Lipids & Signaling on Membrane Surfaces

1537-Pos Board B307
Hypercholesterolemia Induces Upregulation of K_{ACh} Cardiac Currents
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Control of the heart rate is a complex process that integrates the function of multiple proteins. Among them, G protein regulated inwardly rectifying K^+ (GIRK or Kir3) channels play a major role. Kir3 channels are important in regulating membrane excitability in cardiac, neuronal and endocrine cells. In the heart, the atrial K_{ACh} channels are heterotetrameric proteins that consist of two pore-forming subunits, Kir3.1 (GIRK1) and Kir3.4 (GIRK4). Activation of K_{ACh} by acetylcholine (ACh) via the muscarinic M2 receptor and pertussis toxin sensitive G proteins mediates the vagal negative chronotropic effect. Thus, activation of K_{ACh} channels can terminate paroxysmal supraventricular tachycardia, i.e., a rapid cardiac rhythm. On the other hand, vagal stimulation predisposes to atrial fibrillation, which can lead to thromboembolism and stroke. Thus, since hypercholesterolemia is a well-known risk factor for cardiovascular disease, we investigated the role of cholesterol on K_{ACh} currents ($I_{K,ACh}$). We found that in rabbit atrial cardiomyocytes, cholesterol enrichment enhanced channel activity whereas cholesterol depletion suppressed $I_{K,ACh}$. Moreover, a 20-24 week high-cholesterol diet resulted in up to 3-fold increase in $I_{K,ACh}$ in rodents. In accordance, elevated currents were observed in *Xenopus* oocytes expressing the Kir3.1/Kir3.4 heteromer. Interestingly, whereas similarly to the heteromer Kir3.1/Kir3.4, the homomeric pore mutant Kir3.4* (Kir3.4S143T) was also enhanced by cholesterol, Kir3.1* was suppressed by cholesterol. Furthermore, our data suggest that cholesterol affects $I_{K,ACh}$ via a mechanism which is independent of both $PI(4,5)P_2$ and Gbeta gamma. Notably, the impact of cholesterol on $I_{K,ACh}$ is opposite to its impact on I_{K1} in atrial myocytes. The latter are suppressed by cholesterol enrichment and by high-cholesterol diet, and facilitated by cholesterol depletion. These findings indicate that cholesterol plays a critical role in modulating $I_{K,ACh}$ in atrial cardiomyocytes via a mechanism independent of the channel's major modulators.

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Lipid-Dependent Membrane-Mediated Function of Disabled-2
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Disabled-2 (Dab2) targets membranes to trigger a wide range of biological events, including endocytosis and platelet aggregation. The Dab2 PTB domain mediates phosphatidylinositol 4,5-bisphosphate (PtdIns(4,5)P2)-dependent membrane targeting of Dab2 during endocytosis. In addition, Dab2, through its PTB domain, inhibits platelet aggregation by competing with fibrinogen for αIIbβ3 integrin receptor. We have recently showed that the N-terminal region, including its PTB domain (N-PTB), drives Dab2 to the platelet membrane surface by binding to sulfatides through two sulfatide-binding motifs, modulating the extent of platelet aggregation. We have characterized the membrane-binding properties of Dab2 N-PTB using micelles enriched with sulfatides and PtdIns(4,5)P2. We show that both lipid ligands overlap their binding sites and that they trigger protein stability. We also demonstrate that sulfatides, but not PtdIns(4,5)P2, facilitate N-PTB penetration into micelles. Our data show that the surface level of P-selectin, a platelet transmembrane protein known to bind sulfatides and promote cellular interactions, is reduced by N-PTB and that this event is reversed with a mutant form of the protein deficient in sulfatide but not integrin binding. Notably, N-PTB, but not its sulfatide binding-deficient mutant, is able to prevent sulfatide-induced platelet aggregation when tested under microfluidic conditions at flow rates with shear stress levels corresponding to those found in microcirculation. Moreover, the regulatory role of N-PTB can be extended to platelet-leukocyte adhesion and aggregation events suggesting a multitarget role for Dab2 in haemostasis. Lastly, we have solved the NMR structure of the minimal sulfatide-binding Dab2 unit, the sulfatide-binding motif (SBM). Dab2 SBM contains two helices when embedded in micelles, binds sulfatides with moderate affinity, and reduces sulfatide-induced platelet aggregation. Overall, our data exposes a new role for Dab2 in controlling platelet homotypic and heterotypic interactions and unveils the anti-aggregatory properties of its sulfatide-binding region for therapeutic purposes.

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Insight into the Structural Basis and Intermolecular Interactions of the Phytophthora Sojae Avirulence Homolog-5 with Phosphatidylinositol-3-Phosphate
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Oomycetes, such as *P.sojae*, are plant pathogens that employ protein effectors (Avirulence or Avr proteins) to facilitate infection. Plants are able to overcome this infection by their ability to recognize pathogen effectors by the plant defense system. The entry of Avr proteins to plant cells is mediated by external membrane-bound phosphoinositides such as phosphatidylinositol-3-phosphate (PtdIns(3)P). Furthermore, the N-terminal RXLR motif of several Avr proteins is critical for phosphoinositide recognition. However, a recent report indicates that the C-terminal basic domain of related effectors, Avr3a and Avr1b, is relevant for phosphoinositide recognition. Because there is a need to clarify these differences, we have structurally and functionally characterized *P.sojae* Avirulence homolog-5 (Avh5). Using NMR spectroscopy, we demonstrate that Avh5 is helical in nature with a long N-terminal disordered region. HSQC titrations of Avh5 with the PtdIns(3)P head group, inositol 1,3-bisphosphate ($Ins(1,3)P_2$), allowed us to map two lipid-binding regions that comprise the basic-rich second helix and the charged fourth helix. The first residue of the RXLR motif, Arg24, was also perturbed by $Ins(1,3)P_2$. Using both liposome-binding and lipid-protein overlay assays, we demonstrate that whereas mutations in RXLR slightly affect PtdIns(3)P binding; mutations in the basic second helix (residues Lys62, Lys64, and Lys65) almost abolished it. Avh5 exhibited moderate affinity for PtdIns(3)P (K_D=2.3 μM) as determined using surface plasmon resonance. Consistent with our lipid-binding assays, mutations in the RXLR and the basic patch reduced the affinity for the phosphoinositide 1.5- and 44-fold, respectively. Thus, our findings suggest that the central Avh5 basic-rich region has a major role in PtdIns(3)P recognition. Therefore, our identification of PtdIns(3)P-binding site of Avh5 will provide the structural basis to understand the role of the lipid in facilitating entry of Avr proteins into plant cells.

1540-Pos Board B310
Capturing Spontaneous Membrane Insertion and Membrane-Induced Conformational Changes of Talin at an Atomic Resolution
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Integrins are a diverse set of proteins that play a central role in complex biological processes, such as tumor metastasis and thrombus formation. The integrin heterodimer is often expressed in a low-affinity, inactive state, relying on specific cytoplasmic or extracellular signals for its activation. The cytoskeletal-associated protein talin constitutes one of the major activation pathways of integrin through a membrane-mediated mechanism. While the involvement of activated, membrane-bound talin in this process is well established, atomic details of membrane binding of talin and of talin-dependent integrin activation have been lacking. Using our novel, highly mobile membrane mimetic simulation system, we have successfully captured complete insertion of the talin head domain (THD) in a phosphatidylserine membrane in three independent unbiased simulations, revealing key molecular events involved in the process. The THD is initially recruited to the membrane via the documented membrane orientation patch (MOP), consisting of a large number of positively charged residues. Electrostatic potential calculations revealed THD to be highly polarized, providing a potential mechanism explaining how the protein is aligns for optimal encounter with the membrane. We also observe a large, membrane-induced interdomain conformational change (>2.5 nm), which brings the F3 subdomain into contact with the anionic membrane via residues K325, N326, and K327. This result explains how F2 and F3 subdomains can simultaneously bind the membrane, a biochemically established aspect that could not be explained by the crystal structure. Moreover, we characterize a phenylalanine-rich region as the hydrophobic membrane anchor, consisting mainly of F261 and F283, which is released through the snorkeling motion of a few critical lysine residues within the membrane. Although such an anchor has been hypothesized to exist, none had been identified prior to this study.

1541-Pos Board B311
EphA2-Ephrina1 Signaling and PI(4,5)P2 Spatial Organization on Breast Cancer Cells
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EphA2 belongs to the largest subfamily - Eph receptors - of the Receptor Tyrosine Kinase (RTK) superfamily and is over-expressed in many cancer cell lines. The major role of Eph receptors is to regulate the dynamics of cellular

protrusions and cell migration. Previous research reports that activation of EphA2 by its ligand ephrinA1 increases the activity of Phosphoinositide 3-kinase (PI3K). PI3K is one of the key molecules in regulating cell migration by phosphorylating Phosphatidylinositol (4,5)-bisphosphate (PI(4,5)P2) to Phosphatidylinositol (3,4,5)-trisphosphate (PI(3,4,5)P3) at the cell edge facing the highest chemoattractant concentration.
Here, we recapitulate EphA2-EphrinA1 signaling between cells by presenting breast cancer cells expressing EphA2 with an ephrinA1-displaying supported lipid bilayer. Through live cell labeling of PI(4,5)P2 with the fluorescent PLCδ1-PH domain biosensor, we are able to directly monitor PI(4,5)P2 spatial organization and its role in EphA2 signaling pathway. In addition, PI(4,5)P2 signaling and membrane localization are also examined with a spatial mutation strategy, which presents diffusion barriers, disrupting EphA2-ephrinA1 spatial organization. Our study will further clarify the role of PI(4,5)P2 and PI3K in the EphA2 signaling pathway, and help to understand cancer cell progression and metastasis in the long term.

1542-Pos Board B312
Understanding the Nature of Cortical Acto-Myosin Based Active Patterning Machinery and its Implications on the Dynamics and the Organization of Cell Surface Molecules
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We have found that the spatial distribution and the remodeling dynamics of the nanoclusters of outer leaflet glycosylphosphatidylinositol anchored proteins (GPI-APs), is regulated by the activity of the underlying cortical acto-myosin (CA).We have developed a theoretical framework to explain the mechanism of this nanoclustering, based on active hydrodynamics of the CA and its coupling to the membrane. This framework provides insights into the behavior of molecules that interact with the dynamic CA, reflected in their cell surface organization, dynamics and distribution. Here we discuss results from experiments designed to test key assumptions of this theory and major predictions. Using Fluorescence Correlation Spectroscopy (FCS) and single molecule imaging (SM) we provide evidence for a key assumption, namely, a rapidly turning over pool of short and dynamic F-actin based structures at or near the membrane cortex. To test the predictions of CA acting as membrane organizing machinery, we study the cell-surface organization of a model transmembrane (TM) connected to cytosolic actin binding domains (ABD), referred to as TM-ABD. Our results obtained from steady state and time-resolved homo-FRET measurements on TM-ABD indicate that CA-association can drive actin-dependent nanoscale organization of these proteins. The theoretical framework also predicts that cell-surface molecules which interact with this dynamic CA must exhibit anomalous density fluctuations which we are able to confirm in a fluorescence based assay. In addition, using FCS, we explore the temperature dependent diffusion characteristics of these molecules. These studies provide a new paradigm for understanding molecular organization and its spatiotemporal regulation on the plasma membrane.

1543-Pos Board B313
Analysis of the Binding Kinetics of Phosphoinositide Binding Domains Utilizing Voltage Sensing Phosphatases and Total Internal Reflection Fluorescence Microscopy
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Changes in phosphoinositide concentrations ([PI]) in cell membranes constitute important signaling events. Such changes are detected by proteins containing PI binding domains like the pleckstrin homology (PH) domains, e.g. $PI(4,5)P_2$ binding PLCδ1-PH, or PH unrelated PI-binding domains, e.g. the $PI(4,5)P_2$ binding *tubby* domain. These domains can be tagged with a fluorescent label, thereby creating a tool for the detection of [PI] changes in living cells. Since [PI] can change on a sub-second timescale, it is conceivable that the kinetics of PI (un-)binding of these domains become a limiting factor in the detection of such changes. Curiously, for PLCδ1-PH conflicting data on these kinetics have been reported, with dissociation time constants ranging from about 0.25 s to nearly 3 s.
To investigate this problem, we used voltage sensing phosphatases (VSPs) to rapidly deplete $PI(4,5)P_2$ while monitoring the dissociation of GFP-tagged PLCδ1-PH and *tubby* domains from the plasma membrane using total internal reflection microscopy (TIRF-M) imaging at 10 or 20 frames per second. These experiments revealed rapid dissociation of both domains. If Xl-VSP1 was used, the kinetics of $PI(4,5)P_2$ break down were rate limiting for the observed dissociation. In contrast, $PI(4,5)P_2$ break down by Ci-VSP was sufficiently rapid to reveal differences in the time course of dissociation between both domains, with observed time constants below 1 s.
In conclusion, we find the dissociation rates of PLCδ1-PH and *tubby* domains to be fast enough to faithfully track changes in $[PI(4,5)P_2]$ on a sub-second timescale.
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The Interaction of C1B Domains from Novel PKCs with Lipids
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C1 domains are members of the Cys-rich domains superfamily, formed by 50-51 amino acid residues present in many types of proteins., as it is the case of the classical and novel Protein Kinases C (PKCs). Both types of PKCs, classical and novel isoenzymes, possess two C1 subdomains C1A and C1B, although it is not totally clear why two modules are needed. C1 domains are known to interact with diacylglycerol and with exogenous agents like phorbol esters. In this work we have characterized the affinity to anionic phospholipids (1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphate (POPA); 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phospho-L-serine (POPS) and 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphophogycerol (POPG), 1,2-*sn*-dioleilglicerol (DOG) and phorbol 12-myristate-13-acetate (PMA) for C1B from novel isoenzymes PKCε, PKCη, PKCδ y PKCθ and comparing them with a C1B from a classical isoenzyme, namely PKCγ. To carry out this study the different C1B domains were expressed fused with eCFP in HEK295 cells. Fluorescent constructs were obtained from cell lysates and binding to lipid vesicles (LUV) labelled with Oregon Green was monitored through FRET. Results show that binding is enhanced by increasing concentrations of anionic phospholipids in the vesicles but each isoenzyme shows different affinities, so that C1Bγ, C1Bε, C1Bη y C1Bθ have a preference for POPA whereas C1Bδ prefers POPS. Results also show that C1Bε and C1Bη have a higher binding affinity than any of the others C1B domains independently of the lipid composition of LUV. When PMA was included in the vesicles instead of DOG, the binding differences among the C1B domains remarkably decreased and all domains showed an increased affinity for vesicles.

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Computational Analysis of Small GTPase Cycling: Extraction of Parameters and Inference of Pathways
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The Rho family of GTPases control actin organization during diverse cellular responses (migration, cytokinesis and endocytosis). Although the primary members of this family (RhoA, Rac and Cdc42) have different downstream effects on actin remodeling, the basic mechanism involves targeting to the plasma membrane and activation by GTP binding. Our hypothesis is that the details of GTPase cycling between membrane and cytosol are key to the differential upstream regulation of these biochemical switches. Accordingly, we developed a computational modeling framework to analyze experimental data for these systems. We show that experimental data for Rac membrane cycling and activation in cultured cells is well reproduced by a model where Rac dissociates from the membrane prior to binding to GDI; this contrasts with the behavior of Cdc42. The analysis also reveals that the lower apparent affinity of GDI for RacGTP compared to RacGDP can be fully explained by the faster dissociation of the latter from the membrane. A direct consequence is the increased membrane fraction of Rac upon increased GEF/GAP ratio. Non-dimensional steady-state solutions for membrane fraction of GTPases are presented in multidimensional charts. The charts are used to illustrate the effects of GEFs/GAPs and regulated affinities between GTPases and membrane and/or GDI on the amount of membrane bound GTPase. This methodology, is then used to analyze glucose stimulated Rac cycling in pancreatic β-cells. We find that the phosphorylation of GDI alone is not sufficient to translocate Rac. The affinity between Rac and membranes (plasma and granular) must also be increased upon glucose stimulus. In a similar fashion, the charts can be used as a guide in assessing how specific modifications may compensate for altered GTPase-GDI balance in disease scenarios.
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