able to increase wave frequency. On the other hand, FKBP12.6 was not (from 0.12 ± 0.04 Hz to 0.16 ± 0.04 Hz (SEM; n=4; $P>0.35$). Our results indicate that FKBP12 may have an important role as an activator of RyR2 in cardiac cells. Further work is required to determine the individual and combined roles of FKBP12 and FKBP12.6 in cardiac EC-coupling.
Supported by the British Heart Foundation.

1562-Pos
FKBP12 is a High Affinity, Reversible Activator of RyR2, and FKBP12.6 Antagonises Its Actions
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FKBP12.6 binds tightly to RyR2 and evidence suggests that it plays a vital physiological role in regulating channel activity. Moreover, changes in FKBP12.6/RyR2 interactions have been implicated in heart failure. Controversy exists, however, as to how FKBP12.6 affects the single-channel behaviour of RyR2. Furthermore, although higher levels of FKBP12 than FKBP12.6 are present in cardiac cells, the effects of FKBP12 on RyR2 are virtually unresearched. We have therefore compared the effects of FKBP12 and FKBP12.6 on the single-channel function of sheep RyR2 incorporated into bilayers under voltage-clamp conditions. We find that FKBP12 increases RyR2 open probability (Po) in a dose-dependant, reversible manner with an EC_{50} of 51 nM. In the presence of 10 μM cytosolic Ca^{2+}, physiological levels of FKBP12 (3 μM) increased Po from 0.187 ± 0.051 in controls to 0.657 ± 0.111 (SEM; n=14; $P<0.001$). In contrast, under identical experimental conditions, FKBP12.6 did not significantly increase or decrease RyR2 Po, however, it was able to antagonise the actions of FKBP12, shifting the EC_{50} value for FKBP12 to 4 μM. Our experiments demonstrate that FKBP12 has high affinity for RyR2 and that at physiological concentrations (1-3 μM) is an effective activator of the channel thereby suggesting that FKBP12 may have a more important role in cardiac excitation-contraction coupling than previously thought. We hypothesise that FKBP12.6 is a very low efficacy (but high affinity) partial agonist of RyR2 and that the balance between the effects of FKBP12 and FKBP12.6 is crucial for normal EC-coupling in cardiac cells.
Supported by the British Heart Foundation

1563-Pos
Imperatoxin Induces a Biphasic Response in Ca^{2+} Sparks
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Imperatoxin induces a biphasic response in Ca^{2+} sparks
Imperatoxin A (IpTxa), isolated from the venom of the African scorpion *Pandinus imperator,* has been shown to specifically activate ryanodine receptors (RyR) and to be capable of translocating across cell membranes. IpTxa enhances [^{3}H]ryanodine binding to sarcoplasmic reticulum (SR) and stabilizes subconducting states in single channels. We previously demonstrated that IpTxa alters the amplitude of calcium transients in intact field-stimulated cells, causing a rapid increase in transient amplitude. This is followed by a gradual decrement in amplitude to a new steady state at lower amplitude than in control. The current study seeks to clarify how IpTxa acts on RyRs to perturb Ca^{2+} handling in cardiomyocytes. We employed visualization of IpTxa-modified Ca^{2+} sparks in saponin-permeabilized cells to facilitate direct titration of RyRs with known concentrations of IpTxa, ranging from 500pM to 50nM. In addition, we modified our sparks protocol to enable a comparison of the caffeine-releasable SR Ca^{2+} load before and after treatment with the toxin. Our results demonstrate that IpTxa induces a biphasic RyR response, typified by a transient increase in spark frequency, amplitude, FWHM, and FDHM, which is rapidly followed by a sharp decrease in the same parameters. Comparison of pre- and post-toxin caffeine-releasable SR Ca^{2+} consistently reveals that SR content has been reduced as a result of IpTxa perfusion to approximately 75% of control. These results are consistent with the biphasic response observed in Ca^{2+} transient experiments. We believe that IpTxa sensitizes RyR to luminal Ca^{2+}, leading to increased Ca^{2+} release and subsequent depletion of Ca^{2+} from the SR. Our findings have exciting implications for translational research into cardiac diseases such as catecholaminergic polymorphic ventricular tachycardia, in which acute RyR hyperactivity is hypothesized to trigger arrhythmias leading to sudden cardiac death.

1564-Pos
Role of Hydrophobic Interactions in the Block of the Ryanodine Receptor by *Shaker* B Inactivation Peptides
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Shaker B K^+ channel NH_2-inactivation peptides (*Sh*BP) block both sheep and mouse cardiac ryanodine receptor Ca^{2+} release channels (RyR2). We provide new evidence for the presence of hydrophobic residues in the conduction pathway of RyR2 and their role in the block by wild-type (WT) and mutant *Sh*BP. RyR2 proteins were expressed in HEK cells, purified and their single channel activity recorded in lipid bilayers. Four peptides were synthesised and tested: i) WT *Sh*BP MAAVAGLYGLGEDRQHRKKQ, ii) a ''less hydrophobic'' peptide (LHBP) MAQVQGLYGLGEDRQHRKKQ, and 2 ''more hydrophobic'' peptides (MHBP), iii) MHBPI MAVVAGLYGLGEDRQHRKKQ and iv) MHBPII MAAVVGLYGLGEDRQHRKKQ. All peptides blocked the ryanodine-modified RyR2 channel from the cytosolic face in a concentration- and voltage-dependent manner. At a holding potential of +50 mV in symmetrical 210 mM KCl, we found an affinity constant K_D of 39.54 ± 3.90 μM for WT *Sh*BP, 65.90 ± 12.99 μM for LHBP, 27.79 ± 4.29 μM for MHBPI and 44.56 ± 9.38 for MHBPII. The association rates K_{on} of the peptides varied with concentration (2.92 ± 0.08 $s^{-1}.\mu M^{-1}$ for WT *Sh*BP, 3.85 ± 0.32 $s^{-1}.\mu M^{-1}$ for LHBP, 2.96 ± 0.22 $s^{-1}.\mu M^{-1}$ for MHBPI and 2.31 ± 0.25 $s^{-1}.\mu M^{-1}$ for MHBPII). Dissociation rates K_{off} were independent of concentration (115.30 ± 7.90 s^{-1} for WT *Sh*BP, 253.52 ± 24.49 s^{-1} for LHBP, 82.14 ± 5.48 s^{-1} for MHBPI and 102.97 ± 8.30 s^{-1} for MHBPII). Furthermore the block induced by the peptides could be reduced by an increase of the salt concentration at the luminal side of the channel. These findings indicate that hydrophobic interactions between RyR2 and inactivation peptides are necessary for the block, and that the binding sites of the peptides are within the pore. This research was supported by the BHF.

1565-Pos
Crystallographic Insights into the Cardiac Ryanodine Receptor N-terminal Domain and its Disease Mutants
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Ryanodine receptors (RyRs) are large channels governing the release of Ca^{2+} from the sarcoplasmic or endoplasmic reticulum. They are required for the contraction of both skeletal (RyR1) and cardiac muscles (RyR2). Mutations in RyR genes have been associated with severe genetic disorders, but high-resolution data describing the disease variants in detail has been lacking. We have solved the crystal structures of the N-terminal domains of both RyR2 (2.55Å) and RyR1 (3.0Å), along with structures of various RyR2 disease mutants. The N-terminal domain in both RyR1 and RyR2 consists of a core beta trefoil domain flanked by an alpha helix. Two cysteine pairs display a highly increased flexibility, making them ideal candidates to receive redox modifications. Crystal structures of several RyR2 disease mutants (1.7Å - 2.2Å) show that most of the mutations cause distinct local changes to the surface of the protein, highlighting at least two putative binding interfaces required for normal RyR function. One RyR2 disease mutant causes significant changes in the thermal stability of the N-terminal domain, accompanied by large conformational changes in the structure.

1566-Pos
Structural Mapping of the Ryanodine Receptor Type 1 Using A Fret-Based Method
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The type 1 ryanodine receptor (RyR1) mediates excitation contraction coupling in skeletal muscle by releasing stored intracellular calcium in response to cellular depolarization. This 2.2 MDa homotetrameric protein is associated with numerous regulatory proteins that modulate its activity in vivo. Understanding the structure and conformational dynamics of this immense macromolecular complex is an enormous challenge in skeletal muscle biology. In this report, structural determinations of RyR1 were performed using Förster resonance energy transfer (FRET) measurements. In this system, the FRET donor was green fluorescent protein (GFP) fused to RyR1, which could then transfer energy to Cy3NTA, a site-specific FRET acceptor targeted to poly-histidine segments inserted into RyR1. Energy transfer was monitored as a decrease in GFP fluorescence occurring when Cy3NTA was bound to a His tag in close proximity to the GFP donor fused either to position 1 or position 618 of RyR1. Cy3NTA was targeted to each of three ''divergent regions'' (DR) poorly conserved among the three RyR isoforms (DR1; position 4430, DR2; position 1323, DR3; position 1861). While minimal FRET was detected between N-terminally fused GFP and Cy3NTA targeted to these divergent regions, significant energy transfer was detected from GFP at position 618 to Cy3NTA targeted to DR2 or DR3. These experiments indicate that these donor and acceptor sites are in close proximity to each other and also demonstrate the utility of this FRET-based technique for further structural mapping of RyR1. (Supported by NIH grant R21ARO56406).

1567-Pos

FRET Detection of Calmodulin Binding and Structural Rearrangements Within the Cardiac RyR2 Calcium Release Channel

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Calmodulin (CaM) binds to a conserved domain of the ryanodine receptor isoforms expressed in skeletal muscle (RyR1) and cardiac muscle (RyR2) to evoke isoform-specific changes in channel gating. To better understand CaM's interactions with the RyR2 isoform, we are using fluorescence resonance energy transfer (FRET) to define the orientation and kinetics of CaM binding, and to resolve structural rearrangements linked to channel regulation. A FRET donor was targeted to the RyR2 cytoplasmic assembly by preincubating cardiac sarcoplasmic reticulum membranes with a fluorescent-labeled FKBP12.6 (F-FKBP). An acceptor fluorophore was attached within the N-lobe of CaM (F-CaM). A decrease in F-FKBP fluorescence upon addition of F-CaM provided a specific, real-time readout of CaM binding to the RyR2, despite the presence of additional non-RyR CaM targets in the cardiac membranes. FRET demonstrated that the affinity of F-CaM binding to RyR2 was greater in 100 μM than in 30 nM Ca^{2+}. The maximal FRET observed in the presence of saturating [F-CaM] increased as a function of [Ca^{2+}] (30 nM to 1 mM). The Ca^{2+} dependence of this increase in FRET was similar to the Ca^{2+} dependence of [^{3}H]ryanodine binding to RyR2 assayed in equivalent media (K_{Ca} ~5 μM). A marked decrease in FRET between FKBP12.6 and CaM was observed when the acceptor was shifted from CaM's N-lobe to CaM's C-lobe. We conclude that CaM binds to the RyR2 in an extended conformation, with its N-lobe oriented nearest to the FKBP12.6 subunit. CaM's conformation and orientation when bound to the RyR2 are therefore similar to what has been demonstrated previously for the RyR1 isoform (Cornea *et al.*, 2009). Ca^{2+} dependent changes in FRET between FKBP12.6 and CaM may reflect structural changes within the RyR2 linked to channel activation by Ca^{2+}.

1568-Pos

Localization of Potential Calmodulin Binding Sequences onto the Three Dimensional Structure of the Cardiac Ryanodine Receptor Reveals A Binding Pocket for Calmodulin

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Calmodulin (CaM), a 16 kDa ubiquitous calcium-sensing protein, is known to bind tightly to the cardiac calcium release channel/ryanodine receptor (RyR2) at low and high Ca2+ concentrations, and modulate the function of the channel. CaM binding studies using RyR fragments or synthetic peptides have revealed that multiple regions in the RyR's primary sequence may be involved in CaM binding. However, the locations of these potential CaM binding regions in the three dimensional structure of RyRs have yet to be determined. In the present study, we inserted GFP or GST into these proposed CaM binding sequences and mapped some of them onto the three-dimensional structure of intact RyR2 by cryo-electron microscopy and single particle image analysis. Surprisingly, we found that some of these potential CaM binding regions, e.g. Arg-3595 and Lys-4269, are located in close proximity and are adjacent to the CaM binding sites that were mapped previously by 3D cryo-EM. These observations suggest that multiple regions in the RyR2 sequence may form a binding pocket for CaM. (Supported by NIH and CIHR).

1569-Pos

Ryanodine Receptor Channels are Regulated by Specific Binding of A Membrane Phospholipid Metabolite

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Sphingosylphosphatidylcholine (SPC) is metabolized from sphingomyelin (SM) of a minor cell membrane phospholipid during the apoptosis and the hyperlipidemia. Lysophosphatidylcholine (LPC) is produced from phosphatidylcholine (PC) of a major membrane phospholipid during the ischemia. These lipid metabolites are known to modify a variety of ion channels. In the present study, we examined in detail with the planar lipid bilayer method how the cardiac RyR channels are modified by SPC and LPC. The cis-side addition of SPC blocked the channels at the μM level, while the trans-side addition of SPC did not affect. SPC hardly change the membrane capacitance. A kinetic model held in the SPC effect. SPC could thus exert a specific effect via its binding to the cytoplasmic domain of the RyR molecule. On the other hand, both cis-side and trans-side additions of LPC activated the RyR channels at the μM level. LPC significantly increased the membrane capacitance. No kinetic model held in the LPC effect. Unlike SPC, LPC could thus exert a nonspecific indirect effect on the RyR channel via a fusion of LPC into the membrane lipids.

1570-Pos

Molecular Determinants of Ca^{2+} Release Termination in the Cardiac Ryanodine Receptor

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A longstanding question in the field of excitation-contraction coupling in cardiac muscle is how Ca^{2+} release from the sarcoplasmic reticulum (SR) is terminated. Recent studies have suggested that SR Ca^{2+} release terminates as a result of luminal Ca^{2+} dependent inactivation of the Ca^{2+} release channel/ryanodine receptor (RyR2). However, the molecular basis of luminal Ca^{2+} dependent inactivation of RyR2 is unknown. We have previously shown that the pore region of RyR2 is critical for the initiation of spontaneous Ca^{2+} release or store overload induced Ca^{2+} release (SOICR). In the present study, we determined whether the pore region of RyR2 is also important for Ca^{2+} release termination. To this end, we mutated each residue within the inner helix and the helix bundle crossing, and generated stable, inducible HEK293 cell lines expressing theses mutants. Using the fluorescence resonance energy transfer (FRET)-based luminal Ca^{2+} sensing protein, D1ER, we monitored the luminal Ca^{2+} dynamics in HEK293 cells expressing RyR2 wt and mutants during Ca^{2+} overload. Interestingly, we found that the G4871R mutation significantly lowered the critical luminal Ca^{2+} level at which Ca^{2+} release is terminated (the termination threshold), but it had no effect on the critical luminal Ca^{2+} level at which spontaneous Ca^{2+} release or SOICR occurs (the SOICR threshold), as compared with wt. In contrast, the I4862A mutation markedly lowered the SOICR threshold with little impact on the termination threshold. On the other hand, the Q4876A mutation lowered both the SOICR and termination thresholds, whereas the E4872A mutation raised both thresholds. Taken together, our data demonstrate that the pore region of RyR2 is an important determinant of both activation and termination of Ca^{2+} release, and suggest that the pathways for Ca^{2+} release activation and termination are distinct but overlap.

1571-Pos

Modulation of Synchronous Gating in Skeletal Muscle Ryanodine Receptor Channels (RyR1) by Nucleotides or Phosphorylation

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In skeletal muscle fibers, local Ca^{2+} sparks and global Ca^{2+} transients arise from the synchronous activation of arrays of calcium release channels (RyR1) in the sarcoplasmic reticulum. Marx et al. (1998) first described that synchronous Ca^{2+} signaling in cells could be explained by the coordinated gating of neighboring RyR1 channels; i.e. ''coupled gating''. We have previously reported that coupled gating of multiple RyR1 channels requires luminal Ca^{2+} as current carrier and ATP/Mg^{2+} in the cytosolic solution. Here, we have reconstituted into planar lipid bilayers multiple RyR1 channels from skeletal muscle SR microsomes and determined their modulation by different nucleotides. As found for ATP, we determined that ADP and AMP can activate RyR1 and favor coupled gating. Contrarily, ITP, GTP and TTP did not affect channel behavior. A priori, the ATP action seems more robust than that of ADP/AMP (remains after addition of Mg^{2+}). Consequently, we tested the possibility of a phosphorylation-mediated mechanism to explain ATP efficacy. However, we found that addition of PKA, CaMK or phosphatases did not significantly affect channel activity. The lack of effects of kinases/phosphatases was confirmed with macroscopic assays of SR Ca^{2+} release. Thus, our results suggest that nucleotide modulation of RyR1 seems to be specific for adenine nucleotides (especially ATP) and that RyR1 behavior in skeletal muscle does not appear to be significantly modulated by phosphorylation. (Supported by NIH R01 GM078665)

1572-Pos

Modification of Cardiac Ryanodine Receptors by Reactive Carbonyl Species Alter Conductance and Gating

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Previously, we and others found that ventricular myocytes isolated from streptozotocin (STZ)-induced diabetic rats exhibited enhanced spontaneous Ca^{2+} releases. To date, molecular mechanisms underlying this phenomenon remains incompletely understood. This study was designed to determine whether carbonyl adducts previously found on RyR2 during diabetes contribute to its dysregulation. Male Sprague-Dawley rats were injected with STZ. Six weeks later, diabetic rats were divided into two groups: one group was treated with insulin for two weeks while the other group received no treatment. Non-diabetic controls were run alongside. After eight weeks, RyR2 was isolated and proteoliposomes prepared. Following incorporation into the lipid bilayer, diabetic RyR2, which contained elevated levels of carbonyl adducts, activated to a greater