

a common binding site. We interpret these findings in light of an allosteric mechanism whereby SAM interacts with a RyR2 adenine nucleotide binding site. SAM binds to and stabilizes a channel conformation of reduced conductance and we propose the voltage dependence of the SAM-induced subconductance state arises from a voltage-driven alteration in the affinity of the SAM binding site.

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Mapping the Site of RyR2 “Unzipping” Peptide (DPc10) by using Fluorescence Resonance Energy Transfer (FRET) in Permeabilized Cardiomyocytes

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DPc10 is a synthetic RyR2 peptide corresponding to the part of central domain (Gly2460-Pro2495) in cardiomyocytes. It was reported that DPc10 can specifically bind and destabilize RyR2 by causing defective domain interaction between N-terminal and central domains, which is called “domain unzipping”. In this study, we use DPc10 as a marker, through small fluorophore and single site specifically labeled proteins, to map the location of DPc10 binding site in permeabilized cardiomyocytes via different FRET pairs. We first test the DPc10 binding stoichiometry, by measuring the ratio of donor (AF488/568-FKBP12.6) enhancement upon acceptor (HF647-DPc10) photobleaching, which show donor and acceptor stoichiometry is 1:1. Two different R0 FRET pairs (AF488/AF568-FKBP12.6 and HF647-DPc10) are used to assess FKBP12.6-DPc10 FRET efficiency in permeabilized cardiomyocytes via acceptor photobleach (PB) and donor quench (Quench).

The FRET efficiency between AF568-FKBP12.6 and HF647-DPc10 was $90.8 \pm 0.6\%$ (PB), $91.9 \pm 0.6\%$ (Quench), corresponding to a distance of $55.8 \pm 0.6 \text{ \AA}$ (PB), $54.1 \pm 0.8 \text{ \AA}$ (Quench). Furthermore, FRET efficiency between AF488-FKBP12.6 and HF647-DPc10 was $57.9 \pm 1.4\%$ (PB), $62.4 \pm 1.4\%$ (Quench), corresponding to the distance of $53.1 \pm 0.5 \text{ \AA}$ (PB), $51.4 \pm 0.5 \text{ \AA}$ (Quench) which is consistent with the distance measured in FRET between AF568-FKBP12.6 and HF647-DPc10. Moreover, through another FRET pair AF568-CaM and HF647-DPc10, their FRET efficiency was $89.6 \pm 0.6\%$ (PB), which reflects the distance between CaM and DPc10 is $57.2 \pm 0.7 \text{ \AA}$ (PB).

These results provide direct in situ information of DPc10 localization on the functional RyR2.

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Dissecting the Interaction Mode of Calmodulin and Modulating the Regulation of Ryanodine Receptor1 by Tuning Calcium Binding Affinity with Calmodulin

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ABSTRACT

Calmodulin (CaM) is an important intracellular protein which modulates many cell functions by interacting with other enzymes. The skeletal muscle ryanodine receptor (RyR1) is regulated by both Ca^{2+} and CaM. CaM was reported to be able to effectively increase channel opening at low Ca^{2+} and decrease channel opening at high Ca^{2+} . However, which Ca^{2+} binding site of CaM serves as the switch in the biphasic Ca^{2+} dependence of RyR1 activation remains unclear. To address this question, we engineered a series of CaM variants with increased and decreased Ca^{2+} binding affinities. Using various spectroscopic methods such as circular dichroism (CD), fluorescence, high resolution NMR and single channel activity measurements, we have shown that Ca^{2+} binding to CaM's C-terminal acts as the switch converting CaM from a RyR1 activator to a channel inhibitor. These results further indicate that targeting CaM's Ca^{2+} affinity may be a valid strategy to tune the activation profile of ion channels. We then applied peptide model and mini-domain approaches to dissect the possible interaction modes between CaM and RyR1 in the absence and presence of calcium. Using ¹H-¹⁵N HSQC, residual dipolar coupling (RDC), and fluorescence spectroscopy, we have shown that that both Ca^{2+} CaM and Apo-CaM differentially interact with different regions of RyR1 with different binding modes and affinity.

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FRET Detection of RyR2 Calmodulin Binding and Calcium-Dependent Modulation by RyR2₂₄₆₀₋₂₄₉₅ Peptide (DPc10)

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We are using F-FKBP biosensors and FRET to resolve protein-protein interactions and structural changes regulating ryanodine receptor (RyR) channels. We previously showed that this approach resolves the binding of calmodulin (CaM) to its regulatory site on RyR2. Here we examine the modulation of RyR2 CaM binding by DPc10, a peptide derived from the central hot-spot region of RyR2. In 30nM Ca, DPc10 reduced F-CaM binding to RyR2 by more than half, decreasing both the apparent affinity of binding and the maximal FRET at high [F-CaM]. In contrast, in 300 μ M Ca DPc10 had no effect on RyR2 F-CaM binding, suggesting that DPc10 selectively inhibits apoCaM versus CaCaM binding. Accordingly, in experiments using a Ca-insensitive F-CaM (F-CaM₁₂₃₄), DPc10 inhibited RyR2 CaM binding regardless of [Ca], indicating that the loss of DPc10 inhibition in micromolar Ca results from Ca binding to CaM (and not from Ca binding to RyR2). To directly monitor DPc10 binding to RyR2, we attached a nonfluorescent acceptor to the peptide's N-terminus (A-DPc10). Binding of A-DPc10 to RyR2 was shown by a decrease in fluorescence of RyR2-bound F-FKBP donor (ie, FRET). Increasing Ca from 30nM to 300 μ M progressively reduced, but did not abolish, A-DPc10 binding to RyR2. FRET to A-DPc10 was strong when the donor was attached to FKBP at a position adjacent to the RyR2 handle region, and weaker when the donor was attached near the RyR2 clamp region. We conclude that DPc10 binds to a discrete site near the RyR2 handle to inhibit apoCaM binding to the channel. Results suggest new insights into the mechanisms by which DPc10 promotes diastolic Ca leak and myocyte hypertrophy, and new means of probing regulatory interactions between CaM and RyR2 that are crucial for cardiac function and development.

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Stretch-Dependent Sub-Cellular Ca²⁺ Signaling in Atrial Myocytes

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Atrial fibrillation (AF) is a multifactorial disease whose underlying mechanisms are still incompletely understood. Atrial myocytes undergo similar stretch during diastolic filling as ventricular myocytes and acute atrial stretch can induce AF. Our group recently identified a novel mechanism linking cellular stretch to the tuning of Ca²⁺ release from the sarcoplasmic reticulum (SR) in ventricular myocytes (Prosser et al. Science 2011;333:1440-5). This mechanism, “X-ROS signaling”, depends on NOX2 (NADPH oxidase) to generate reactive oxygen species (ROS), which oxidizes the local RyR2 Ca²⁺ release channels of the SR and increases their Ca²⁺ sensitivity. Stretch mediates X-ROS signaling through microtubules, which interact with NOX2 located in transverse tubules and the surface sarcolemmal membranes.

Interestingly, in atrial myocytes from AF patients both increased ROS levels and arrhythmogenic Ca²⁺ signaling instability are present. We evaluated the effect of acute and repeated stretch on sub-cellular Ca²⁺ sparks in murine atrial myocytes (C57/B6) with instrumentation (WPI, Sarasota, FL) and methods of Prosser et al. (2011). Ca²⁺ sparks were recorded before and after acute and repeated stretch (8% of cell length). Here, we report an increase in stretch-dependent Ca²⁺ spark frequency in single murine atrial myocytes. Thus, stretch-dependent Ca²⁺ release mechanisms are also operative in atrial myocytes and likely to play a role in Ca²⁺ dependent arrhythmias like AF. Our ongoing work will interrogate the components of the X-ROS pathway to determine the relative contribution of this pathway in atrial myocytes.

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Effect of Scorpion Toxins on the CRC/RyR Function

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It was shown previously that maurocalcin (MCA) induces long lasting subconductance states (LLSS) investigating the RyR function by single channel electrophysiology. These LLSSs are polarity and potential dependent and caused by the distinct positively charged surface formed by 5 amino acids