, CSQ2 activated both RyR2 and RyR1 with 1mM Ca^{2+}, and CSQ1 activated RyR2 with 1 mM and $\leq$100μM Ca^{2+}. Therefore the inhibition of RyR1 by CSQ1 is both CSQ and RyR isoform-specific. The isoform-specific regulation of RyR1 by CSQ1 and RyR2 by CSQ2 may contribute to the different Ca^{2+} store and Ca^{2+} release properties in cardiac and skeletal muscle.

2629-Plat Functional Importance Of Type-1 Ryanodine Receptors In Smooth Muscle Cells

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Ca^{2+} release from the sarcoplasmic reticulum through ryanodine receptors (RyRs) plays a critical role in regulating numerous cellular responses in smooth muscle cells (SMCs). Three subtypes of RyRs (RyR1, RyR2 and RyR3) are all expressed in SMCs; however, the functional roles of each individual subtype of RyRs are incompletely understood. In the present study, we attempted to determine the potential importance of RyR1 in local and global Ca^{2+} release in pulmonary artery SMCs (PASMCs) using RyR1 gene deletion ($RyR1^{-/-}$) mice. Our data indicate that the frequency of spontaneous local Ca^{2+} release events (Ca^{2+} sparks) is dramatically decreased in PASMCs from $RyR1^{-/-}$ mice, although the amplitude is not affected. Global Ca^{2+} release induced by the RyR agonist caffeine is significantly reduced as well in $RyR1^{-/-}$ cells. We have also found that an increase in $[Ca^{2+}]_i$ following membrane depolarization with high K^+ is markedly attenuated in $RyR1^{-/-}$ PASMCs; and application of the neurotransmitter ATP results in a much smaller increase in $[Ca^{2+}]_i$ release in $RyR1^{-/-}$ cells. Furthermore, hypoxia-induced increase in $[Ca^{2+}]_i$ is significantly inhibited in $RyR1^{-/-}$ PASMCs. Collectively, our findings point to the functional importance of RyR1 in local and global Ca^{2+} release, which may contribute to cell contraction and other cellular functional events in smooth muscle.

2630-Plat Redox-Modification of Ryanodine Receptors Underlies Sarcoplasmic Reticulum Ca leak in Chronic Heart Failure

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Although, abnormal cardiac ryanodine receptor (RyR2) function resulting in leaky sarcoplasmic reticulum (SR) Ca stores is recognized as an important contributor to altered intracellular Ca handling in heart failure (HF), the specific molecular causes underlying these changes in RyR2s remain poorly understood. The present study was designed to test the hypothesis that the HF-related alterations in RyR2s are caused by post-translation modification of the RyR2 channel by reactive oxygen and/or nitrogen species generated in the failing heart. Experiments were performed using methods of cellular electrophysiology and imaging in intact and permeabilized myocytes isolated from normal and failing canine hearts. In HF, SR Ca leak measured directly with SR-loaded Fluo5N was markedly enhanced in permeabilized myocytes, resulting in reduced [Ca]SR compared to controls. Both SR Ca leak and [Ca]SR could be normalized by treating the myocytes with the reducing agents MPG and DTT (1 mM). Conversely, the oxidizing agents DTDP (30 microM) and thimerosal (0.5 mM) accelerated SR Ca leak and decreased [Ca]SR in cells from normal hearts. Moreover, exposure to MPG significantly improved intracellular Ca handling parameters in intact HF myocytes. Activities of single RyR2 channels isolated from HF and control hearts were recorded using the lipid bilayer technique. In both groups two functional types of RyR2s could be distinguished: high-Po (open probability) and low-Po. In control, the majority (~ 90%) of RyR2s were low-Po, whereas in HF most RyR2s (~70%) were of high-Po type. High-Po channels from HF samples could be partially normalized by DTT; whereas low-Po control RyR2s could be converted to high-Po by treatment with DTDP. These findings suggest that altered redox-modulation contributes to abnormal function of RyR2s in HF and that improving RyR2 redox-balance presents a potential therapeutic target for treating HF.

2631-Plat Hadrucalcin, A Novel Member Of The Calcin Scorpion Toxin Family, Rapidly Penetrates Cellular Membranes To Bind Ryanodine Receptors And Alter Calcium Release

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Members of the calcin scorpion toxin family, including Imperatoxin A ($IpTx_a$), Maurocalcin (MCa), Opicalcin 1 & 2 (Opi 1 & 2), and

Hemicalcin (HCa), are highly basic 33-mer peptide toxins that fold along an inhibitor cysteine knot (ICK) motif, and whose pharmacological activity evokes activation of ryanodine receptors (RyR). We previously described $IpTx_a$, the first calcin toxin known to activate RyR, and demonstrated its ability to traverse cell membranes and alter Ca^{2+} release in intact cardiomyocytes. We introduce Hadrucalcin (HdCa), a new calcin toxin isolated from the venom of *Hadrurus gertschi*, a scorpion endemic to Guerrero, Mexico. Like other calcin toxins, HdCa is an amphipathic molecule with a stretch of positively-charged residues resembling the protein translocation domain of some cell-penetrating peptides. HdCa is distinguished from previously described congeners of the calcin family by two additional amino acids in its primary sequence. In the present study, we show that HdCa is capable of enhancing Ca^{2+} release from the sarcoplasmic reticulum (SR) of intact cardiomyocytes. Perfusion of field-stimulated cardiomyocytes with HdCa elicits three discernable effects:

1. up to 105% increase in fractional Ca^{2+} release compared to control;
2. up to 109% increase in Ca^{2+} transient amplitude compared to control, followed by a decrease to a new steady state as low as 63% of control; and
3. spontaneous Ca^{2+} release.

Significantly, HdCa perfusion of resting cardiomyocytes elicits discharge of SR Ca^{2+} stores and trains of trigger activity, both novel effects not previously observed with calcin toxins. Our results suggest that HdCa is a cell-penetrator and a powerful activator of RyRs, which has exciting translational potential for targeted delivery of drugs to RyR as novel therapeutic intervention in arrhythmogenic disease.

Platform BB: Excitation-Contraction Coupling

2632-Plat Structural and Functional Characterization of Ryanodine Receptor-Natrin Toxin Interaction

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Cysteine-rich secretory proteins (CRISPs) are found in many sources, especially in the mammalian reproductive tract and in salivary glands from reptile venoms. Some CRISPs can inhibit ion channels, such as the cyclic nucleotide-gated ion channel, potassium- channel, and calcium-channel. Natrin is a member of CRISPs that has been purified from snake venom, and its targets including the calcium-activated potassium channel and the calcium release channel/ryanodine receptor (RyR). Immuno-precipitation experiments showed that natrin binds specifically to type 1 RyR (RyR1) from skeletal muscle, inhibits the binding of $[^3H]$-ryanodine to RyR1, and blocks calcium release. Cryo-electron microscopy and single-particle image reconstruction analysis revealed that natrin binds to the clamp domains of RyR1. Further analysis showed that the binding of natrin altered the conformation of RyR1, and that binding of natrin to the four subunits of the RyR1 tetramer exhibited a positive cooperativity. Docking the crystal structure of natrin into the cryo-EM density map of RyR1+natrin complex indicated that the cysteine-rich domain of natrin is crucial for the binding. These findings reveal how natrin toxin blocks the RyR, and suggest a common interaction mode between CRISPs and RyR1 calcium release channel.

2633-Plat Dynamic FRET Signals between DPA and the α1s and β1a Subunits of the DHPR of Mammalian Skeletal Muscle Fibers

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Fluorescence resonance energy transfer (FRET) is a useful tool to investigate proximity between donor fluorescent molecules and Foster-energy-transfer acceptors. We took advantage of the rapid (τ~0.6 ms) voltage-dependent translocation of the non-fluorescent lipophilic anion dipicrylamine (DPA) across the transverse tubular system (TTS) membranes to investigate this molecule's FRET interaction with specific domains of the α1s and β1a subunits of the dihydropyridine receptor (DHPR). To this end, plasmids encoding for N- tagged EGFP α1s-DHPR (EGFP-α1s-DHPR) and N- or C-tagged ECFP β1a-DHPR were transfected into adult FDB muscles by *in vivo* electroporation. Two-photon microscopy demonstrated that N-tagged α1s-DHPR (EGFP-α1s-DHPR) was targeted to the surface and TTS membranes of the muscle fibers. In the absence of DPA voltage-clamped enzymatically-dissociated fibers expressing EGFP-α1s-DHPR did not show voltage-dependent optical transients. Staining with 5 μM DPA allowed the recording of signals whose sign and amplitude were consistent with the voltage-dependent translocation of DPA molecules across the surface and TTS membranes and their subsequent FRET interaction with the N-terminal of the α1s-DHPR (to within ~6–7 nm). Furthermore, the kinetic features of these FRET signals suggest that, in response to step depolarizations, the N-domain of α1s-DHPR possibly moves away from the internal membrane plane within ~20 ms after the onset of the pulse. In contrast with what was observed with the α1s-DHPR, the expression of fluorescently tagged β-DHPR failed to report voltage-dependent FRET signals, which suggests that this subunit may be located relatively far away with respect to the membrane plane.

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