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.

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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.

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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.

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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.