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Symposium 18: Calcium Flickers and Motility at the Leading Edge Membrane

3221-Symp
Imaging Neutrophil Migration in Vivo using Zebrafish
Anna Huttenlocher.
Univ Wisconsin Madison, Madison, WI, USA.
Cell migration is crucial for diverse biological processes. Here we have visualized the dynamics of PI(3)K activity during neutrophil migration in intact tissues, revealing that PI(3)K activation at the leading edge is critical for neutrophil motility. Rac was activated locally in living zebrafish using genetically encoded photoactivatable Rac, demonstrating that Rac activation is sufficient to direct cell migration in vivo. In PI(3)K-inhibited cells, Rac activation at the leading edge rescued membrane protrusion but not cell migration or polarity in PI(3)K-inhibited cells. Uncoupling Rac-mediated protrusion and polarization suggests a new paradigm of two-tiered PI(3)K-mediated regulation of cell motility. This work exemplifies a broadly applicable new approach for examining spatio-temporal regulation of signaling within multicellular organisms.

3222-Symp
Second Messengers at the Leading Edge: Calcium Joins PIP3 as an Essential Signal
Joseph J. Falke.
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The leading edge of a chemotaxing cell is the engine that drives the cell up an attractant gradient. In one of the most dramatic spatial redistributions in cell biology, local second messenger signals recruit dozens of regulatory proteins from cytoplasm to the leading edge membrane. In turn these proteins control the actin polymerization and membrane remodeling processes propelling migration. It has long been known that the signaling lipid PIP3 is a crucial second messenger at the leading edge where it recruits PH domain proteins. Recent evidence has revealed that calcium is also an essential second messenger at the leading edge, where the local calcium signal recruits protein kinase C and other C2 domain proteins. The leading edge PIP3, calcium, and PKC are all required components of the positive feedback loop that maintains leading edge structure and activity. This positive feedback may well play a central role in the compass that guides cell migration.

3223-Symp
Calcium Flickers in Cell Migration
Heping Cheng.
Peking Univ, Beijing, China.
No Abstract.

3224-Symp
Signaling Control of Collective Cell Migration
Tobias Meyer.
Stanford Univ Sch Med, Stanford, CA, USA.
No Abstract.

Symposium 19: The 'Un' in Unconventional Molecular Motors

3225-Symp
Opening Up, Coming Together and Reaching Out: More Surprises from Myosin VI
Lee Sweeney.
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Myosin VI challenges the prevailing theory of how myosin motors move on actin: the lever arm hypothesis. While the reverse directionality and large powerstroke of myosin VI can be attributed to unusual properties of a subdomain of the motor (converter with a unique insert), these adaptations cannot account for the large step size on actin. Either the lever arm hypothesis needs modification, or myosin VI has some unique form of extension of its lever arm. We determined the structure of the region immediately distal to the lever arm of the motor and show that it is a three-helix bundle. Based on C-terminal truncations that display the normal range of step sizes on actin, CD, fluorescence studies, and a partial deletion of the bundle, we have demonstrated that this bundle unfolds upon dimerization of two myosin VI monomers to generate an extension of the lever arm of myosin VI. Furthermore, based on the ability of myosin VI monomers to dimerize when held in close proximity, we postulated that cargo binding normally regulates dimerization of myosin VI. We tested this hypothesis by expressing a known dimeric cargo adaptor protein of myosin VI, optineurin, and the myosin VI-binding segment from a monomeric cargo adaptor protein, Dab2. In the presence of these adaptor proteins, full-length myosin VI has ATPase properties of a dimer, appears as a dimer in EM, and moves processively on actin filaments. The results support a model in which cargo binding exposes internal dimerization sequences within full-length myosin VI. Since unexpectedly a monomeric fragment of Dab2 triggers dimerization, it would appear that myosin VI is designed to function as a dimer in cells.

3226-Symp
Novel Functions for Myosin-I in Microvillar Membrane Motility and Mechanics
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Class I myosins are evolutionarily ancient, monomeric unconventional myosins that interact with highly charged acidic phospholipids in cellular membranes. Myosin-1a, one of eight class I myosins expressed in vertebrates, is found almost exclusively in epithelial cells that line the intestinal tract (also known as enterocytes). Here, myosin-1a resides in the brush border - an array of actin-rich microvilli that extend from the enterocyte apical surface. Within the microvillus, myosin-1a links the inner leaflet of the plasma membrane to a supporting parallel bundle of actin filaments. Although the functional consequence of myosin-1a/membrane interactions remained unclear for many years, our recent studies indicate that this motor can power the plus-end directed movement of microvillar membrane along core actin bundles. This novel form of subcellular motility leads to the shedding of small vesicles from microvillar tips, into the intestinal lumen. Careful analysis of vesicle components suggests that shedding activity may represent a previously unidentified aspect of epithelial cell biology, which serves to protect enterocytes from microbes in the intestinal lumen. Parallel biophysical studies indicate that myosin-1a (and other class I myosins) also contributes to membrane-cytoskeleton adhesion, which enables the apical membrane to resist deformation, and in turn, allows a single enterocyte to stabilize a staggering ~1000 microvilli on its apical surface. Together, these findings position myosin-1a as an important player in microvillar membrane motility and mechanical stability. Because they also highlight the unique multifunctional nature of this motor, our current efforts are focused on defining the molecular mechanisms and regulatory switches that enable cells to coordinate these functions.

3227-Symp
Mechanism and Regulation of Cytoplasmic Dynein
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Cytoplasmic dynein is a molecular motor responsible for nearly all minus-end directed microtubule-based transport in cells. We use the model system *S. cerevisiae* to purify recombinant cytoplasmic dynein and its associated complexes and subunits (dynactin, lis1 and nudel). Using this recombinant system we are able to easily engineer point mutations, truncations and genetic tags that allow us to add a variety of handles that can be used for purification or fluorescent labeling. We then measure the activity of these complexes using a number of assays including single molecule fluorescence microscopy. Our results reveal that there are a variety of mechanisms that can be used to regulate the motor activity of cytoplasmic dynein including two mechanisms that enhance dynein processivity. Using purified recombinant dynein-dynactin complexes, we find that dynactin is a dynein processivity factor, but does not use its microtubule-binding domain to enhance processivity. We also find that regulation of ATPase activity is another possible mechanism of regulation, as mutants that cannot hydrolyze ATP at one of dynein's four ATP (AAA4) binding sites are also more processive. Current studies in the lab are focused on determining additional points of regulation in the dynein mechanochemical cycle.

3228-Symp
Unconventional Model for Dynein-Driven Movement
Keiko Hirose[1], Hironori Ueno[1], Takuo Yasunaga[2], Chikako Shingyoji[3].
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Previous electron microscopy (EM) and FRET analyses have shown that the tail of dynein swings with respect to the head and the stalk between the two nucleotide states that are thought to correspond to the pre- and post-powerstroke states (Burgess et al., 2003; Kon et al., 2005). However, how these structural changes observed in isolated dynein molecules are related to the minus-end-directed movement along a microtubule has not been clear. To understand how dynein changes with respect to microtubules, we studied the structures of sea urchin, outer-arm dynein bound to microtubules in the two nucleotide

states. By our new method, 'cryo-positive stain EM', the microtubule-bound stalks, as well as the ring-like heads and curved tails, were clearly observed.
In the no nucleotide state (with apyrase; the post-powerstroke state), the majority of dynein images showed two rings superimposed, suggesting close association of the two heads. When ATP and vanadate were added (the ADP•Vi, pre-powerstroke state), one of the heads moved with respect to the other. There was no detectable difference in the orientation of the stalks between the two nucleotide states; the stalks always pointed at the same angle towards the minus end of the microtubule to which they were bound (Ueno et al., 2008). The results disagree with models in which the stalk rotates on the microtubule and acts as a lever arm to amplify structural changes.
Rotation of the tail relative to the head would change the distance between the stalk tip and the tail-microtubule attachment point. In fact, some ADP•Vi images clearly showed the tail in a more extended state. Based on these results, we propose a new model, in which dynein pulls a microtubule by shortening the distance between its head/stalk and the tail-microtubule attachment, without rotating the stalk.

Platform AZ: Voltage-gated K Channels-Gating

3229-Plat
Thermodynamic Properties of Ionic Currents in *shaker* K^+ Channel Heterotetramers with Different Stoichiometries of ILT Mutations and/or Quadruple Gating Charge Neutralization
Dominique G. Gagnon, Francisco Bezanilla.
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The concurrent substitutions V369I, I372L, and S376T (ILT) in the S4 segment N-terminal end of voltage-dependent *Shaker* K^+ channels uncouple gating currents from ionic conduction (Smith-Maxwell *et al.*, 1998). This ILT mutation is believed to affect the last concerted step for channel activation. We aimed at determining the effect of temperature on steady-state and kinetics of activation of heterotetramers with different stoichiometries of ILT mutations. In addition, we also studied the single voltage sensor *Shaker* containing the ILT mutation in its voltage sensor. The heterotetrameric channels were encoded by concatemerized cDNA of *Shaker* zH4 Δ(6-46), expressed and studied in *Xenopus* oocytes using cut-open oocyte voltage-clamp under temperature ranging from 5-20 °C. The concatemeric channels $4wt_{ILT}$, $3wt_{ILT}$/wt, $2wt_{ILT}$/2wt, wt_{ILT}/3wt and wt_{ILT}/3mut (mut=R362Q/R365Q/R368N/R371Q) were studied. For all constructs, the amplitude of the ionic currents decreased by about 50-75% and the weighted time constants of activation increased by ten to fifteen fold when going from 19 to 5 °C, in a reversible manner. We also measured the steady-state voltage dependence of the conductance to estimate the entropic change during the final transition. The enthalpic and entropic components estimated from the temperature dependence of kinetics of activation for the different constructs provide essential information about the nature of the conformational changes and interactions between subunits. This information is used to refine the proposed model of independent voltage sensors followed by a concerted opening step.
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3230-Plat
A New Approach to the Structural Investigation of the Voltage-Sensitive Domain of Voltage-Gated Cation Channels as a Function of the Transmembrane Voltage
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Two fundamentally different approaches, self-assembly from solution and directed-assembly at the water-gas interface, are effective in the vectorial immobilization of the expressed voltage-sensitive domain of the KvAP channel on a suitably alkylated surface of silicon. The solvation of the immobilized protein was subsequently exchanged to form a phospholipid bilayer by incubating in a phospholipid-detergent solution in the presence of BioBeads. The formation of the protein monolayer and the vectorial-orientation of the protein molecules therein were investigated via interferometric X-ray reflectivity. The electron density profile of the tethered protein monolayer is consistent with the profile computed from the crystal structure, irrespective of the preparation procedure. Formation of lipid bilayer will require confirmation via neutron reflectivity using deuterated phospholipid. This approach enables an investigation of the structure of the VSD itself, as well as within the intact Kv-channel, as a function of the applied transmembrane voltage via a number of time-resolved techniques.

3231-Plat
The Importance of Ion Binding for Potassium Channel Inactivation and Recovery
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Potassium channels control the flow of ions across cell membranes with gating mechanisms involving conformational changes at the intracellular gate and the selectivity filter. Opening of the intracellular gate via external stimuli (activation) results in a transient period of conduction before the selectivity filter undergoes a conformational change, which constricts the permeation pathway (inactivation). When the applied stimulus is removed and the lower gate closes (deactivation), the filter slowly resets to a conductive conformation (recovery from inactivation). Using the KcsA channel as a prototypical model system to examine these issues, a combination of computer simulation (all-atom free energy and potential of mean force computations as well as transition pathway determination using the string method with swarms-of-trajectories) and experiment (electrophysiology and X-ray crystallography) is used to provide new insight into the microscopic mechanism that underlies inactivation and recovery from inactivation. An ion binding event is revealed as a crucial step in resetting the inactive filter during the recovery from inactivation.

3232-Plat
Charge Reversion to Charge Carrying Positions of S4 in Voltage Gated Shaker K-Channels
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Voltage gated Shaker potassium channels increases open probability (Po) by 20-fold with a ~6 mV depolarization. Such high voltage sensitivity is mostly due to the electrophoretic transmembrane relocation of four arginines residues in each of their four voltage sensing protein domains (VSD). These arginines movement across the electric field make possible channel opening upon membrane depolarization. We tested if the positions occupied by the voltage sensing arginines could carry acidic residues. We mutated three of these positions to aspartate: Arg362, Arg365, and Arg368 on an N-type inactivation removed background Shaker channel. All mutations were introduced with the use of the QuikChange kit and the mutation was verified by sequencing. Heterologous expressed in Xenopus oocytes, all mutants showed levels of expression comparable to that of the native channel. To determine voltage sensitivity of these charge reverting modified channels, we measured the voltage dependency of Po at voltages negative enough to observe only sporadic single channel openings in membrane patches containing hundreds of channels. From the exponential relation between Po and voltage we estimated the effective valence of opening in the range of Po ~10-6. All charge mutants showed an effective valence ~50% of that of the native Shaker. These results together with the comparable level of channel expression in oocytes are consistent with the idea the voltage sensitive positions in S4 are not specific for basic residues.
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3233-Plat
Sequential Electrostatic Interactions between E160 in S2 and Arginines in S4 During Voltage Dependent Activation of Kv7.1 Channels
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The fourth transmembrane segment of Kv channels, S4, contains a series of positively charged residues that imparts voltage sensitivity to the channel. Because the insertion of a highly charged peptide into a hydrophobic lipid environment is energetically unfavorable, electrostatic interactions with countercharges in the protein and phospholipids are required to lower this energy barrier. However, once the protein has been inserted into the membrane, what further role do these interactions play? In functional channels, electrostatic interactions are assumed to stabilize voltage sensor movement from a resting to an activated conformation. Although this assumption is at the crux of many models of voltage dependent gating, experimental evidence specifically examining these interactions in functional channels is incomplete. Here, we demonstrate in Kv7.1 channels that the first glutamate in S2, E160 (E1), form state dependent electrostatic interactions with arginines in S4. We used charged MTS reagents to directly probe the environment around E1 after mutating E1 to cysteine. We found that $MTSES^-$ but not $MTSET^+$ modifies E1C, suggesting a positively charged environment around E1. Mutations neutralizing or reversing the charge of the first or fourth arginine in S4 (R1 or R4) change the polarity of the environment around E1C such that $MTSET^+$ modifies E1C in the