1557-Pos

Association of Triadin to the Junctional Sarcoplasmic Reticulum of Skeletal Muscle Cells

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The junctional sarcoplasmic reticulum (jSR) of skeletal muscle cells contains several proteins that participate to the mechanisms of Ca2+ release in the process of excitation-contraction coupling. Among these proteins, ryanodine receptor, triadin, junctin and calsequestrin have been found to associate into a stable complex. We recently reported that assembly of jSR domains is accompanied by a strong decrease in the mobility fraction of jSR proteins (Cusimano et al., PNAS 2009). In particular, we found that the mobility of triadin appeared to be mediated by its intraluminal region (aa 232-729). In order to identify the minimal regions required for association of triadin to the jSR, deletion mutants of the luminal domain (triadin Δ232-440 and triadin Δ441-729) were generated and expressed in primary muscle myotubes. Analysis of the mobility fraction of these mutants showed that they do not differ from wild type triadin, indicating that either one of the two regions is sufficient to provide a strong association of the protein to the jSR. Interestingly, we found that the luminal region of triadin contains several defined domains, including a coiled coil region and short amino acids repeats, that are present in the region between aa 232-440 and aa 441-729, where they may mediate protein-protein interactions. Results on the role of these amino acid repeats in mediating triadin association with the jSR will be reported.

1558-Pos

Time-Resolved FRET Detection of Structural Distributions Involving FKBP12.6 and Calmodulin Bound Within Macromolecular RyR Channels

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We used multi-exponential analysis of time-resolved (TR) fluorescence decays to investigate the array of distances underlying fluorescence resonance energy transfer (FRET) between donors and acceptors bound within functional RyR channels in sarcoplasmic reticulum membranes. Previously, we have used steady-state (SS) FRET to demonstrate that CaM is oriented with the N-lobe proximal to the FKBP12.6 subunit on each lateral face of the RyR tetrameric complex (Cornea et al. 2009). Here, we used cysteine mutagenesis and sulfhydryl-specific fluorescent labeling to attach Alexa Fluor (AF) donor probes to single-cysteines at FKBP12.6 sites 49 or 85 (denoted D49 and D85), and acceptor probes to calmodulin (CaM) N-lobe sites 26 or 34 (denoted A26 and A34). The fluorescence of RyR-bound donor-labeled FKBP12.6 was decreased in the presence of saturating acceptor-labeled CaM, indicating FRET. Förster analysis of TR- and SS-FRET data of donor/acceptor pairs AF350/AF488 and AF488/AF568 (with different ranges of sensitivity) yielded similarly-ranked energy transfer efficiencies: D85/A26 $\geq$ D49/A26 $>$ D49/D34 $>$ D85/34. Distances calculated from these FRET efficiencies are remarkably similar for the two different donor/acceptor probe pairs. However, this kind of analysis can extract only averaged distances from an RyR sample in which distinct biophysical states are known to co-exist. Further multi-exponential analysis of the donor-only (AF350-FKBP12.6) and donor+acceptor (AF350-FKBP12.6/AF488-CaM) TR-FRET data sets resolved at least two simultaneous distance populations for each pair of labeled sites. These distances and their distribution responded to changes in [Ca^{2+}]. We conclude that TR-FRET provides a powerful approach for resolving dynamic transitions between structural states within functional, multimeric RyR channels. Further studies will aim to determine whether these findings reflect transitions between different conformations of CaM itself, or of the underlying RyR channel.

1559-Pos

FKBP12.6 Inhibits Resting Ryanodine Receptor Activity but PKA-Dependent Phosphorylation Does Not Alter FKBP12.6-RyR2 Binding in Rat Permeabilized Myocytes

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FK506-binding proteins FKBP12.6 and FKBP12 are associated with the cardiac ryanodine receptor (RyR2), and PKA dependent hyperphosphorylation of RyR2 has been proposed to interrupt the FKBP12.6-RyR2 interaction and activate RyR2 opening. However, the physiological function of FKBP12.6/12.0 in cardiac myocytes and the role of PKA-dependent RyR2 phosphorylation are controversial. We used permeabilized rat ventricular myocytes, and fluorescently-labeled FKBP12.6/12.0 to directly measure in situ binding of FKBP12.6/12.0 to RyR2, with simultaneous Ca sparks measurements as an RyR2 functional index. We found that both FKBP12.6 and FKBP12 concentrate at the Z-line, consistent with RyR2 binding. However, only FKBP12.6 inhibits resting RyR2 activity. Assessment of fluorescent FKBP binding at the Z-line of permeabilized myocytes revealed a high affinity of FKBP12.6-RyR2 (Kd = 0.97 ± 0.1 nM) and much lower affinity of FKBP12-RyR2 (Kd = 206 ± 70 nM). Fluorescence recovery after photobleach of FKBP in myocytes confirmed these different affinities and showed that the main difference was in koff. Activation of RyR2 phosphorylation by PKA had no significant affect on either binding kinetics or affinity of FKBP12.6/12-RyR2. Using quantitative immunoblots, we determined the concentration of endogenous FKBP12 in intact myocytes was ~1 μM, while FKBP12.6 is at most ~150 nM. Taken together, our data suggest that FKBP12.6 binds to and stabilizes the resting RyR2 but cAMP-dependent RyR2 phosphorylation does not dissociate FKBP12.6 (or FKBP12) from RyR2 in the myocyte environment. More important, this study highlights the importance of in situ binding properties measurement and clarifies some aspects of controversy.

1560-Pos

FKBP12 and FKBP12.6 Exert Opposing Actions on the Single-Channel Behaviour of Both RyR1 and RyR2

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It is widely believed, that in striated muscle, FKBP12.6 selectively binds to RyR2 but that FKBP12 is the binding partner for RyR1. Few studies have addressed whether FKBP12 can modulate RyR2 function or whether FKBP12.6 can affect RyR1. Our recent single-channel studies show that FKBP12 activates RyR2 with nanomolar affinity but because binding is rapidly reversible, this interaction is unlikely to be detected by Western blot analysis. We have therefore compared how FKBP12 and FKBP12.6 affect the single-channel properties of RyR1 and RyR2 reconstituted into bilayers. Using Ca^{2+} as the permeant ion, cytosolic addition of 500 nM FKBP12 significantly decreased the open probability (Po) of RyR1 from 0.021 ± 0.005 (SEM; n = 4) in controls to 0.001 ± 0.001 (SEM; n = 4; $P<0.05$). This effect was irreversible after perfusing away the FKBP12. In contrast, 200 nM and 1 μM FKBP12.6 increased RyR1 Po. This effect was also irreversible after washout of FKBP12.6 but could be antagonised by addition of FKBP12. In comparison, FKBP12 is a reversible activator of RyR2 whereas FKBP12.6 has little intrinsic action itself but can antagonise FKBP12-induced activation. The presence or absence of FKBP12/12.6 did not lead to the appearance of sub-conductance gating states in RyR2 but, after FK-506 treatment or addition of FKBP12.6 to RyR1, sub-conductance states were frequently observed. Since FKBP12 activates RyR2 but is antagonised by FKBP12.6, and FKBP12.6 activates RyR1 but is antagonised by FKBP12, the ratio of FKBP12/FKBP12.6 levels in the cytoplasm will be critical in determining RyR activity *in situ*. Changes in the cytoplasmic FKBP12/FKBP12.6 ratio may be important in heart failure, where it has been suggested that less FKBP12.6 binds to RyR2.

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1561-Pos

Use of Rapamycin Reveals Evidence of the Physiological Roles of FKBP12 and FKBP12.6 In Cardiac Excitation-Contraction Coupling

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A huge body of evidence suggests that FKBP12.6, which binds tightly to RyR2, is an important physiological regulator of cardiac excitation-contraction (EC) coupling yet its mechanism of action remains elusive. Our recent single-channel studies now reveal that RyR2 activity may be controlled by the opposing actions of FKBP12.6 and FKBP12. To investigate how these proteins regulate RyR2 function in cardiac cells we have used rapamycin to dissociate FKBP12 and FKBP12.6 from RyR2 in isolated rat permeabilised ventricular myocytes. In control myocytes perfused with Fluo-5F, spontaneous waves of Ca^{2+}-induced Ca^{2+}-release were induced by 234 nM Ca^{2+} in the mock cytoplasmic solution. Treatment for 4-6 min with 20 μM rapamycin, reduced the frequency of the Ca^{2+}-waves from 0.57 ± 0.07 Hz to 0.18 ± 0.05 Hz (SEM; n=5; $P<0.002$) and led to the appearance of 'mini-waves' that did not propagate throughout the cell. Rapamycin treatment also increased baseline Fluo-5F fluorescence intensities by 204 ± 47% (SEM; n=8; $P<0.05$), an effect that was completely reversed by perfusion with a physiological level of FKBP12 (3 μM; n=4; $P<0.001$) but not with 200 nM FKBP12.6 (n=4). In rapamycin-treated cells, FKBP12 (from 0.15 ± 0.06 Hz to 0.27 ± 0.07 Hz (SEM; n=6; $P<0.025$) was

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).