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Tracking Voltage-Sensitive Movements of the M2 Muscarinic Acetylcholine 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 quenchers of the fluorescent signal also alter the electrochromic signal, suggesting 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 activated rat 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 (Szczepek et.al. Nature Comm 2014), or underneath the TMH5/6 IC3 loop based on a beta-arrestin-1 K77C/beta-2 adrenergic receptor TMH5 K5.78C 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. [Support: RO1 DA003934, KO5 DA021358 (PHR)]

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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 prototypic 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 upon TCR-pMHC binding involve 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 JCaM1.6 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 B cell receptor clusters is depleted of probes bearing unsaturated and branched geranylgeranyl 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