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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+}.

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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).

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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.

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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.

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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