Platform M: Excitation-Contraction Coupling

1043-Plat

Simultaneous Phosphorylation of RyR2 by PKA and Camkii is Required for Induction of CA-Dependent Arrhythmia Caused by MIR-1 Overexpression

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We recently showed that disruption of localization of PP2A phosphatase activity to the ryanodine receptor (RyR2) complex by overexpression of the muscle-specific microRNA, miR-1, stimulates excitation-contraction coupling and results in increased arrhythmogenic potential in cardiac myocytes. In the present study, we examined the role of PKA and CAMKII as mediators of the effects miR-1 on Ca signaling and arrhythmogenesis using cellular electrophysiology and Ca imaging complemented with quantitative measurements of RyR2 phosphorylation at PKA site S-2808, and at CAMKII site S-2814. Adenovirally-mediated 2-fold overexpression of miR-1 resulted in 10-fold increase in arrhythmogenic potential measured as a frequency of spontaneous Ca waves and DADs in myocytes exposed to 100 nM isoproterenol (ISO). Quantitative imminoblotting using site-directed phosphospecific-antibodies showed that RyR2 phosphorylation in miR-1 overexpressing cells was low at S-2808 under basal conditions. Exposure of myocytes to ISO maximized phosphorylation at S-2808. Phosphorylation at S-2814 under basal conditions was maximal and did not further increase in the presence of ISO. Additionally, ISO increased SERCa-mediated SR Ca uptake and SR Ca load through phosphorylation of phospholamban (PLB). To define which of these factors (increased CaSR content or increased RyR2 PKA phosphorylation) mediated the increased arrhythmogenic potential we infected myocytes with viral constructs of a dominant-negative PLB mutant that accelerates SERCa-mediated SR Ca uptake by displacing endogenous PLB from SERCa. Myocytes coexpressing miR-1 and dnPLB did not exhibit enhanced predisposition to Ca-dependent arrhythmia in the presence of ISO despite maximal SR Ca load. Importantly treatment of cells with either PKA or CAMK inhibitors completely abolished increased arrhythmogenic activity. We conclude that neither CAMKII nor PKA phosphorylation alone is sufficient to produce the changes in RyR2 activity that underlies the arrhythmogenic disturbances caused by miR-1 overexpression.

1044-Plat

Defective RYR2 Channels Trigger Ventricular Arrhythmias in Duchenne Muscular Dystrophy

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Duchenne muscular dystrophy (DMD) affects one in 3500 newborn males and usually leads to death from respiratory or cardiac failure by age 30. Interestingly, the severity of cardiomyopathy is not proportional to the severity of skeletal muscle disorder. Among DMD patients, the cardiac phenotype varies with age from no discernable cardiac left ventricular enlargement or dysfunction to early onset of dilated cardiomyopathy (DCM) with heart failure. The incidence of DCM in DMD patients has been estimated to be 25% by 6 years of age, 59% at 10 years of age and ~100% in adults. DMD patients often exhibit electrocardiographic abnormalities and frequent premature ventricular contractions. As the cardiomyopathy progresses, ventricular arrhythmias (VA) increase, often leading to sudden death. Most of the electrical and functional abnormalities have been attributed to cardiac fibrosis. However, electrical abnormalities may occur in the absence of overt cardiac histopathology and ECG changes are similar in patients with DMD regardless of presence of DCM.

Here we show that structural and functional remodeling of the cardiac sarcoplasmic reticulum (SR) Ca2+ release channel/ryanodine receptor (RyR2) occurs in the mdx mouse model of DMD. RyR2 from mdx hearts were S-nitrosylated and depleted of calstabin2 (FKBP12.6) resulting in "leaky" RyR2 channels and diastolic SR Ca2+ leak. Inhibiting the depletion of calstabin2 from the RyR2 complex with the calcium channel stabilizer, S107 ("rycal"), inhibited the SR Ca2+ leak, restored normal Ca2+ transients, inhibited isoproterenol induced aberrant depolarizations in isolated cardiomyocytes and prevented arrhythmias in vivo.

Thus, diastolic SR Ca2+ leak via RyR2 due to S-nitrosylation of the channel and calstabin2 depletion from the channel complex likely triggers cardiac arrhythmias. Prevention of the RyR2-mediated diastolic SR Ca2+ leak may provide a novel cardiac therapeutic approach in DMD.

1045-Plat

Sodium Current-Induced Release of Calcium from the Sarcoplasmic Reticulum in Rabbit Ventricular Myocytes

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The hypothesis that Na current (INa) can induce release of Ca from the sarcoplasmic reticulum (SR) by activating reverse Na-Ca exchange (NCX) has been debated since 1990. We tested this hypothesis with epi-fluorescence imaging of adult rabbit ventricular myocytes loaded with the Ca indicator fluo-4. Ca release was triggered with an action potential clamp with and without an initial voltage ramp from −80 to −40 mV, for a duration of 1.5s. We confirmed that this protocol selectively blocked INa without altering Ca influx through L-type Ca channels (LCCs) and SR Ca load. With 0 mM Na in the pipette (to reduce intracellular Na), inactivating INa reduced SR Ca release flux by 27% ± 4% (n=9). With 5 mM Na in the pipette, the Ca release upon inactivation of INa was reduced by 33% ± 5% (n=4). We suggest that increased activation of reverse NCX by increased intracellular Na concentration mainly produced by INa explains these findings. These conclusions are in agreement with studies on normal and NCX knockout mice, which show that INa affects SR Ca release only in normal, but not in NCX knockout mice. In similar experiments, we applied 100 nM TTX to selectively block brain isoforms of Na channels. In the presence of TTX, the SR Ca release flux was reduced by 35% ± 3% (n=6). This effect of INa on Ca release can be explained by early reverse NCX, activated by TTX sensitive INa, which could prime the dyadic cleft with Ca. Furthermore, the results can be explained if INa activation of NCX, and subsequent priming of the dyadic cleft with Ca, increases the coupling fidelity between LCCs and ryanodine receptors within a couplon. Thus the presence of INa increases the likelihood that couplons are activated.

1046-Plat

Single Cell Measurements of Isometric Force and Cytosolic Calcium in Intact Mammalian Cardiomyocytes

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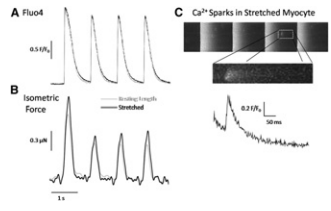
Single cell measurements of contractile force in ventricular cardiomyocytes were pioneered in the early 1990s (Le Guennec et al, 1990) and recently advanced by the Kohl laboratory (Iribe et al, 2006). Here we have modified their technique to directly record isometric force in adult mammalian ventricular myocytes. Using a high sensitivity force transducer, we have simultaneously recorded contractile force with cytosolic Ca2+ transients and sparks in intact cells subject to sarcomeric stretch. Consistent with previous reports and the Frank-Starling law of the heart, stretch led to an increase in active contractile force (Fig. 1B, normalized to passive tension) without significantly altering cytosolic Ca2+ transients (Fig. 1A). Stretch also promoted an increase in Ca2+ spark activity (Fig. 1C), consistent with recent work from our group (Iribe et al, 2009). This technique will be utilized to evaluate the effects of sarcomeric stretch on Ca2+ signals, membrane electrical properties, and contractile force in healthy and diseased or genetically altered heart cells.



1047-Plat

Na/K-ATPase α2-Subunit Preferentially Modulates Ca Transients and SR Ca Release in Cardiac Myocytes

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Na/K-ATPase (NKA) is essential in regulating $[Na]_i$, and thus cardiac myocyte Ca and contractility via Na/Ca exchange. Different NKAα-subunit isoforms are present in heart and may differ functionally, depending on differential membrane localization. In smooth muscle and astrocytes, NKA-α2 is located at the junctions with endo(sarco)plasmic reticulum, where they could regulate local Na, and indirectly junctional cleft [Ca]. In contrast, NKA-α1 is ubiquitously

distributed and may regulate bulk $[Na]_i$. It is controversial whether this model holds for cardiac myocytes. We measured the effect of selective NKA-α2/NKA-α1 inhibition with low concentrations of ouabain on $[Na]_i$, Ca transients and fractional SR Ca release in cardiac myocytes from wild-type (WT) mice and transgenic mice in which NKA-α1 is ouabain-sensitive and NKA-α2 is ouabain-resistant (SWAP mice), respectively. Different [ouabain] (5 and 0.1 μM, respectively) were used to attain a similar level of total NKA inhibition in WT and SWAP mice. Ouabain increased Ca transients in WT (F/F_0= 1.84 ± 0.13 *vs*. 1.54 ± 0.12 under control conditions) but not in SWAP mice (1.49 ± 0.15 *vs*. 1.46 ± 0.12), despite a similar and modest increase in $[Na]_i$ (≤ 2 mM). Ca transient increase in WT mice was mediated specifically by NKA-α2 inhibition, because 5 μM ouabain had no effect in transgenic mice where both NKA-α1 and NKA-α2 are ouabain-resistant. Ouabain also significantly increased the fractional SR Ca release in WT mice (from $23 \pm 2\%$ to $28 \pm 2\%$) but not SWAP mice ($23 \pm 3\%$ with and without ouabain). Dual-color immunofluorescence measurements coupled with spatial cross-correlation analysis revealed that NKA-α2 is co-localized to a significant extent with both Na/Ca exchanger (43% co-localization) and ryanodine receptors (43% co-localization) in cardiac myocytes. In conclusion, our data indicate that NKA-α2 has a more prominent role (*vs*. NKA-α1) in modulating Ca transients and SR Ca release in cardiac myocytes.

1048-Plat

Molecular Mechanism of Store Operated Ca^{2+} Entry in Adult Mammalian Skeletal Muscle

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In skeletal muscle, store-operated Ca^{2+} entry (SOCE) is a trans-sarcolemmal Ca^{2+} influx pathway activated when sarcoplasmic reticulum (SR) Ca^{2+} stores are depleted. However, the mechanism of activation and physiological role of SOCE in adult skeletal muscle remains largely unknown. We recently reported that both STIM1 and Orai1 proteins are required for SOCE in skeletal myotubes. Here we investigated the mechanism of SOCE in adult skeletal muscles using mouse FDB fibers following electroporation of either cherry-tagged dnOrai1 cDNA or STIM1 siRNAs. Using a Mn^{2+} quench assay, we found that thapsigargin-induced SR Ca^{2+} store depletion activates a Ca^{2+} influx pathway in adult FDB fibers that is inhibited by: 1. multiple SOCE channel blockers (La^{3+}, BTP-2 or SK&F96365) (3321 ± 598 counts/sec, n=16 and 477 ± 274 counts/sec, n=4 in the absence and presence of La^{3+}, respectively), 2. expression of cherry-tagged dnOrai1 (571 ± 130 counts/sec, n=12), or 3. STIM1 knockdown (7565 ± 1590 counts/sec, n=3 and 217 ± 247 counts/sec, n=5 in control and after STIM1 knockdown, respectively). To further assess the role of SOCE in muscle, we generated skeletal muscle-specific HA-tagged dominant negative Orai1 transgenic mice (HSA^{dnOrai}) using a transgene driven by the human skeletal muscle actin (HSA) promoter (provided by Dr. J. Molkentin). HSA^{dnOrai} mice survive beyond weaning and develop/breed normally. Western blot analysis using an HA antibody confirmed dnOrai1 transgene expression in skeletal muscle, but not in heart, lung, brain, spleen kidney, or liver. Primary myotubes derived from HSA^{dnOrai} mice show significantly decreased SOCE following store depletion as assessed in Mn^{2+} quench (>92%) and Ca^{2+} influx (>95%) assays These results demonstrate that STIM1-Orai1 coupling mediates SOCE in adult skeletal muscle and that HSA^{dnOrai} transgenic mice are a valuable tool for future studies of the physiological role of SOCE in skeletal muscle.

1049-Plat

Developmental Changes of the Sarcoplasmic Reticulum

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In ventricular myocytes, excitation-contraction coupling (ECC), or the process by which an action potential leads to myocyte contraction, is reliant upon external influx of calcium (Ca^{+2}) via sarcolemmal voltage-gated L-type Ca^{+2} channels to trigger Ca^{+2} release through nearby ryanodine receptors located in the sarcoplasmic reticulum (SR) via the mechanism of Ca^{+2}-induced Ca^{+2} release. This causes a global increase in intracellular in Ca^{+2} concentration ($[Ca^{+2}]_i$) that activates contractile filaments. The level of expression of proteins and cellular organelles involved in cardiac ECC changes during development. These differences arise from adult myocytes being larger, having smaller surface-to-volume ratio and a developed transverse (T)-tubule system that tightly couples to the junctional SR. To investigate ECC developmental changes, we developed an adenovirus encoding a tagged red fluorescent protein with a retention signal for the sarcoplasmic reticulum (tRFP-SR). This approach allowed us for the first time to track in real-time SR structure and $[Ca^{+2}]_i$ dynamics in neonatal and adult ventricular myocytes. Using confocal and total internal reflection fluorescence (TIRF) microscopy, we found that the SR within neonatal and adult ventricular cardiomyocytes forms a vast network that spans virtually the entire cell. Interestingly, we found that the SR is a highly dynamic network with relatively rapid changes in morphology in neonatal myocytes. Calcium sparks were observed from these SR structures, suggesting they harbor functional ryanodine receptors. In contrast to neonatal myocytes, the SR of adult ventricular myocytes is highly stable. Our findings suggest, that the fidelity of the $[Ca^{+2}]_i$ transient during ECC in neonatal myocytes is not simply the result of ryanodine receptors in a static SR network. Instead, our data indicate it is the result of the activation of numerous couplings that form and dissolve rapidly between the sarcoplasmic reticulum and plasma membrane.

1050-Plat

Superfast Confocal Imaging of Ca^{2+} Reveals the Spread of Excitation Through the Tubular Network and the Ca^{2+} Release Waveform in Skeletal Muscle

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In skeletal muscle, rapid spread of excitation is required for uniform release of Ca^{2+} and subsequent contraction. Excitation leads to a large release of Ca^{2+} from sarcoplasmic reticulum (SR), where the rising phase is very brief, due to fast release termination. The longitudinal spread of excitation within the tubular (t-) system network occurs in spontaneously excited mechanically skinned fibres, suggesting the involvement of longitudinal tubules. In such a large cell every transverse tubule may not be excited following depolarization at the cell surface. The rapid rising phase of the action potential-induced Ca^{2+} transient and any longitudinal spread of excitation between sarcomeres cannot be adequately measured with conventional imaging techniques. By imaging Ca^{2+} transients with Oregon Green Bapta 5N at 15.5 μs $line^{-1}$ on a Zeiss 5 LIVE confocal system we resolved the rising phase of Ca^{2+} release from SR and also tracked the longitudinal spread of excitation along the t-system from the subsequently released Ca^{2+}. Following field stimulation of skinned fibres, we observed that in areas where transverse tubules failed to be excited by the initial stimulus, Ca^{2+} release propagated in from the adjacent regions at a rate of ~16μm ms^{-1}. The rise time of the Ca^{2+} transient showed two phases, it initially rose rapidly at x F/F_o ms^{-1} and then continued at a slowing rate until the peak of the transient indicating the rapid onset of release inactivation. Nav1.3 immunostaining identified a complex subsarcolemmal t-system network which may help ensure the synchronous spread of excitation throughout the fibre from the surface membrane. However uniform calcium release in skeletal muscle also requires longitudinal tubules within the t-system network to pass action potentials between excited and "failing" transverse tubules.

Platform N: Emerging Single Molecule Techniques I

1051-Plat

Split-GFP Complementation for Targeting and Imaging Single Molecules in Living Cells

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Current limitations for studying intracellular dynamics of biomolecules by single molecule fluorescence include the necessity to express a molecule of interest at very low concentration or to target it very specifically with a small number of probes. Inside cells, both conditions are hard to meet. In the first case, very low expression levels of a molecule of interest might influence the cell physiological responses. In the second case, probes with high specificity and very high affinity binding constants for their target are required. To circumvent these issues we introduce the use of split-GFP fusions and their complementation by small synthetic peptides to image the dynamics of single proteins in live cells. Cells expressing the split-GFP fusions are non-fluorescent and a controlled subset of the GFPs can be "lit up" when providing the complementary peptide at different concentrations. With this approach, we imaged the plasma membrane diffusion of single CD4-split GFP and GPI-split GFP proteins in different cell lines. We also demonstrate intracellular single molecule imaging using caveolin-1-split GFP proteins as an example. In all cases, background free tracking of single proteins could be achieved by TIRF microscopy within minutes of complementation. Split-GFP fusions and the development of other split-fluorescent protein variants provide a generic method for multicolor single molecule imaging in living cells even at elevated protein expressions. Split-fluorescent protein fusions also complement the toolbox of intracellular targeting strategies by providing a unique way to verify the specific targeting of molecules introduced in living cells. Toward this aim, we present early efforts at employing split-GFP fusions for addressable targeting of single fluorophores and single quantum dots.