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Tracking Voltage-Sensitive Movements of the M2 Muscarinic Acetylcho-
line Receptor...

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Numerous G protein-coupled receptors (GPCRs) have been shown to change their activity levels in response to changes in membrane potential. More recently, it has been directly demonstrated that these changes in activity reflect underlying changes in conformational state. However, the nature of the motion undergone by G protein-coupled receptors during voltage changes remains poorly understood. By combining site-directed fluorometry of the M2 muscarinic acetylcholine receptor with site-directed mutagenesis of residues that may serve as quenchers of the fluorescent signal, we attempt to refine our understanding of the voltage-dependent conformational changes that occur in a GPCR. When using tetramethylrhodamine-5-maleimide (TMRM), voltage-sensitive fluorescence changes display an electrochromic response in addition to the component that provides information on the voltage-sensitive conformational changes of the receptor. Interestingly, some mutations of potential quenching fluorophores do not alter the electrochromic signal allowing that alterations at these sites may result in a change in the landscape of the electric field within the protein. This work was supported by R01-GM030376 and F31-NS081954.

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Beta-Arrestin Biased Signaling at a Class a GPCR: Modeling the ORG27569 Induced CB1/Beta-Arrestin 1 Complex

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The CB1 allosteric modulator, ORG27569 (ORG), is an inverse agonist of the G-protein signaling pathway and agonist of the beta-arrestin-1 pathway (Ahn et al JBC 2013). The intracellular conformational change associated with arrestin-biased signaling is outward movement of the intracellular TMH7/HX8 elbow region away from TMH2 (Rahmeh et al PNAS 2012). We report here a 1.4µs molecular dynamics study of ORG interacting with CB1R via the lipid bilayer that revealed productive binding: ORG entered CB1 via the TMH6/7 interface, interacting with Y6.57 and F7.35. Subsequently, direct interactions between EC3 loop and ORG induced an outward movement of the TMH7/HX8 elbow region creating an opening between TMH2/7 that permitted docking beta-arrestin-1 with CB1. The active human beta-arrestin-1 structure used for docking was derived from the phosphorylated V2R C-terminal peptide (Shukla et al JBC 2013). The intracellular conformational change associated with beta-arrestin activation includes phosphorylation of the conserved 404-Tyr residue of beta-arrestin-1 crystal structure (Shukla et al Nature 2013) via peptide removal and mutation to the human sequence. CB1 residues, T460/S462/S464/T465/T467/S468, in the distal C-terminus, important for beta-arrestin association (Daigle et al J Neurochem 2008), were phosphorylated and placed to interact with beta-arrestin-1 N-domain positively charged residues, including critical lysines K10/K11 (Ostermaier et al PNAS 2014). The arrestin finger loop residues 66-70(EDLDV) were modelled as helical, based on photoactivated rhodopsin and visual-arrestin peptide NMR studies (Feuerstein et al Biochemistry 2009). Two orientations of beta-arrestin-1 relative to CB1 were modelled to have the N-domain underneath the TMH7/HX8 region based on a recent visual-arrestin-1 fingerloop crystallized in Opsin (Szczepak et al. Nature Comm 2014), or underneath the TMH5/6 IC3 loop based on a beta-arrestin-1 K77C/beta-2 adrenergic receptor TMH5 K3.78C crosslinking study (Shukla et.al Nature 2014). MD simulations, in AMBER14 with the CHARMM36 forcefield, addressing stability of the two CB1/beta-arrestin-1 complexes in fully hydrated POPC bilayers will be presented.

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Molecular Determinants and Kinetic Parameters of Ligand Binding to G Protein-Coupled Receptors using Markov State Model Analysis

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G protein-coupled receptors (GPCRs) continue to hold leading positions as drug targets. However, many GPCR drug candidates fail in clinical trials because of limited in vivo efficacy. While binding affinity, i.e. the strength of association of a drug to its receptor, has traditionally been viewed as an appropriate surrogate for in vivo efficacy, retrospective analyses of marketed drugs suggest that kinetic parameters, such as the rates at which the drug associates with or dissociates from the target, may play a role that is as important as, or even more important than, binding affinity in determining in vivo efficacy. Thus, predictions of both kinetic and thermodynamic parameters of ligand binding to GPCRs are highly desirable because they may inform the rational discovery of improved therapeutics.

The main challenge in studying molecular recognition by a GPCR using molecular dynamics (MD) is that ligand binding and unbinding are rare events on microscopic time-scales, and as such, they are difficult to observe using unbiased simulations. In this work, we present a general strategy that employs biased MD simulations to build Markov State Models (MSMs) of the binding of small molecules to a prototypical family A GPCR. Using Perron cluster analysis and transition path theory we were able to identify the kinetic basins of the binding process, and to characterize both the metastable and the transition states between bound and unbound conformations. By investigating the role of hydrophobic interactions, dewetting, and conformational changes in the binding pocket, we were able to characterize the microscopic determinants that influence association and dissociation rates of the ligand. This information has a direct applicability in rational drug design approaches.

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Lck Cluster Dynamics in Live Cells

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T-Lymphocytes initiate an adaptive immune response after specific binding of the T-cell receptor (TCR) to an antigenic peptide bound to the major histocompatibility complex (peptide-MHC) on an antigen-presenting cell. Key events on TCR-MHC binding include the phosphorylation of intracellular tyrosine residues of the TCR and recruitment of adapter molecules, which results in downstream signaling. Initial TCR phosphorylation is primarily carried out by lymphocyte specific kinase (Lck) making it a central molecule for T-cell signaling.

Lck has been shown to form nano-scale clusters that can be visualized by super-resolution microscopy techniques. However, neither the molecular determinants nor the function of Lck clustering are clearly understood. In principal, local lipid heterogeneities of the plasma membrane, actin-driven compartmentalization and protein-protein interactions between clustered molecules could account for non-random distribution of Lck on the plasma membrane.

We carried out single molecule fluorescent microscopy of mEos3.2-tagged Lck expressed in Jcama1.6 cells to elucidate the mechanisms that lead to Lck clustering in the plasma membrane. Our single molecule tracking data show transient immobilization of Lck molecules in clusters of about 150 nm. Strikingly, these clusters appear to coincide with topological protrusions of the membrane as indicated by the increased brightness of clustered Lck molecules as well as a fluorescent membrane probe. We further find that neither disruption of the actin cytoskeleton nor cholesterol depletion influence Lck clustering.

Finally, we map the region of Lck responsible for its immobilization to the N-terminal unique domain (SH4) using truncation mutants of Lck.

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The B Cell Receptor Dictates its Local Lipid Environment

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The B cell receptor (BCR) is responsible for sensing and responding to intact antigen during the immune response and is hypothesized to nucleate a unique composition of lipids surrounding receptor clusters during antigen binding and signaling. This unique lipid composition, sometimes referred to as a "lipid raft," is predicted to be enriched in glycosphingolipids and cholesterol. An altered lipid composition surrounding the BCR could influence the partitioning and activity of regulatory proteins and lipids, acting as a mechanism for the B cell to regulate signal transduction following stimulus by antigen. However, without direct observations of this phenomenon, theories surrounding lipid compositional heterogeneity in B cells remain controversial. In this work, we utilize two-color super-resolution localization microscopy (STORM and PALM) to directly observe the lipid composition proximal to B cell receptors, using the photoactivatable fluorescent protein mEos3.2 anchored to the plasma membrane through posttranslational lipid modifications. These probes mimic important regulatory proteins involved in BCR signaling by their membrane anchor but lack the protein interaction domains or biological activity found in the native protein. By analyzing the co-distributions of the B cell receptor and lipid probes using correlation functions, we find that the membrane surrounding the B cell receptor clusters is depleted of probes bearing unsaturated and branched geranylnerylarnyl modifications and is enriched in probes bearing saturated palmitoyl modifications. Quantification of lipid probe distributions reveals that B cell compositional heterogeneity influences the partitioning of...
these lipid modifications with order 1kBt of effective potential, as expected from predications of composition fluctuations in critical systems. These findings provide definitive evidence of membrane compositional heterogeneity in an important biological signaling system. Our results suggest that compositional fluctuations contribute to cellular responses by influencing the spatial distribution of specific components in the plasma membrane.

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FcerI Signal Propagation is Regulated through Transient Binding of Syk
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The high affinity IgE receptor, FcεRI, serves as the primary immunoreceptor on mast cells and basophils. Crosslinking of IgE-bound FcεRI via multivalent antigen induces phosphorylation of the immunoreceptor tyrosine-based activation motifs (ITAMs) followed by recruitment of the kinase Syk and further signal propagation. While the sequence of these signaling events has been well studied using biochemical techniques, the biophysical mechanisms that regulate FcεRI signaling are unclear. Using two-color single molecule imaging in live cells, we quantify how FcεRI aggregation influences Syk recruitment and mobility. We imaged the basal surface of RBL-2H3 cells expressing mNeon-Syk using TIRF microscopy upon crosslinking of FcεRI observed an increase in the membrane localization of mNeon-Syk. Interestingly, while ensemble measurements showed that FcεRI and mNeon-Syk were colocalized over minutes, single molecule imaging revealed that mNeon-Syk clusters are in fact maintained through a continuous exchange of transiently bound mNeon-Syk. We quantify the residency time of individual mNeon-Syk molecules at FcεRI aggregates and explore the relationship between FcεRI aggregate size and Syk residency time. Previously, it has been shown that high concentrations of stimulating cross-linking antigen induce a drastic reduction in FcεRI mobility. However, when antigen is added at low concentrations, low valency, or presented from a mobile bilayer, this mobility change is less substantial. We use single particle tracking to characterize the mobility of mNeon-Syk and find a similar antigen dependence on mobility. We show that at high concentrations of crosslinking antigen, Syk is highly immobilized at the membrane, yet when antigen is presented at lower concentrations or from a mobile bilayer Syk remains mobile at the membrane while still exhibiting an increased residency time. These results demonstrate that mobile FcεRIs are capable of recruiting Syk and that receptor immobilization is not a requirement for signaling.

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Establishing the Structural Rules for Ligand Recognition, Signaling and Assembly in Innate Immune Receptors
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Type-I transmembrane Toll-like receptors (TLRs) are central to host defense against pathogens. Of the ten human TLRs, TLR4 is of particular interest given the complex mechanism required to recognize lipopolysaccharide (LPS) from the outer membranes of Gram-negative bacteria, culminating in transfer of LPS from bacterial LPS aggregates to the CD14 coreceptor, and find a similar antigen dependence on mobility. We use single particle tracking to characterize the mobility of mNeon-Syk and explore the relationship between FcεRI aggregate size and Syk residency time. Previously, it has been shown that high concentrations of stimulating cross-linking antigen induce a drastic reduction in FcεRI mobility. However, when antigen is added at low concentrations, low valency, or presented from a mobile bilayer, this mobility change is less substantial. We use single particle tracking to characterize the mobility of mNeon-Syk and find a similar antigen dependence on mobility. We show that at high concentrations of crosslinking antigen, Syk is highly immobilized at the membrane, yet when antigen is presented at lower concentrations or from a mobile bilayer Syk remains mobile at the membrane while still exhibiting an increased residency time. These results demonstrate that mobile FcεRIs are capable of recruiting Syk and that receptor immobilization is not a requirement for signaling.

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Reinforcement of Integrin-Mediated T-Lymphocyte Adhesion by TNF
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The mammalian inflammatory response significantly relies on integrin-mediated T-lymphocyte adhesion to endothelial cells. Whereas the outside-in signalling pathway of integrins in response to the proinflammatory cytokine tumor necrosis factor (TNF) has already been studied in detail, little knowledge exists about the inside-out signalling pathway of integrins in lymphocyte activation by TNF. We here show single-cell force spectroscopy (SCFS) data of T-lymphocyte (Jurkat E6-1) adhesion to fibronectin. Fibronectin is present on top of endothelial cell layers and is therefore a crucial player in mediating T-lymphocyte binding to endothelial cells. Our results show that activating integrins with TNF significantly increases the maximum adhesion force and detachment energy in Jurkat cell adhesion to fibronectin-coated surfaces. Analysis of single-molecule ruptures further proves that TNF reinforces integrin binding strength, particularly at sub-second timescales. Hence, our results provide quantitative evidence for the significant impact of TNF-induced inside-out signalling in the T-lymphocyte adhesion machinery.