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

extent with 1.0 μM Ca^{2+} than control RyR2 (P_o was 5X greater in diabetic RyR2). Two weeks of insulin treatment blunted the enhanced Ca^{2+} responsiveness. When added to the *cis* chamber the potent reactive carbonyl species (RCS), 80 μM methylglyoxal increased the open probability (P_o) of RyR2 3-fold (0.05 to 0.16) within 10 min and this increase was independent of holding potential. Increasing [MGO] further to 160 μM, reduced the conductance of RyR2 by 25% without changing P_o. Incubating RyR2 with MGO (5-500 μM with 200 μM free Ca^{2+} in buffer) dose-dependently reduced its ability to binding [3H]ryanodine. Singly mutating R1611, R2190 and K2888 to W or Y, to mimic adducts previously found on them during diabetes, resulted in gain-of-function of RyR2 (P_o increased >2-fold at 3.3 μM Ca^{2+}). Mutating c-terminal R4462, and R4683 to W or Y resulted in loss-of function of RyR2. We conclude that modification of RyR2 by RCS during diabetes is responsible in part for its dysregulation. (This work was funded by NIH and Nebraska Redox Biology Center)

1573-Pos

Gating of the Purified Human Cardiac Ryanodine Receptor (hRyR2) in the Absence of Regulatory Accessory Proteins

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The cardiac ryanodine receptor (RyR2) mediates Ca^{2+} efflux from intracellular stores to effect myocyte contraction during excitation-contraction coupling. Mutations in this channel perturb Ca^{2+} release function, leading to triggered arrhythmias that may cause sudden cardiac death (SCD). The exact molecular mechanisms by which SCD-linked RyR2 dysfunction occurs constitutes a burgeoning area of cardiac research. Most studies so far have concentrated on the secondary effects of mutation on channel function by virtue of affecting channel modification by phosphorylation and accessory protein binding, with no great emphasis on elucidating the gating mechanisms of the channel itself. Our aim is to elucidate the mechanistic basis of wild-type (WT) hRyR2 activation by its primary activating ligand, Ca^{2+}, under precisely controlled conditions in the absence of any accessory proteins with a view to determining the effect of mutation on hRyR2 gating in the same way. hRyR2 channels, recombinantly expressed in HEK293 cells, were purified and studied at the single channel level in symmetrical 210mM KCl under reducing conditions. *Trans* (luminal) Ca^{2+} was buffered at 50nM using EGTA, while *cis* (cytosolic) Ca^{2+} buffering was stringently controlled using EGTA, HEDTA and NTA to achieve free Ca^{2+} concentrations in the range of 0-500 μM. Preliminary data obtained from sigmoidal dose-response curves of P_o vs pCa for 10 WT hRyR2 channels yields an EC_{50} of 3.25 ± 1.04 μM, resulting in a maximum P_o greater than 0.8 (in 5 out of 10 channels). This increase in P_o resulted from an increase in the frequency of channel openings, until P_o of 0.8 - above which any increases in P_o resulted from an increase in open times. Likely gating mechanisms will be discussed with a view to mutant channel analysis.
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1574-Pos

S-Adenosyl-L-Methionine Activation of Cardiac Ryanodinr Receptors is Associated with an Increased Frequency of Subconductance Sates

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The biological methyl group donor, S-adenosyl-L-methionine (SAM) activates the cardiac ryanodine receptor (RyR2). Previously we investigated the mechanism underlying SAM regulation of RyR2 with [^{3}H]ryanodine binding to cardiac SR vesicles. SAM enhances Ca^{2+} -activation of RyR2 and increases the apparent affinity of ryanodine for the channel. Notably, methyltransferase inhibitors have no effect on SAM-activation, and SAM-mediated methylation of RyR2 is not detected. Furthermore, the concentration dependence for SAM and ATP-induced increase in [^{3}H]ryanodine binding overlap. Presently, we investigated the affect of SAM on native RyR2 channels incorporated into planar lipid bilayers. Channel were grouped according to initial P_o values under control conditions (10 μM cytosolic Ca^{2+}), those with $P_o < 0.2$ (n=7), and $P_o > 0.2$ (n=5). For channels with an initial $P_o < 0.2$, SAM caused a rapid (within seconds) increase in P_o ($p < 0.05$). The SAM-induced increase in P_o was due primarily to an increase in mean open time ($p<0.05$; n=3). Interestingly, SAM activation was associated with an increased frequency of subconducatance states. In contrast, the increase in channel P_o caused by 2mM ATP was not associated with the appearance of subconductance states. Thus, the effect on subconductance states appears specific to SAM. This work highlights the complexity underlying SAM regulation of RyR2. The data suggest ligand binding is among the multiple mechanisms responsible for SAM-activation of RyR2.

1575-Pos

Molecular Interplay between the Heart Lim Protein (HLP) and RyR2 in Murine Heart

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HLP is a heart-specific LIM-only protein having two LIM domains each consisting of two zinc fingers. Through the bacterial 2 hybrid screening and a following LC-MS/MS study, we have found that HLP interacts with the cytosolic divergent region of mouse heart RyR2. The direct interaction between RyR2 and HLP was confirmed by GST pull-down and co-immunoprecipitation assays. HLP was partially co-localized with RyR2 in HL-1 cells and rat adult cardiomyocytes. siRNA or Adenovirus-mediated knock-down of HLP in HL-1 cells and neonatal cardiomyocytes led to more than 70% decrease in the expression of HLP, without a concomitant change of other Ca^{2+} handling proteins (e.g. SERCA, RyR2, calsequestrin and DHPR). Ca^{2+} transient measurement of fura2-loaded cardiomyocytes by 1Hz field stimulation demonstrated that silencing of HLP decreased the peak amplitude of Ca^{2+} transient (~ 15%) in HL-1 cells and in neonatal cardiomyocytes. Currently, various deletion-mutants of LIM protein are being used to characterize the RyR2 binding sites in HLP. (This work was supported by the Korean Ministry of Science and Technology grant, Systems Biology Research Grant, M1050301001-6N0301-0110, and the 2009 GIST Systems Biology Infrastructure Establishment Grant).

1576-Pos

Mitsugumin-29 Regulates RyR1 Activity In Mouse Skeletal Myotubes

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Canonical-type transient receptor potential cation channel type 3 (TRPC3) in plasma membrane allows the entry of Ca^{2+} ions into various cells. In skeletal myotubes, functional interaction between TRPC3 and RyR1 (ryanodine receptor1, a Ca^{2+} channel in sarcoplasmic reticulum (SR) membrane) regulates the gain of skeletal excitation-contraction coupling (*J. Biol. Chem*., 2006). Mitsugumin-29 (MG29) is a four membrane-spanning protein and is found in both plasma and SR membrane. MG29 has been known as a TRPC3-interacting protein in skeletal myotubes (*Biochem. J.*, 2008).
To identify critical region(s) of MG29 that participate in binding to TRPC3 or the role of MG29 in skeletal muscle, N-terminus, three intervenient loops among four transmembrane regions, and C-terminus of MG29 were expressed in E. coil as N-terminal GST-fused forms, and subjected to co-immunoprecipitation assay with intact TRPC3 from rabbit skeletal muscle. Cytoplasmic N-terminus and a loop between first and second transmembrane domains of MG29 effectively bound to TRPC3. Two deletion mutants of MG29 (missing the TRPC3-binding sites: deleting the N-terminus only or longer N-terminus covering the loop region) was expressed in mouse skeletal myotubes, and the myotubes was subjected to the measurement of Ca^{2+} transients with Fura-2 or Fluo-4. The later mutant showed significantly decreased responsiveness of RyR1 to caffeine, suggesting that MG29 may be a mediator between the functional interaction between TRPC3 and RyR1.

1577-Pos

Phosphorylation of Excitation-Contraction Coupling Components in a Guinea-Pig Model of Heart Failure

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Phosphorylation status appears to be a key determinant of excitation-contraction coupling ion channel and pump function. Dysfunction of the ryanodine receptor (RyR) secondary to catecholaminergic drive and phosphorylation has been proposed as a factor in contractile dysfunction and arrhythmia patho-physiology in the failing heart. The phosphorylation states of RyR, along with those of phospholamban and troponin I have been investigated by immunoblotting, and quantitated by comparing levels in failing hearts with basal levels, minimum levels after beta-blocker treatment and maximal levels achieved by ex vivo treatment with isoprenaline. We found that RyR residue Ser2809 was phosphorylated to 124 ± 11 % ($n = 5$, $P > 0.05$) of control (sham-operated, basal) in heart failure under basal conditions and 143 ± 12 % ($n = 6$, $P < 0.05$) with isoprenaline treatment, and residue Ser2030 was 94 ± 10 % ($n = 8$, $P > 0.05$) for heart failure and 199 ± 9 % (n=6, $P < 0.05$) for isoprenaline treatment. Phosphorylation levels at Ser16 of phospholamban were higher: 159 ± 17 % (heart failure, $n = 7$, $P < 0.05$) and 366 ± 95 % (isoprenaline treatment, $n = 5$, $P < 0.05$). At Ser23/24 of troponin I there is no significant