The average pore radius decreased from 3.6 to 2.4 angstroms. Without water in the pore, the PLN pentamer is highly unlikely to exhibit significant ion conductance. To quantitatively demonstrate this conjecture, steered molecular dynamics was employed to induce permeation of chloride and calcium ions through the pore. During each passage, the forced ions were observed to partially lose their hydration shells. A chain of water formed transiently in the pore after each ion passage. The results of our study strongly suggest that a PLN pentamer, in the conformation resolved by NMR, does not form an ion channel.

Ryanodine Receptors

2105-Pos Generation of the GFP-tagged Type 1 Ryanodine Receptor Mutants by Transposon-based Random Insertion Approach

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Board B220

Ryanodine receptor (RyR) is a family of intracellular Ca^{2+} release channels in the sarcoplasmic reticulum in skeletal muscle and plays an important role in excitation-contraction coupling. A relatively small C-terminal region (500–1,000 residues) of the RyR constitutes an ion channel and the other N-terminal region forms a large cytoplasmic assembly which serves as the regulatory domain. The structure-function relationship of RyR, especially in the cytoplasmic assembly, however, remained still unclear because of its large size. A tansposon-based random insertion approach is a powerful tool to study the structure-function relationship of large proteins. In this study, we applied this method to investigate the domain structure and function of the type 1 RyR (RyR1) by inserting GFP. The Tn5 transposon encompassing the kanamycin-resistant gene (*Kan*^{r}) which was flanked by two unique restriction enzyme sites (*Asc*I and *Pac*I) was randomly inserted into full-length RyR1 cDNA via in vitro transposon reaction using the EZ::TN transposase. Clones with an in-frame insertion of transposon were selected by kanamycin resistance, followed by DNA sequencing. Then, *Kan*^{r} was replaced by genes encoding GFP variants by *Asc*I / *Pac*I digestion. The resultant clones were transfected into Flp-In T-REx HEK293 cells to screen GFP fluorescence. The GFP-positive clones were tested by caffeine-induced Ca^{2+} release and some of these clones showed a reduced caffeine sensitivity. We are currently investigating [^{3}H] ryanodine binding and single channel activity of the GFP-tagged RyR1 mutants which was stably expressed in HEK293 cells. The transposon-based random insertion approach should be a powerful tool to study structure-function relationships of the RyR channels.

2106-Pos Interaction Of The K3614-N3643 Calmodulin-binding Domain With The C4114-N4142 Region Of The Ryanodine Receptor (RyR1) Is Involved In The Mechanism Of Ca2+/agonist-induced Channel Activation

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We tested the hypothesis that the interaction of the K^{3614}-N^{3643} calmodulin (CaM) binding domain (CaMBD) with its neighboring domain (e.g. CaM-like domain) serves as an intrinsic regulator of RyR1 by performing the following experiments (a and b). (a) We raised antibodies against synthetic peptides corresponding to the CaMBD and the C^{4114}-N^{4142} region, which is a part of the CaM-like domain. Both antibodies produced significant inhibition of [^{3}H] ryanodine binding activity, and the antibody concentration required for inhibition increased with an increase of [Ca^{2+}] from 0.1 to 1.0 μM. These data suggest that the binding of antibody to either side of the interacting domains interfered with Ca^{2+}-dependent formation of a 'channel activating link' between the two regions. (b) In order to monitor the state of inter-domain interaction and its changes during the Ca^{2+}/agonist-mediated channel activation, we employed the Stern-Volmer fluorescence quenching analysis of the accessibility of the fluorescent probe MCA, which was incorporated to the partner domain of CaMBD, to a large size fluorescence quencher, BSA-QSY. The quencher accessibility of the attached probe (namely, the size of the gap between the interacting domains) decreased with an increase of [Ca^{2+}] from 0.03 μM to 2.0 μM. The Ca^{2+}-dependent decrease in the quencher accessibility was more pronounced in the presence of 4-CmC, and was reversed by 1 mM Mg^{2+} (well-known inhibitor of Ca^{2+}/agonist-induced channel activation). These results suggest that the K^{3614}-N^{3643} and C^{4114}-N^{4142} regions of RyR1 interact with each other in a Ca^{2+}/agonist-dependent manner, which serves as a mechanism of Ca^{2+}/agonist-dependent activation of the RyR1 Ca^{2+} channel.

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2107-Pos Transient Relief Of Mg2+ Inhibition Of RyR1 Ca2+ Channel Is A Part Of The Mechanism Of 'Domain Switch'-mediated Channel Activation

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Relatively high concentrations of Mg^{2+} (~1 mM), normally present in the myoplasm, inhibit Ca^{2+}/pharmacological agonist (PA)-dependent channel activation, but the voltage sensor-mediated physi-

ological activation of RyR1 channels is not affected, unless [Mg^{2+}] increases to an abnormally high level (Lamb *et al.*). According to our hypothesis, a 'domain switch' (DS), composed of the interacting N-terminal and central domains of RyR1, plays a key role in e-c coupling. In order to test whether DS-mediated channel activation can overcome Mg^{2+} inhibition, we examined the effect of various concentrations of Mg^{2+} (0.16–3.0 mM) on the stopped-flow time course of SR Ca^{2+} release induced by two types of agonist: (a) domain peptides that activate the channel *via* the DS (DP4, DP1) and (b) PAs (4-CmC, caffeine). In the case of PA-induced Ca^{2+} release, both the magnitude and the initial rate of Ca^{2+} release decreased sharply in a range of [Mg^{2+}] from 0.3 mM to 1 mM. However, in the case of DS-mediated activation of Ca^{2+} release, there was virtually no change in both parameters of Ca^{2+} release up to 1.0 mM Mg^{2+}. In contrast to the Ca^{2+} release experiment, Mg^{2+} (1 mM) produced a severe inhibition of [^{3}H]ryanodine binding (the assay that requires a long incubation) not only in the presence of 4-CmC, but also in the presence of DP4. These data suggest that like the case of physiological activation of e-c coupling, the DS-mediated channel activation overcomes Mg^{2+} inhibition in a transient manner.

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2108-Pos The RyR1 SPRY2 Domain Binds to the DHPR α1S II-III Loop and to the RyR1 Binding Site for the β1a Subunit

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Board B223

Excitation-contraction coupling (ECC) links electrical signals to Ca^{2+} from the sarcoplasmic reticulum (SR) and depends on a physical interaction between the DHPR α_{1S} II-III loop and RyR1 in skeletal muscle, but the binding binding site for the II-III loop on RyR1 remains elusive. Skeletal ECC also depends on a variably spliced ASI region of RyR1 (Thr^{3471} - Gly^{3500}), which bind to the β_{1a} subunit of the DHPR (Cheng et al., 2005). Leong & Maclennan (1998) identified a 37 residue sequence in RyR1 that interacted with the II-III loop. Most of these residues are located within the SPRY2 region which is the second of three RyR1-SPRY domains. Our aims were to characterise binding of the RyR1-SPRY2 domain to the II-III loop and identify regions of the II-III loop involved in the interaction. We used nuclear magnetic resonance (NMR) and spectrofluorimetry to identify these regions. SPRY2 binds to the A and C regions of the II-III loop with affinities of 8.3 ± 0.3μM and 20.6 ± 0.4μM respectively. Mutations showed that acidic residues in SPRY2 (including Leong & Maclennan's residues)) bind to basic residues in the A region. The ASI region also contains a sequence of basic residues that are very similar in structure to those of α_{1S} II-III loop. A peptide corresponding to the ASI region interacts with the SPRY2 domain with an affinity of 1.4 ± 0.1μM. We provide compelling evidence that SPRY2 is a functional domain, which binds to the loop and to the ASI domain of RyR1.

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2109-Pos A Ca^{2+} Dependent Interaction of the Carboxy Terminal Tail with the Putative Ca^{2+} Binding Domain of RyR1

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A domain within RyR1 encompassed by amino acids 4062–4210 that binds Ca2+ and interacts with the calmodulin binding domain (amino acids 3614–3643) is now shown to also interact with the carboxyterminal tail of RyR1, suggesting that these regions could form a pivotal series of interactions for transducing ligand binding to cytoplasmic domains into changes in the activity of the channel in the transmembrane domain.

YFP tagged R4064–4210 was expressed alone and with CFP tagged C-terminal fragments. Coexpression of R4064–4210 with R4559–5037 (M1–> end), R4837–5037 (M5–> end), or R4938–5037 (the C-terminal tail) all yielded FRET. However, no FRET was observed between R4064–4210 and fragments missing the last 100 amino acids (R4559–4937 and R4837–4937). While Ca2+ does bind to R4064–4210 we were unable to alter [Ca2+]-dependent activity of RyR by mutating predicted -z position EF hand amino acids, implying that Ca2+ binds by an alternative mechanism.

2110-Pos β-Adrenergic Agonists Accelerate Heat-Induced Episodes in the Y522S Mouse Model of Malignant Hyperthermia

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Board B225

The skeletal muscle calcium release channel (RyR1) mutation of tyrosine 522 to serine (Y522S) is linked to Malignant Hyperthermia (MH) with cores in humans. The Y522S mouse displays the hallmark signs of MH as determined by sensitivity to volatile anesthetics and *in vitro* contracture test (IVCT). In addition to these defining characteristics the mice undergo heat-induced sudden death and may provide insight into the putative linkage between RyR1 mutations and Exertional Heat Illness. We established that intracellular calcium leak through the mutant RyR1 in response to heat involves oxidative stress and associated modifications of RyR1. Additional mechanisms contribute to the heat sensitivity. β-adrenergic mediated stress increases the probability of sudden death in

response to elevated environmental temperatures. We analyzed the effects of β-adrenoceptor agonist, isoproterenol, and antagonist, propranolol on heat-induced episodes in the Y522S mouse. The agonist hastened and antagonist attenuated the mutant animals' response to heat. IVCTs of solei muscles from mice treated with isoproterenol displayed an increased rate of contracture to passive heating. Calcium imaging of myotubes revealed that isoproterenol increased cytosolic calcium in the mutant derived myotubes. RyR1 phosphorylation at serine 2844 was equally enhanced in both genotypes. Membranes from isoproterenol and control treated animals did not enhance [^{3}H]-ryanodine binding when performed under reducing conditions. In the absence of DTT, phosphorylation enhanced [^{3}H]-ryanodine binding to the mutant membranes compared to the *wildtype.* The results indicate that phosphorylation of the oxidatively modified mutant channel increased the Ca^{2+} leak. We propose that β-adrenergic activation increases the probability of a MH episode in susceptible mice by increasing cytosolic calcium in skeletal muscle of mutant animals. Oxidative and phosphorylation modifications of mutant RyR synergize to enhance the probability of sudden death in response to elevations in environmental temperature.

2111-Pos Transient Expression Of Exogenous Ryanodine Receptor 1 And Ryanodine Receptor 3 Generates Different Calcium Release Events In Mouse Skeletal Muscle

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Mammalian skeletal muscles express two isoforms of RyR with different functional properties. RyR1 is the predominant isoform in the adult. RyR3 is widely distributed through most types of skeletal muscle during development, but is only found in limited amount in diaphragm and slow-twitch limb muscle in the mature animal. We showed that transient expression of RyR3 in adult skeletal muscle results in frequent spontaneous calcium release events and voltage-activated calcium sparks. The two types of events appear to be spatially segregated, in a pattern that presumably depends on the expression level of RyR3. Overexpression of wild-type RyR1 is associated with the occurrence of infrequent,spontaneous, slow calcium waves, but no voltage-activated Ca2+ sparks. An N-terminal EGFP-tagged RyR1 was also used to study the expression pattern of RyR. 3-D reconstructions of deblurred image stacks show targeting of the EGFP-RyR1 to the terminal cisternae of the sarcoplasmic reticulum, TC. 5 days after transfection the expression was heterogeneous: TC-located EGFP fluorescence was observable in the entire fiber, with marked concentration of fluorescence in a 50 μm area around individual nuclei.Calibrated with a standard, expression levels varied between 0.5 and >10 μM, a large excess over the native density. Ca2+transients and release flux were evaluated in voltage-clamped fibers by confocal scanning of fluorescence of rhod-2, introduced through the patch pipette. Dual scanning of EGFP and Ca2+ indicator showed that Ca2+ release flux was affected in high expression areas. There were no spontaneous Ca2+ release events, but high expression areas were bent, indicating Ca2+ leak. Work is in progress to further characterize the properties of the Ca2+ events mediated by the different isoforms.

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2112-Pos Inhibition of Ryr1 by Different Lanthanides Might Reveal Fine Details of the Ion Conducting Pore

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Effect of Europium on the gating of the RyR1 was investigated using Müller-type artificial bilayer system. Europium applied on the *trans* side inhibited the RyR1 channel by $K_d = 4.7 \pm 0.1$ μM (similarly to the Gd^{3+} effect), with a high cooperativity, as characterized by a Hill-coefficient of $N = 5.9 \pm 0.9$ in contrast to Gadolinium, which exhibited N = 4. Inhibition of the RyR1 activity from the *cis* side was also different from that of Gadolinium, characterized by $K_d = 167 \pm 5.0$ nM and $N_{hill} = 2.0 \pm 0.1$. The Eu^{3+} inhibition was potential independent if the charge carrier moved according to the physiological direction of calcium release, while it was potential dependent (and proportional with the driving force) if the current was opposite to the current during calcium release. We assume, that the Europium binding site is in or near to channel pore because of voltage dependence of the Europium blockade. Effect of Europium was similar on the Ryanodine binding of HSR vesicles with slightly different affinity probably due to the unspecific Europium binding by the associated junctional proteins. Ryanodine exerted its characteristic effect locking the channel into its half-conductance state on the Europium modified channel independently that Europium was applied from the *cis* or from the *trans* side. A model explaining these data - considering the different ionic diameters for calcium, gadolinium and europium - is proposed to explain these findings and to propose further investigation of the ion conducting pore of the RyR1, based on the different ion-diameters of the lanthanides (lanthanide contraction).

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2113-Pos Partial Phenotypic And Functional Characterization Of Leukocytes From Ryr1 R163c MH Mice

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Board B228

Ryanodine receptors (RyRs) are ER calcium channels expressed in all nucleated cells. The three RyR isoforms are differentially

expressed in cells of hematopoietic origin. RyR1 is preferentially expressed in peripheral monocytes, dendritic cells and B lymphocytes. To understand the role of the RyR1 on the development and function of these cells, we compared bone marrow and splenic myeloid and lymphoid cells from knock in mice expressing the R163C gain-of-function mutation in the RyR1 gene to those from wild type littermates. The R163C mutation predisposes heterozygous human carriers and knockin mice to the disease malignant hyperthermia (MH). Flow cytometry assays revealed differences in the prevalence of murine B cell precursors in R163C bone marrow. In the spleen, R163C mice exhibited significant differences vs wild type littermates in (1) the overall cell number and (2) the proportion of leukocyte subsets. In proliferation assays R163C splenocytes displayed an unexpected pattern of reactivity to mitogens. These findings suggest that RyR1 has had a previously underappreciated role in the development and function of myeloid and lymphoid immune cells.

2114-Pos Reactive Cysteines C1040 and C1303 of Ryanodine Receptor Type 1 Influence Responses to Oxidative Stress Produced by 1,4-Naphthoquinone and GSH Depletion

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Board B229

Redox modulation of the skeletal muscle ryanodine receptor1 (RyR1) plays a key role in determining the responsiveness of the Ca^{2+} release channel to physiological modulation. The sensitivity of RyR1 to 1,4-napthoquinone (NQ) and glutathione (GSH) may be conferred by previously identified hyper-reactive cysteines, including positions C1040 and C1303. Wild type RyR1 (WT), C1040S, and C1303S mutations were stably expressed in HEK-293 cells and their sensitivity to NQ-induced cytotoxicity was determined with the MTS assay. NQ decreased cell viability in a does-dependent manner, but WT cells were significantly less sensitive than null cells lacking RyR1 expression. HEK-293 cells expressing WT and C1303S were significantly less susceptible to NQ compared to C1040S expressing cells. GSH depletion was induced by inhibiting γ-glutamyl cysteine synthase with buthionine sulfoximine (BSO, 100 μM) for 24 hr, but depletion by itself did not impair cell viability for any of the genotypes tested. Challenge of null and WT cells following depletion of GSH revealed no difference in sensitivity to NQ. However both C1040S and C1303S mutants showed reduced sensitivity to the cytotoxic effects of NQ in the presence of GSH depletion. These results indicate a protective role of RyR1 towards the cellular toxicity induced by a redox cycling and arylating quinone (NQ). The data also suggests an involvement of hyper-reactive cysteines C1040 and C1303 in determining responses to oxidative stress.

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2115-Pos Myoplasmic Resting Ca2+ Reveals Enhanced RyR1 Basal Activity Of Triadin-null Myotubes

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Board B230

Previously we have shown that deletion of triadin in mouse skeletal muscles results in a significant increase in myoplasmic resting Ca^{2+} concentration ($[Ca^{2+}]_r$) of both primary myotubes and adult fibers. To identify the mechanism responsible for this Ca^{2+} elevation we used Ca^{2+} sensitive microelectrodes and several pharmacological tools to assess the contribution of different Ca^{2+} pathways on the overall resting calcium of cultured myotubes. Under control conditions $[Ca^{2+}]_r$ of wt- and triadin-null myotubes was of 117±3 nM and 212±3 nM (mean ± SEM), respectively. Inhibition of sarcolemmal calcium entry with 0.5mM Cd^{2+}/0.1 mM La^{3+} reduced $[Ca^{2+}]_r$ in both wt- and triadin-null myotubes, to 99±1 nM and 150±3 nM, respectively. Pre-treatment of the triadin-null myotubes with 2APB (50μM) had similar effect as Cd^{2+}/La^{3+}, reducing $[Ca^{2+}]_r$ to 151±2 nM. Inhibition of DHPR activity with Nifedipine (5μM), in the other hand, had no significant effect on $[Ca^{2+}]_r$. Pretreatment with 15μM ryanodine, a condition that effectively blocks caffeine-induced Ca^{2+} release in Fluo-4 loaded myotubes, resulted in significant reduction of $[Ca^{2+}]_r$ in triadin-null cells from 212±3 nM to 171±3 nM, but had no major effect in wt-myotubes (127±3 nM vs 117±3 nM). Treatment with Cd^{2+}/La^{3+} in ryanodine pre-treated cells further reduced $[Ca^{2+}]_r$ of triadin-null myotubes compared to either treatment separately to 146±1 nM. These data suggest that the increased intracellular resting Ca^{2+} observed in triadin-null myotubes is a combination of increased basal RyR1 channel activity and enhanced extracellular Ca^{2+} entry. This supports an inhibitory role for triadin on RyR1 activity.

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2116-Pos Catechins - Polyphenol Compounds of Green Tea Extracts Regulate Both Skeletal and Cardiac Type Ryanodine Receptors

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Board B231

Catechins are the major components of polyphenols in the leaves of green tea. The active principles are epicatechin (EC), epicatechin gallate (ECG), epigallocatechin (EGC) and epigallocatechin gallate (EGCG). Catechins have protective effects against atherogenesis,

carcinogenesis, and neurodegeneration. Epidemiological studies have shown that intake of tea catechins is associated with a lower risk of cardiovascular disease. Some studies with EGCG have suggested that its protective effects are at least in part attributable to its direct influence on intracellular Ca^{2+} homeostasis, possibly by targeting Ca^{2+} entry pathways, and/or release from internal Ca^{2+} stores. Here we report that tea catechins directly react with both skeletal and cardiac ryanodine receptors (RyR1 and RyR2) and potently regulate the activities of these channels. [^{3}H]ryanodine binding assay and measurement of purified RyR single channels reconstituted in planar lipid bilayer were used to assess the functional influences of catechins. [^{3}H]ryanodine binding analysis revealed that EGCG, ECG and EGC act as potent activators of sarcoplasmic reticulum membrane-bound RyR1 and RyR2, whereas EC has negligible effect. Compared to a saturating concentration of caffeine (30mM), EGCG or ECG at 30-fold lower concentration (10μM) produced 5-fold higher efficacy toward RyR channel activity. EGCG, ECG or EGC (300–500nM) significantly affected channel gating kinetics - increasing open probability, prolonging mean open time and shortening mean closed time. The current findings reveal that both RyR1 and RyR2 Ca^{2+} release channels are a sensitive target of the active principles green tea extracts - the polyphenol catechins. These studies provide a framework for understanding the protective actions of these catechins in physiological and pathophysiological states.

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2117-Pos Intracellular Distribution of Ryanodine Receptors in Rainbow Trout Cardiomyocytes

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Board B232

In cardiomyocytes from rainbow trout, it is uncertain how important Ca^{2+}-induced Ca^{2+}-release via ryanodine receptors (RYRs) from the sarcoplasmic reticulum (SR) is in excitation-contraction (e-c) coupling. Compared with mammalian cardiomyocytes, the SR is more sparsely developed, and the effect of inhibiting RYR Ca^{2+}-release with ryanodine varies with experimental conditions. Further, it has been suggested that the SR becomes more important for e-c coupling with acclimation to cold. The aim of this study was to investigate the distribution of RYRs in rainbow trout cardiomyocytes, and how the expression changes with temperature acclimation (7, 14 and 21°C). For cellular localization, we used an immunocytochemistry approach. Because trout cardiomyocytes are relatively thin (~ 3 μm) and the antibody signal faint, we found imaging with widefield microscopy followed by deconvolution gave a better result than confocal imaging. The distribution of RYR varied significantly between individuals. In cells from some animals, we observed only a dense labeling in the central core, where the mitochondria are. In cells from other animals, we also observed a striated pattern that aligned with the sarcomeric m-band. The great individual variation was supported by western blotting used to quantify RYR expression. We found that individual variation was greater than any potential effect of temperature acclimation. This high variability may explain the uncertainty about the functional importance of Ca^{2+}-induced SR Ca^{2+}-release via RYRs during e-c coupling.

2118-Pos Structural studies on the Cardiac Ryanodine Receptor by Neutron Reflectometry and Small Angle Neutron Scattering

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Board B233

The ryanodine receptor (RyR) is a large ion channel of approximately 2.3Mda. Due to lack of crystallographic data, the 3-dimensional structure of the RyR has been proposed by electron microscopy (EM). Reconstructions of ligand-bound RyR complexes have reached resolutions better than 30 Angstroms, although they can be difficult to interpret due to the small size of the ligands (<20kDa). Neutron scattering using deuterium labelling is a powerful method for the solving of interacting complex biological structures. We have therefore examined structural characteristics of the purified cardiac isoform (RyR2) by neutron reflectometry (NR) and small angle neutron scattering (SANS). Our neutron measurements give complimentary information to EM studies, with the aim of providing evidence for the localization of ligand binding sites on RyR2, relative to each other and to the membrane. Preliminary NR data, collected on ILL instrument D17, gave insight into the integration of RyR2 into lipid bilayers. The binding of deuterated GST-FKBP12.6 was monitored by changes in the diffraction signal. Subsequently, though it required much larger quantities of material (milligram range), we were able to measure the size of solubilized RyR2 by SANS on ILL instrument D22 (Radius of gyration ~30nm).

Supported by the British Heart Foundation

2119-Pos Regulation of Ryanodine Receptors from Cardiac Muscle by Luminal Ca^{2+} and Mg^{2+}

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Board B234

Luminal stimulation of cardiac RyRs (RyR2) involves three Ca^{2+} sensors on both the luminal and cytoplasmic side of the RyR; the activating luminal *L*-site (40 μM affinity) and cytoplasmic *A*-site (1 μM) and the inactivating cytoplasmic I_2-site (1 μM). The *L*-site can initiate channel openings allowing luminal Ca^{2+} to the *A*- and I_2-sites. Cytoplasmic Mg^{2+} inhibits RyRs by displacing Ca^{2+} from the *A*-site but the possibility that similar processes occur at the *L*- and I_2-sites has not been explored.

Sheep heart RyRs were incorporated into artificial lipid bilayers. A novel inhibition of RyR2 by luminal Mg^{2+} was observed, pointing to an important role for luminal Mg^{2+} in cardiac muscle. At diastolic cytoplasmic $[Ca^{2+}]$ ($[Ca^{2+}]_C$ = 100 nM) luminal Mg^{2+} inhibition was *voltage-independent* and was alleviated by increasing $[Ca^{2+}]_L$ from 0.3 mM (K_i for Mg^{2+} = 90 μM) to 1 mM (K_i = 1 mM). At systolic $[Ca^{2+}]_C$ (1 – 10 μM), Mg^{2+} inhibition was substantially reduced and its properties were consistent with luminal Mg^{2+} flowing through the channel and binding to the cytoplasmic *A*-site. Under these conditions K_i was *voltage-dependent*; 13 mM at −40 mV and >100 mM at +40 mV.

The data was accurately fitted by a model in which Mg^{2+} and Ca^{2+} compete at both the *L*- and *A*-sites and where the *L*-site has similar affinities for both ions. The model predicts that under physiological divalent ion concentrations (1 mM free Mg^{2+} in the cytoplasm and lumen) and membrane potential (0 mV), $[Ca^{2+}]_L$-activation of Ca^{2+} release is primarily due to displacement of Mg^{2+} from the *L*-site and that luminal Mg^{2+} is an essential cofactor for the phenomenon. Therefore competition between luminal Ca^{2+} and Mg^{2+} may play an essential role in store-load dependent Ca^{2+} release.

2120-Pos Mechanisms for Amitriptyline Activation of Cardiac RyRs and SR Ca^{2+} Release

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Board B235

Epidemiologic studies have linked the antidepressant amitriptyline (AMT) to increased risk of sudden cardiac death. AMT binds to cardiac calsequestrin (Casq2), a sarcoplasmic reticulum (SR) protein that regulates ryanodine receptor Ca^{2+}-release channels (RyR2). We measured AMT effects on RyR2 in lipid bilayers and on SR Ca^{2+} release in cardiomyocytes both lacking and containing Casq2. In bilayers, AMT on either side of the membrane induced trains of long channel openings (bursts) with 60–90% of normal conductance. AMT induced similar bursts in RyR2 from Casq2 null (Casq2−/−) mice indicating that bursts were not mediated by AMT binding to Casq2. However, AMT at low (2–3 μM) but not at high concentrations (5–10 μM) produced more activation in Casq2+/+ RyRs than Casq2−/− RyRs, suggesting that Casq2 modulates AMT action. In sheep RyR2, the [AMT]-, voltage- and P_o-dependencies of burst frequency and duration indicates that AMT binds primarily to open RyR2 at a site within the bilayer. The AMT EC_{50} (2 mM ATP, 1 μM Ca^{2+}) was 20 μM at +40 mV and 200 μM at −40 mV. EC_{50} was proportional to basal P_o and variations in P_o brought about by changing cytoplasmic Ca^{2+} and Mg^{2+} altered EC_{50} accordingly.

In intact mouse cardiomyocytes of either genotype, AMT increased the rate of spontaneous Ca^{2+} releases and decreased the SR Ca^{2+} content. Interestingly, low AMT concentrations (0.5–1 μM) produced significantly greater effects in Casq2+/+ compared with Casq2−/− cells. Taken together, these data suggest that AMT activates SR Ca^{2+} release by acting directly on RyR2. However, the presence of calsequestrin *sensitizes* RyR2 to the effects of AMT.

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2121-Pos Ryanoids and Imperatoxin affect the modulation of cardiac Ryanodine Receptors by Dihydropyridine Receptor Peptide A

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During cardiac stimulation, Ca^{2+}-entry via L-type Ca^{2+} channels (DHPR) is known to trigger ryanodine receptor (RyR)-mediated Ca^{2+}-release from sarcoplasmic reticulum (SR). Recently, DHPR have also been found to inhibit Ca^{2+} sparks in resting cells via a Ca^{2+}-entry independent mechanism. A DHPR peptide of the loop II–III (PepA) was reported to modulate isolated skeletal and cardiac RyRs in a complex manner. Here, we found that PepA induced "flicker block" and transition to subconductance states (substates) of fully-activated cardiac RyRs reconstituted in lipid bilayers. PepA-induced substates and block events had different voltage dependences. Substates did not represent occupancy of a ryanoid site. However, the increased affinity for ryanodol in the presence of PepA and the increased occurrence of PepA-induced substates upon addition of ryanoids suggest cooperative interaction between sites. Imperatoxin-A competitively excluded PepA from the substate site(s) and prevented the access of PepA to the block site. The action of PepA on RyR2 was mimicked by inactivating gate moieties ("ball peptides") from K^+ and Na^+ channels (ShakerB and KIFMK, respectively). The actions of ShakerB and KIFMK (well resolved substates and "slow-block" events) allowed, by comparison, better understanding of the action of PepA on RyR2. We hypothesize that the cytosolic RyR2 conduction pathway may interact with PepA through a two-step mechanism, reminiscent of the inactivation of voltage-gated channels. The first step includes binding to an outer "vestibular" region (substates). Then PepA moves deeper into the RyR2 transmembrane field and blocks the channel. Therefore, it would be of interest to address the physiological significance of a "PepA-like action" for local and global RyR2-mediated Ca^{2+} release in cells.

2122-Pos Endurance Training Reduce Diastolic SR Ca^{2+} Leak From RyR And Restores Contractile Function In Mice With Type 2 Diabetes

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Purpose: Increased diastolic SR Ca^{2+} leak from ryanodine receptors (RyR) have been proposed to be the mechanism behind depressed contractility in the diabetic heart. In this study, contractile performance and calcium handling was studied in isolated cardiomyocytes in trained and sedentary db/db and in wild-type mice.

Methods: Male db/db mice and wildtypes were randomized to sedentary control or regular interval training on a treadmill for 12 weeks, 5 days/week, 1.5 hours/day, at 85–90% of maximal oxygen uptake (VO2max). Cardiomyocytes were stimulated (1Hz) on an inverted epi-fluorescence microscope. SR Ca^{2+} leakage was determined using tetracaine and caffeine.

Preliminary results: VO2max increased 23% in trained db/db-mice ($p<0.01$). Cardiomyocyte fractional shortening (3.5±1.6% vs. 8.4±2.3%) and calcium amplitude (Fura-2 ratio 0.07±0.02 vs. 0.15±0.03) was lower, the rate of Ca^{2+} decay 47% slower, and Ca^{2+} leakage (normalized to total SR Ca^{2+} load) significantly higher (31% vs. 7%) in db/db mice compared with wild-type controls ($p<0.05$). Endurance training restored fractional shortening, Ca^{2+} amplitude, and Ca^{2+} decay to a level not significant from wild-type controls. In trained db/db mice, Ca^{2+} leakage from RyR (9%) was similar to that observed in wild-type mice, and probably explains increased SR Ca^{2+} content and increased calcium amplitude. Furthermore, improved Ca^{2+} decay was due to increased activity of the SR- Ca^{2+} pump and not increased sodium-calcium exchanger activity. The protein analysis showed normalised SR- Ca^{2+} pump expression, reduced total pCaMKII, increased pPLB by PKA and reduced pPLB by CaMKII in trained db/db ($p<0.05$). More functional data when blocking CaMKII and PKA together with exercise training effects on wildtypes will be presented at the meeting.

Conclusions: Endurance training improves cardiomyocyte function in diabetes type-2 mice through reduced RyR-leakage and increased SR- Ca^{2+} pump activity.

2123-Pos Mutation-Linked Structural Instability Underlies RyR2 Channel Hypersensitivity To Serum Factors

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The stress-induced nature of arrhythmias in individuals with mutations in the cardiac ryanodine receptor (RyR2) is consistent with a 'triggering' event that acutely promotes RyR2 dysfunction. We previously showed that mutant RyR2 (R4497C) mediated augmented Ca^{2+} fluxes in single HL-1 cells following exposure of cells to serum obtained from 'exercised' normal and arrhythmia-susceptible cohorts. However, profiling serum-induced RyR2 Ca^{2+} release determined that 'exercised' serum at concentrations $>$ 100μg/ml triggered global Ca^{2+} transients in cells expressing WT or mutant RyR2 (21±9% and 57±23% total cell populations, respectively). Ca^{2+} transients were larger in cells expressing mutant RyR2 (146 ± 21% compared to WT RyR2 (100%)). In all populations, global Ca^{2+} transients were only evoked in cells that had previously been exposed to $>$10μg/ml 'resting' or 'exercised' serum. Studies using recombinant RyR2 channels containing an intra-sequence cyan and yellow fluorescent protein FRET tandem that exquisitely reports intra-RyR2 structural rearrangements revealed that cellular exposure to 10–50μg/ml 'resting' serum resulted in persistent conformational changes in RyR2 that were significantly greater in the mutant channels (128±7% versus 100% (WT)). There was a remarkable correlation between the extent of serum-induced conformational rearrangement of RyR2, and the propensity of cells for triggered global Ca^{2+} transients. Incubation of cells in physiological concentrations of adrenaline (Adr), noradrenaline (NAdr) and dopamine (Dopa) (pg/ml: Adr, 60; NAdr, 300; Dopa, 150; determined in cohorts' 'resting' serum) resulted in small but equivalent structural rearrangements in WT and mutant RyR2, suggesting that circulating catecholamines may not directly promote the augmented structural rearrangement and lower threshold for activation that characterises mutant RyR2. Our data supports a model in which mutations induce the persistent structural instability in RyR2 that increases the likelihood of abnormal Ca^{2+} release in response to stress-induced, pro-arrhythmic triggers.

2124-Pos Suppression Of Arrhythmogenic Ca^{2+} Fluxes In Cardiac Cells Using Fragments Of Human RyR2

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Physical interaction between discrete domains of the cardiac ryanodine receptor (RyR2) is a pivotal mechanism of channel regulation. Acquired or genetically-induced perturbations in intra-RyR2 interactions promote channel dysfunction associated with heart failure and arrhythmia pathogenesis. As a result, stabilisation of RyR2 conformation has emerged as an important therapeutic strategy for normalising Ca^{2+} cycling in cardiac disease. We have previously identified a surface-accessible, hinge-like region of RyR2, termed the I-domain (residues 3722–4610) that is a key determinant of channel stability and a major locus for arrhythmia-linked mutations. We investigated the acute effects of microinjected fragments corresponding to distinct regions of the I-domain on intracellular Ca^{2+} handling and cellular phenotype in beating monolayers of fluo4-loaded HL-1 cardiomyocytes. The SALVO program that analyses the Ca^{2+}-handling basis of cellular rhythmicity, synchronicity and contractility (Barberini et al., this meeting) revealed that I-domain fragments potently and rapidly suppressed caffeine-induced increases in cellular Ca^{2+} fluxes. Remarkably, Ca^{2+} fluxes were also reduced in adjacent non-microinjected cells. This 'bystander effect' extended to both physically coupled and spatially separated cell populations (within 12,000μm^2) and is consistent with roles for direct cell-to-cell communication via gap junctions, and the intercellular transfer of diffusible effectors. Recombinant expression of I-domain fragments in RyR2-deficient HEK cells

suppressed carbachol-induced Ca^{2+} fluxes in transfected and non-transfected cells, directly corroborating the bystander effect determined in HL-1 cells, and also implicating the possible involvement of IP_3R in this phenomenon. Our results suggest that epitopes within the RyR2 I-domain represent feasible and attractive targets for acutely manipulating cardiac Ca^{2+} handling and reducing arrhythmogenic Ca^{2+} fluxes. The suppression of Ca^{2+} fluxes in non-transduced adjacent cells via a bystander effect has significant implications for the future therapeutic modulation of Ca^{2+} signalling in the myocardium.

2125-Pos MicroRNA *miR-1* Overexpression Enhances Intracellular Ca Release and Promotes Cardiac Arrhythmogenesis by Targeting PP2A Regulatory Subunit B56alpha and Causing CAMKII-Dependent Hyperphosphorylation of Ryanodine Receptor

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MicroRNAs are small endogenous noncoding RNAs that regulate protein expression by hybridization to complementary sequences of target mRNAs. Changes in abundance of muscle-specific microRNA, *miR-1*, have been implicated in cardiac disease, including arrhythmia and heart failure. However, the specific molecular targets and cellular mechanisms involved in the action of *miR-1* in the heart are only beginning to emerge. In this study we investigated the effects of increased expression of *miR-1* on excitation-contraction coupling and Ca cycling in rat ventricular myocytes by using methods of cellular electrophysiology, Ca imaging and quantitative immunoblotting. Adenoviral-mediated overexpression of *miR-1* in myocytes resulted in a marked increase in the amplitudes of the inward Ca current (ICa) and cytosolic Ca transients and enhanced the frequency of spontaneous Ca sparks while reducing the sarcoplasmic reticulum (SR) Ca content as compared with control. In the presence of isoproterenol, rhythmically paced, *miR-1*-overexpressing myocytes exhibited spontaneous arrhythmogenic oscillations of intracellular Ca and membrane potential, events that occurred only rarely in control myocytes under the same conditions. The effects of *miR-1* were completely reversed by the CAMKII inhibitor KN93. Although phosphorylation of phospholamban was not altered, *miR-1* overexpression selectively increased phosphorylation of RyR2 at CAMKII Ser-2814 but not PKA Ser-2808 site. Overexpression of *miR-1* was accompanied by a selective decrease in expression of the PP2A regulatory subunit b56alpha involved in PP2A targeting to specialized subcellular domains. Luciferase reporter assays demonstrated that *miR-1* could directly interact with the 3′-UTR of the PP2A regulatory subunit b56alpha mRNA. We conclude that *miR-1* enhances cardiac excitation-contraction coupling by selectively increasing phosphorylation of the L-type and RyR2 channels via disrupting localization of PP2A activity to these channels.

2126-Pos Modulation of Resting Ca Sparks in Intact Myocytes by Endogenous CaMKIIδc

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Acute activation of both exogenous and endogenous Ca-Calmodulin dependent protein kinase (CaMKII) in permeabilized phospholamban knockout (PLB-KO) mouse myocytes can phosphorylate RyR and activate Ca sparks independent of SR Ca load (Circ Res 2006;*99*:398–406). To assess the role of CaMKII on resting Ca release in intact myocytes (independent of SR Ca content or PLB effects), we compared Ca sparks in PLB-KO and novel double transgenic mice (DTG) that are produced by crossbreeding PLB-KO with CaMKIIδC overexpressing mice. Compared with PLB-KO mice, these DTG cardiomyocytes exhibited: (1) increased twitch Ca transient and fractional release (by ~40%), but unchanged SR Ca load; (2) increased resting Ca spark frequency (300%), despite the lower diastolic [Ca2+]i, which implies enhancement of RyR Ca sensitivity by CaMKII-dependent phosphorylation; (3) elevated Ca spark amplitude and rate of Ca release (which might suggest that more RyR channels participate in a single spark); (4) prolonged Ca spark rise time (which indicates that CaMKII either delays RyR closure or prolongs the time when openings can occur); (5) repetitive sparks at a single release site occurred much more frequently in DTG vs. in PLB-KO myocytes. Analysis of repetitive sparks from individual Ca release sites indicates that CaMKII enhanced Ca sensitivity of RyR channel, but not intrinsic restitution properties. These results were consistent with a positive modulation of RyR Ca release via a regulation of both RyR activation and the Ca spark termination process.

2127-Pos FRET between FKBP and CaM located on the Ryanodine Receptor in the Cardiac Myocyte Environment

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We have used site-directed labeling and FRET to evaluate distance relationships between FKBP and CaM bound to functional ryano-

dine receptors (RyR2) in situ in cardiac myocytes. Prior cryo-EM studies suggest the apoCaM and FKBP RyR binding sites are within ~9 nm of each other. To test this in the cellular environment, we measured FRET between fluorescently labeled FKBP and CaM in permeabilized rat cardiomyocytes. Single-cysteine FKBP mutants were labeled with fluorescein-5-maleimide or Alexa Fluor 488 maleimide as FRET donors (D), obtaining functionally-silent D-FKBP variants. Single-cysteine CaM variants were labeled with Alexa Fluor 568 as FRET acceptor (A), obtaining functionally-silent A-CaMs. Both D-FKBP and A-CaM were highly localized to the Z-line. Three methods were used to assess FKBP-CaM FRET in myocytes where intracellular [Ca]i was clamped at 10 nM. First, the addition of D-FKBP produced a robust FRET signal detected as an increase in the A-CaM fluorescence. FRET was abolished by rapamycin (due to dissociation of D-FKBP donor from its sites). Second, addition of A-CaM quenched direct D-FKBP fluorescence. The fractional decrease in donor signal upon acceptor addition reflects the amount of FRET. Third, after attaining steady state in the D-FKBP and A-CaM channels, we photobleached the acceptor and calculated FRET from the enhancement of D-FKBP fluorescence. The FRET efficiency between D-FKBP and A-CaM labeled in the N-lobe was 35.9 ± 1.8% (n= 12), corresponding to a distance of 6.9 ± 0.1 nm. This is identical to the distance measured in parallel determinations we carried out in isolated SR. These results are for CaM labeled at the N-lobe, but FRET between C-lobe labeled CaM and FKBP is substantially less. These results provide direct in situ measurements of FKBP-CaM localization on the functional RyR2.

2128-Pos The Effects of Ca^{2+}/ Calmodulin-Dependent Protein Kinase Phosphorylation of Single RyR2 Channels

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CaMKII phosphorylation of RyR2 has been reported to increase RyR2 channel activity (Witcher *et al.* J Biol Chem. 1991; Wehrens *et al.* Circ Res. 2004) but the phosphorylation site/s involved are disputed. We have therefore correlated the effects of CaMKII-dependent phosphorylation of RyR2 channels reconstituted into planar lipid bilayers with the phosphorylation state of S2809. Under voltage-clamp conditions, RyR2 channels incubated for 10 minutes in the presence of 12.5 units/ml of CaMKII (in the presence of 1μM CaM, 1mM ATP, 5mM Mg^{2+}) (P_o 0.269 ± 0.153 (SEM; *n*=6)) exhibited a small but non-significant increase in P_o compared with control channels (P_o 0.092 ± 0.032 (SEM; *n*=6)). This contrasts with the effects of PKA-dependent (10 units/ml) phosphorylation of RyR2 channels under similar experimental conditions, where P_o rose significantly from 0.126 ± 0.035 to 0.574 ± 0.106 (SEM; *n*=10; $p<0.01$). Using antibodies specific for the phosphorylated and dephosphorylated states of S2809 (Badrilla Ltd, Leeds), Western blot analysis demonstrated that both CaMKII and PKA treatment increases RyR2 phosphorylation at S2809. Thus, while both PKA and CaMKII phosphorylate RyR2 at S2809, only PKA treatment is effective at activating the channel. These results suggest that PKA phosphorylation at a site other than S2809 may be primarily responsible for the increase in P_o.

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IP_3 Receptors

2129-Pos Isoform-specific Modulation of Inositol (1,4,5)-trisphosphate Receptors by Cytosolic ATP

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Inositol (1,4,5)-trisphosphate receptors (InsP3R) are the predominant route of calcium release in non-excitable cells and they play a major role in regulating calcium signaling in numerous physiological systems. There are three known isoforms (InsP3R-1, InsP3R-2 and InsP3R-3) and multiple splice variants of InsP3R expressed in mammalian cells. This sequence diversity along with varied tissue distributions hints at important isoform-specific regulatory mechanisms. One such regulatory mechanism is the modulation of calcium release from InsP3R by cytosolic free ATP. All three isoforms contain putative ATP binding domains, with InsP3R-1 expressing two such domains (ATPA, and ATPB) in the S2+ isoform and three sites (ATPA, ATPB and ATPC) in the S2- splice variant while InsP3R-2 and InsP3R-3 each express a single ATPB site. Functionally, ATP has been shown to positively regulate InsP3R-1 and InsP3R3 while InsP3R-2 is thought to be insensitive to ATP modulation. The purpose of this study was to examine the contributions of ATP binding sites to the calcium release properties of the individual isoforms. TNP-ATP binding assays using GST-fusion proteins containing the ATP binding domains were used to confirm ATP binding. Calcium release assays from permeabilized cells were used as a means of measuring the effects of ATP on endogenous InsP3R in native exocrine acinar cells and on individual wild type or mutant isoforms expressed in DT40-3ko cells. The results presented here demonstrate that, contrary to prior studies, InsP3R-2 can indeed be modulated by ATP. In addition, even though InsP3R-2 and InsP3R-3 contain identical ATP biding sites, they exhibit dramatically different sensitivities to ATP. The impact of this differential modulation can therefore depend on the metabolic state of the cell and on the relative abundance and localization of the three InsP3R isoforms.

2130-Pos Constructive Use Of Noise In Intracellular Calcium Oscillations

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