we used yeast as a reconstitution system to identify the minimal components sufficient for in vivo uniporter activity. First, we considered Dictyostelium discoideum and showed that it has a highly simplified uniporter machinery: the expression of DdMCU, a single transmembrane component alone is sufficient to reconstitute mitochondrial calcium uniporter activity. Second, to establish human uniporter activity, the coexpression of MCU and - the animal specific protein - EMRE is necessary, whereas expression of MCU alone is insufficient. Our work established yeast as a powerful in vivo reconstitution system for the uniporter to study the evolution and function of this channel.

895-Plat
Fluctuations in Calcium Concentration Alter the Temporal Dynamics of Calcium-Dependent Signaling Cascades
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Calcium signaling is often localized in spatially restricted ''microdomains,'' which may involve only 1-100 calcium ions. Fluctuations in the local calcium concentration can arise from calcium influx and association/dissociation with calcium buffers [Weinberg and Smith. Biophys J 106(12): 2693 (2014)]. However it is unclear to what extent these fluctuations alter calcium-dependent signaling cascades. We construct a Markov model of a calcium-dependent signaling cascade and compare the first hitting time distribution for a Markov model that accounts for calcium fluctuations, a phase-type distribution that can be calculated from the infinitesimal generator matrix, with the corresponding model that neglects these fluctuations. In general, when calcium fluctuations are much faster than the characteristic time for the signaling cascade, the distributions for the two processes are similar. However, when the time scale of calcium fluctuations is on the same order as the signaling cascade or slower, the mean and variance of the hitting time is increased, in particular when the number of calcium ions is small, a consequence of a long-tailed hitting time distribution. These ''rare events'' comprising the long tail can be significant and have a physiological impact. We further study calcium fluctuations in two settings: calcium-dependent synaptic vesicle release [Bollmann et al. Science 289, 953 (2000)] and a calcium-release site model composed of calcium-activated calcium channels [DeRemigio and Smith. Cell Calcium 38: 73 (2005)]. In these models, we demonstrate the conditions for which calcium fluctuations alter the distribution, mean, and variance of the timing for synaptic vesicle release and calcium-release site activation, respectively. Under physiological conditions, the mean hitting time can be increased orders of magnitude when calcium fluctuations are accounted for, demonstrating a significant influence on intracellular signaling.

896-Plat
Visualizing Calcium Influx through Single Orai1 Channels
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Orai is the pore-forming subunit of two-component channels that mediate store-operated calcium entry (SOCE). When activated by the ER resident calcium sensor STIM, Orai channels possess high selectivity for calcium but an extremely small conductance (~10 fS in 2 mM Ca^{2+} from noise analysis) that has precluded direct recording of single channel currents. We have developed an approach to visualize the activity of single Orai1 channels by fusing human Orai1 to a fluorescent, genetically-encoded calcium indicator (GECI). Whole-cell recording in HEK 293 cells co-transfected with STIM1 showed that GECI-Orai1 is fully functional as a CRAC channel, with normal activation rates, current amplitudes, ion selectivity and rectification, and Ca^{2+}-dependent inactivation. When GECI-Orai1 and the CRAC-activating domain (CAD) of STIM1 were co-expressed at low levels and imaged using a TIRF microscope, cells exhibited sporadic fluorescence transients the size of diffraction-limited spots and the brightness of a few activated GECI proteins. Transients typically rose to a maximum within 100-200 milliseconds and fell into two classes according to duration: briefer ''flickers'' lasting only a few hundred milliseconds, and longer ''pulses'' lasting ~1 to several seconds. The frequency of both classes increased as CAD expression levels were increased. GECI-Orai1 transients exhibited physiological characteristics expected for single Orai1 channels in the plasma membrane, including rapid response to changes in extracellular calcium, enhancement by valinomycin-induced hyperpolarization, and block by trivalent cations. When single molecule GECI-Orai1 traces were aligned by the rise in green fluorescence, a corresponding rise in mCherry-CAD fluorescence, indicative of binding, was observed. These data indicate that GECI-Orai1 transients correspond to the opening of single Ora1 channels gated by CAD. These first recordings of single channel Orai1 currents reveal unexpected dynamics, and when paired with CAD association, support multiple single channel states.

897-Plat
Functional Reconstitution and Structural Flexibility of the CRAC Channel Orai
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Calcium (Ca^{2+}) release-activated Ca^{2+} (CRAC) channels mediate Ca^{2+} influx across the plasma membrane in response to Ca^{2+} store depletion in the endoplasmic reticulum (ER). CRAC channel function requires two components: the plasma membrane Ca^{2+} channel Orai and its regulator located in the ER membrane, the Ca^{2+} store sensor STIM. Using liposomes reconstituted with a purified fusion protein of human Orai1 and cytosolic fragments of human STIM1 (hO1-SS), we show that Orai1 and STIM1 are sufficient to form active CRAC channels in vitro. Reconstituted hO1-SS recapitulates CRAC channel properties as shown by detection of sodium (Na^{+}) flux in the absence of Ca^{2+} and by direct detection of Ca^{2+} flux. 2-APB, a known CRAC channel inhibitor, blocks both fluxes. Our findings confirm that human STIM1 gates the pore of Orai1 and demonstrates that the two proteins are sufficient to form functional channels in the absence of other cellular factors. Previously, we published the crystal structure of drosophila Orai in a closed state. Here we present low-resolution X-ray diffraction data of human Orai1, which indicate an overall structure that is indistinguishable from drosophila Orai. In addition, a new 4.25 Å resolution X-ray structure of drosophila Orai reveals an extended conformation of the fourth transmembrane helix (M4) at the periphery of the channel that extends into the cytosol and is strikingly different from the arrangement of these helices in the previous structure. The comparison of structures reveals conformational flexibility that starts from the M4 helices and continues into the cytosolic M4 extension helices. In all of the structures, the pore adopts the same conformation and remains closed. The conformational flexibility observed for the M4 and M4 extension helices may have a role in the binding of STIM and in signal transduction from the ER.

898-Plat
Thermodynamic and Structural Analysis of Calmodulin Interaction with the Skeletal Muscle Ryanodine Receptor
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In skeletal muscle, the calcium sensor calmodulin (CaM) plays a key role in excitation-contraction coupling, the process of translating neuronal stimuli into mechanical contraction of muscle, by regulating the opening and closing of the calcium channel ryanodine receptor (RyR1). By interacting with this channel differently at high and low calcium, CaM acts as a feedback regulator of calcium levels during muscle contraction: at low calcium, CaM weakly activates RyR1, while calcium-CaM inhibits it. We are investigating the interaction between CaM and its established binding site on RyR1 (CaMBD, residues 3614-3640) using a multi-faceted approach combining biophysical (fluorescence spectroscopy) and structural (solution NMR) methodologies. Förster resonance energy transfer (FRET) experiments in an auto-fluorescent biosensor construct (YFP-CaMBD-CFP) enabled us to determine Gibbs free energies of binding in the absence and the presence of calcium. Using this system, we systematically explored the thermodynamics of molecular recognition between the two biomolecules in high and low calcium environments, as well as the roles played by individual RyR1 residues and each CaM lobe at the interface. To gain additional insights into the interplay between the processes of calcium- and target-binding to CaM, we analyzed the interaction between wild-type or mutated RyR1 CaMBD sequences and CaM mutants in which the calcium-binding sites in one domain had been rendered non-functional. Overall, these experiments show that CaM C-domain binding to molecular determinants in the N-terminus of RyR1 CAMBD dominates the interaction, both in the presence and the absence of calcium. However, the interaction is three orders of magnitude stronger at high calcium levels. To obtain residue-specific information of the binding interface, we are currently undertaking NMR studies of isotopically labeled CaM (wild-type and calcium-binding mutants) in the absence and presence of wild-type and mutated RyR1 sequences.

899-Plat
Stretch-Induced Changes in Atrial Ca Signaling
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Atrial myocytes undergo stretch during diastole as do ventricular myocytes. We have identified a novel mechanism that links cellular stretch in ventricular myocytes to the tuning of Ca^{2+} release from the sarcoplasmic reticulum (SR)(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 appears to oxidize ryanodine receptors (SR Ca^{2+} release channels) and increases their sensitivity to $[Ca^{2+}]i$. Stretch mediates X-ROS signaling through microtubules, which appear to interact with NOX2 to enable it to generate ROS.

We evaluated X-ROS signaling in murine atrial myocytes (C57/B6). Ca^{2+} sparks were recorded before and after stretch (10-20% of cell length). Ca^{2+} spark frequency increased during stretch and returned to pre-stretch values during relaxation. Interestingly, microtubule density was higher and protein expression levels were increased in murine atria compared to ventricles (Fig 1 A). Similarly, protein expression levels of the catalytic NOX2 subunit gp91phox were higher in atria compared to ventricles (Fig 1 A). Inhibition of gp91phox with the inhibitory peptide gp91ds-tat prevented stretch-induced Ca^{2+} spark increases in atrial myocytes (Fig 1 B). Thus, X-ROS signaling is also operative in atrial myocytes.

900-Plat

Novel Genetically Encoded Ratiometric Calcium Indicators

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Genetically encoded calcium indicators (GECIs) have recently undergone dramatic improvements in brightness, dynamic range, kinetics, and calcium sensitivity and now are capable of reporting single neuronal action potentials - comparing favorably to synthetic calcium dyes. Nevertheless, GECIs still lag behind synthetic calcium dyes in applications requiring quantitation of absolute intracellular calcium concentration. Here, we describe a novel approach to achieve dual-color ratiometric calcium measurements.

When expressed individually, GCaMP and mCherry, respectively, exhibit calcium-dependent and -independent fluorescence intensities; and when present at the same level, their fluorescence intensity ratio should provide an estimate of absolute calcium concentration. Unfortunately, the two proteins do not always express equally in all locations in a cell. A simple tandem construct ensures equal levels everywhere of the two proteins, but non-radiative energy transfer from GCaMP to mCherry reduces the dynamic range of GCaMP signals and leads to apparent calcium-dependent fluorescence changes of mCherry. We introduced a rigid alpha helix, the ER/K helix, between the fluorophores, in order to prevent significant Förster resonance energy transfer (FRET). In parallel experiments, we demonstrated in vitro and in cellulo that the tandem GCaMP-ER/K-mCherry construct provides quantitative estimates of calcium concentration, without suffering reduction in dynamic range due to FRET activity.

Platform: Cell Mechanics, Mechanosensing, and Motility I

901-Plat

Coupling of Apical Contractions and Adherens Junction Maturation by Synaptopodin-Depedent Recruitment of A-Actinin-4

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Cell-cell adhesive contact is a central player in mechanotransduction, providing both mechanical support and a hub for signaling. Yet, the molecular mechanism is poorly defined, partly due to a lack of cell culture model to mechanically manipulate tension at cell-cell contacts. Here, using live cell imaging of α-actinin, we showed that MDCK cells exhibit many cellular behaviors of epithelial cells in vivo, including (1) oscillating contractility of the adherens junction, (2) pulsatile centripetal cellular contractions, and (3) ratchet constrictions of apical junctional domain. We hypothesized that pulsatile contractions participate in the maturation of junctional complexes. To test our hypothesis, we applied cyclic pulsatile tension to cell-cell contacts in confluent cell monolayers. We found that pulsatile intercellular tension induces recruitment of α-actinin-4 to the adherens junction in a time and tension-dependent manner without changing the localization of canonical adherens junction proteins, suggesting that a missing factor is involved. We have identified this missing factor as synaptopodin. Synaptopodin recruitment to the adherens junction is also tension-sensitive, which is necessary for generation of contractility at cell-cell adhesions. Recruitment of a-actinin-4 strengthens cell-cell adhesion and promote epithelial permeability barrier. Thus, by controlling the mechanical force generated through apical contractility, epithelial cells can adjust the strength of cell-cell adhesion and the maturation process of adherens junction maturation. Conversely, synaptopodin dictates the efficiency of mechanical-biochemical coupling between contractility and junction assembly through the recruitment of a-actinin-4. Our studies reveal a tunable molecular system with both mechanical and biochemical inputs and underscore the complexity of junction assembly in epithelial monolayers in vivo and in cultured cell systems.

902-Plat

Two Distinct Actin Networks Mediate Traction Oscillations to Confer Mechanosensitivity of Focal Adhesions

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Adherent cells actively sense the mechanical stiffness of their extracellular matrix (ECM) by exerting traction force through focal adhesions (FAs), which are integrin-based protein assemblies. Also, FAs control cell spreading, proliferation, survival, differentiation, and migration. FA-mediated mechanosensation underlies cell durotaxis - the tendency of most cell types to migrate toward stiffer microenvironment. Strikingly, FA-mediated traction forces oscillate in time and space, and this oscillation governs durotaxis. The interactions underlying this intriguing spatio-temporal pattern of FA traction force are unknown, as are the contributions of these interactions to this mechanosensation. To address these questions, we established the first coherent, experimentally validated model of FA formation. The model integrated the spatiotemporal coordination between a branched actin network and stress fibers during FA growth. Our model predicted that retrograde flux of branched actin network contributed to a traction peak near the FA distal tip and that stress fiber-mediated actomyosin contractility generated a second traction peak near the FA center. Furthermore, a negative feedback loop involving formin-mediated stress fiber elongation and actomyosin contractility developed and resulted in oscillation of the center traction peak. This oscillation competed with the distal traction peak, and the competition underpinned oscillation of the FA traction maximum in time and space. More importantly, this negative feedback loop broadened the substrate stiffness range, over which the FAs could accurately adapt with traction force generation. Our findings shed light on the fundamental mechanism of FA mechanosensation and durotaxis.

903-Plat

Mechanical Activation of α-Catenin and Vinculin

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Shaping, maintenance and repair of adult tissues require fine-tuning of cell-cell adhesion. α-catenin, a cytoplasmic adapter links the actin cytoskeleton to cell-cell junctions, plays a central role in regulation of the cell-cell adherence. This regulation requires binding of α-catenin to vinculin in a force dependent manner. By stretching single α-catenin construct using magnetic tweezers, we find that force in physiological range can expose the vinculin-binding sites buried in α-catenin, drastically promoting subsequent binding of the head domain of vinculin with a nanoMolar affinity. The bound vinculin head then irreversibly locks α-catenin in its unfolded conformations after force is released. The bound vinculin head can however be displaced at high forces > 30 pN, resulting in a biphasic force dependent binding of α-catenin to vinculin head. Further, we find that full-length vinculin also binds to mechanically unfolded α-catenin, implying release of the auto-inhibition conformation of full-length vinculin. Together, these results provide important insights into mechanosensing at cell-cell adherence.

904-Plat

Molecular Regulation of Actin Turnover at the Leading Edge of Migrating Cells

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Actin couples molecular motors to extracellular adhesions and its turnover regulates cell polarization and protrusion efficiency. Actin filament turnover is regulated by polymerization and depolymerization at the ends, which is increased by severing filaments internally. To determine how these reactions affect F-actin turnover rate we simulated the molecular mechanisms revealed by experiments and determined the ODEs for changes in the average filament length and number of filaments that determine total F-actin. These results