Membrane Receptors & Signal Transduction I

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Retinal Changes Conformation during the Early Stages of Rhodopsin Activation

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G protein-coupled receptors (GPCRs) are integral membrane proteins that account for one third of drug targets. Although they are well studied biophysically, the details of their activation mechanism remain unknown. Here we combine solid-state 2H NMR and molecular dynamics simulations to study activation of the prototypical GPCR rhodopsin in a membrane environment. Our NMR data showed that rhodopsin's ligand, retinal, changed conformations as the protein transitioned between the dark-state and the Meta-I intermediate. To better understand ligand dynamics, we conducted three separate all-atom simulations of rhodopsin in explicit solvent, totaling 4.5 microseconds. These simulations examined dark-adapted rhodopsin bound to 11-cis retinal and two separate simulations tracking the formation of Meta-I. The Meta-I simulations began with a dark-state structure bound to the activating ligand, all-trans retinal. We introduced minor differences between these two simulations to track the role of two glutamates in Meta-I formation. We then computed a set of experimental observables, the deuterium line-shape for individual methyl groups in retinal, from each simulation. The results showed that the dark-state simulation and NMR data matched each other but not Meta-I. Further, the spectra of both Meta-I simulations differed from the dark-adapted NMR spectra, but only one of these matched the experimental Meta-I NMR spectra. The matching simulation exhibited concerted motion between retinal and its Schiff base-linked Lysine-296, and the highly conserved Triptophan-265, distinguishing it from the dark-state simulation.

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On the Relative Stability of Dimeric Interfaces of the Mu-Opioid Receptor Inferred from Recent Crystallographic Studies

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The mu-opioid (MOP) receptor is the predominant target for most clinically used analgesics. In response to demonstrated correlations between opioid dependence and the MOP receptor, researchers have focused on alternative targets. Opioid receptor oligomers are among those that have been suggested to mediate analgesia without the common opioid-related adverse effects. Thus, obtaining a molecular-level understanding of the nature of receptor-receptor interactions in the membrane, either within, or between receptor subtypes, can both create new opportunities for drug discovery, and address the role of oligomerization in receptor function. The recent MOP receptor crystal structure has inspired hypotheses of dimerization contacts, specifically: a closely packed interface involving transmembrane (TM) helices TM5 and TM6, and a less compact one involving TM1, TM2, and helix 8 (H8). These interfaces exhibit similar arrangements to those found in crystals of the chemokine receptor CXCR4 and the kappa-opioid receptor, respectively. While it is tempting to speculate that the tighter TM5/TM6 arrangement has physiological relevance, additional studies are necessary to a) understand the relative dimer stability at TM5/TM6, TM1/TM2/H8, and other interfaces in the membrane, b) evaluate the contribution of the engineered T4 lysozyme (T4L) to the TM5/TM6 association, and c) investigate possible variability across different receptors. To begin to address these questions we have performed umbrella sampling molecular dynamics simulations of coarse-grained representations of the MOP receptor interacting at the TM5/TM6 (with and without T4L) or TM1/TM2/H8 interfaces in an explicit lipid-water environment. We have derived relative estimates of the dimerization free energy at the two specific interfaces and from these we suggest relative dimer lifetimes and dimeric fractions in a lipid mimetic environment. This information can help design future experiments aimed at understanding the role of dimerization in receptor function.

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A Computational Investigation of the Effect of Membrane Curvature on G-Protein Coupled Receptor Oligomerization

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Typical cellular membranes display wide structural and rheological diversity. Such properties have been suggested to impact the spatial organization of membrane proteins with consequent functional implications. Indeed, oligomers of G protein-coupled receptors (GPCRs) are actively investigated for their role in cell physiology and as promising new targets of improved clinical relevance. Inspired by recent experimental findings, we used microsecond-scale molecular dynamics (MD) simulations to investigate the effect of membrane curvature on the mobility and spatial organization of coarse-grained (CG) representations of the prototypical GPCR beta2 adrenergic receptor (B2AR) in a mixture of 80% dioleoylphosphatidylcholine (DOPC), 10% dioleoylphosphatidylglycerol (DOPG), and 10% cholesterol used as a membrane mimetic. Specifically, we explored the self-association of 16 CG B2AR molecules in either a flat lipid bilayer or a closed vesicle with a ~32 nm diameter at a protein/lipid-cholesterol ratio of ~1:450. All simulations were performed with the GROMACS simulation package using the 4 atoms-to-1 bead MARTINI force field. In agreement with simple hydrodynamic models, we find that receptor mobility is hindered at large curvature. A detailed analysis of receptor collisions and residence time of dimeric complexes during simulations yields results consistent with an increased, more stable self-association of receptors in the planar bilayer compared to the small vesicle system.

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Retinal Conformation Governs pKa of Protonated Schiff Base in Rhodopsin Activation

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We have explored the relationship between conformational energetics and the protonation state of the Schiff base in retinal, the covalently bound ligand responsible for activating the G protein-coupled receptor rhodopsin, using quantum chemical calculations. Guided by experimental structural determinations and large-scale molecular simulations on this system, we examined rotation about each bond in the retinal polyene chain, for both the protonated and deprotonated states that represent the dark and photoactivated states, respectively. Particular attention was paid to the torsional degrees of freedom that determine the shape of the molecule, and hence its interactions with the protein binding pocket. While most torsional degrees of freedom in retinal are characterized by large energetic barriers that minimize structural fluctuations under physiological temperatures, the C6-C7 dihedral defining the relative orientation of the β-ionone ring to the polyene chain has both modest barrier heights, and a torsional energy surface that changes dramatically with protonation of the Schiff base. This coupling between conformational degrees of freedom and protonation state is further quantified by calculations of the pKa as a function of the C6-C7 dihedral angle. Notably, pKa shifts of greater than two units arise from torsional fluctuations observed in molecular dynamics simulations of the full ligand-protein-membrane system, implying significant changes in the acidity of the Schiff base prior to forming the activated MII state. These new results shed light on important mechanistic aspects of retinal conformational changes that are involved in the activation of rhodopsin.

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Fluorescence Studies of the Bradykinin 2 and μ-Opioid Receptors Suggest that Caveolae Localization of GPCRs is Mediated by their Attached G Proteins

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Caveolae are 50-100 nm plasma membrane invaginations that have several proposed functions. Some studies suggest that caveolae may influence cell signaling by sequestering certain signaling proteins such as G-protein coupled receptors. We have previously found that Caveolin-1, the main structural protein of caveolae specifically binds to Gαq and that their binding is strengthened upon Gαq activation. Here, we have used fluorescence methods to determine the effect of caveolae on the functional properties and localization of two G-protein coupled receptors: the bradykinin receptor type 2 (B2R), which is coupled to Gαq, and the μ opioid receptor (μOR), which is coupled to Gαi. While caveolae do not affect cAMP signals mediated by μOR, they prolong Ca^{2+} signals mediated by B2R. In A10 cells, down-regulation of Caveolin-1 ablates the prolonged calcium signal in accord with idea that caveolae binds to Gαq. Immunofluorescence and FRET studies show that a significant fraction of B2R resides at or close to caveolae domains while none or very little μOR resides in caveolae domains. FRET between B2R and caveolae is reduced by down-regulation of Gαq or by addition of a peptide that interferes with Gαq/Caveolin-1 interactions suggesting that Gαq promotes localization of B2R to

caveolae domains. Fluorescence Correlation Spectroscopy studies on live cells show that the presence of caveolae changes the distribution of the apparent diffusion coefficients of B2R and Gαq but not μOR and Gαi. Our results suggest that Gαq can localize its associated receptors to caveolae domains to enhance their signals.

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Probing the Stoichiometry and Geometry of M3 Acetylcholine Receptors at the Plasma Membrane

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G-protein-coupled receptors (GPCRs) are the largest family of transmembrane proteins in nature. GPCRs cascade signals from outer environment into cells and hence, are the target of more than 60% of modern clinical drugs. Determination of oligomerization of GPCRs is subject to significant controversy in the literature. We have investigated the quaternary structure of human muscarinic acetylcholine receptor type 3 (hM3) in living cells using Förster Resonance Energy Transfer (FRET) and two-photon excitation in an optical microspectroscopic set-up [Raicu et al, Nature Photonics, 2009]. The wild-type form of the hM3 receptor was fused to Cerulean while its mutated form, activated solely by a synthetic ligand (RASSL), was fused to Citrine and expressed constitutively in Flp-InTM T-RExTM 293 cells [Alvarez-Curto et al, Journal of Biological Chemistry, 2010]. When WT-hM3-Citrine and RASSL-hM3-Cerulean were co-expressed in same cells, excited donor (Cerulean), transferred its energy to nearby acceptors (Citrine) through FRET. Apparent FRET efficiency (Eapp) distribution maps were obtained for the imaged section of the cells by unmixing acquired spectrally resolved images using elementary donor and acceptor spectra. By selecting pixels in the Eapp image corresponding to the plasma membrane, a FRET efficiency histogram was obtained displaying the number of pixels in a particular range of Eapp values versus the corresponding Eapp value. The Eapp histograms were analyzed using a FRET theory [V. Raicu, Journal of Biological Physics, 2007], predicting various numbers and positions of peaks in the histograms for various sizes and geometry of oligomers. Eapp histograms of all cells were best fitted by a rhombus tetramer model. By also simulating the amplitudes of the peaks, we determined that hM3 receptors form both dimers and rhombus tetramers at the plasma membrane, and that their proportion remained largely unaffected by CNO binding.

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GPCR Activation on the Microsecond Timescale in MD Simulations

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G-protein coupled receptors (GPCRs) play a central role in signal transduction and consequently represent a major drug target. Structural similarities suggest a common working mechanism, which is typically deduced from crystal structures of activation intermediates. The latter have become available recently, in particular for the light sensing GPCR rhodopsin and other members of the rhodopsin-like GPCR family. These crystal structures provide snapshots along the activation pathway, however, the transitions between those remain speculative.

Time resolved spectroscopy is able to provide crucial insights into dynamic details. However, another promising route to study receptor dynamics was opened by the recent occurrence of special purpose computers for molecular dynamics (MD) simulations, which allow to observe conformational transitions related to receptor activation/deactivation directly in unbiased MD simulations on timescales of tens of microseconds.

The activation kinetics for GPCRs, such as rhodopsin, typically feature time constants on the order of milliseconds, which is still beyond reach for direct simulation. However, here we present microsecond simulations for a special case, squid rhodopsin from Todarodes pacificus. While it shares many structural features with its bovine counterpart, activation occurs with a time constant of 12.5 microseconds at room temperature. We analyze simulations of the light activated receptor spanning up to 20 microseconds, allowing us to identify activation events and focus specifically on the interactions between the retinal ligand and the receptor, that trigger the activation process.

This work was supported by the German Academy of Sciences Leopoldina (fellowship to M.H.) and the NSF (grant CHE-0750175). We are grateful for an allocation of computer time on Anton at the Pittsburgh Supercomputing Center (supported by the NIH).

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Characterizing the Effect of $A_{2A}R$-D_2R Heteromeric Complex Formation on Homomeric $A_{2A}R$ and D_2R Signaling

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G protein-coupled receptors (GPCRs) are the targets of many drugs used in clinical practice because G proteins mediate a plethora of physiological functions. Appreciation of the existence of oligomeric GPCR complexes with distinct signaling properties from their monomeric counterparts is growing. Yet, the effect of heteromerization on the pharmacology and signaling of many GPCR monomers remains unknown. We have undertaken the task to examine the effect of heteromerization on the G_s signaling through the adenosine 2A receptor ($A_{2A}R$) and G_i signaling through the dopamine receptor type 2 (D_2R). Signaling through the $A_{2A}R$-D_2R heteromeric complex is of great interest as this heteromer is a pharmacological target for pathologies associated with dysfunctional dopaminergic signaling, such as in Parkinson's disease. In order to analyze $A_{2A}R$-D_2R heterocomplex cross signaling through G_i, we are using an electrophysiological assay with heterologously expressed channels serving as reporters for GPCR signaling. Preliminary data suggest that heteromer formation decreases the dopamine elicited G_i signaling through the D_2R. We are in the process of assessing the effect of D_2R on the G_s signaling of the $A_{2A}R$, using a tritiated cAMP assay. We hypothesize that G_s and G_i signaling through the $A_{2A}R$-D_2R heterocomplex are inversely coupled, in a manner similar to the serotonin 2A- metabotropic glutamate type 2 receptor heteromer (Fribourg et al. 2011). Thus, if our hypothesis is correct, heteromerization is expected to increase adenosine elicited G_s signaling through the $A_{2A}R$. Furthermore, we expect that dominant agonists and inverse agonists for the $A_{2A}R$ and D_2R can be used to manipulate the G_i and G_s signaling through the heteromer. Characterization of the signaling pathway through the $A_{2A}R$-D_2R heteromer could lead to novel therapeutics for Parkinson's disease.

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Incorporation of Fluorescently Tagged Chemokine Receptor 5 (CCR5) into Membrane Nanoparticles

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Nanoscale apolipoprotein bound bilayers (NABBs) are soluble phospholipid bilayers encapsulated by the amphipathic helical protein apolipoprotein A-I (Apo A-I). NABBs, unlike detergent micelles and liposomes, provide a homogenous, native-like membrane environment to study structure-function relationships of membrane proteins. NABBs have previously been employed to study G protein-coupled receptors (GPCRs) such as rhodopsin and CCR5, but the approach was limited by poor expression of Apo A-I and low yields of GPCR incorporation. To address these shortcomings, we have engineered several synthetic genes of Apo A-I derived from zebrafish that express at higher levels in E. coli as compared to older Apo A-I variants. The size of the NABBs can be controlled by changing the length of Apo A-I, an additional advantage that will enable study of receptor oligomerization. We intend to use this platform in conjunction with single molecule fluorescence techniques to study GPCR-ligand interactions, focusing on the chemokine receptors CCR5 and CXCR4. The endogenous ligands of these receptors - including RANTES (CCR5) and CXCL12 (CXCR4) - have been shown to prevent HIV particle entry, but the kinetics and structural determinants of binding are poorly characterized. We show receptor labeling with Alexa 488 and 647 using unnatural amino acid mutagenesis and SNAP-tag technologies followed by incorporation into NABBs. The assemblies have been characterized by fluorescence correlation spectroscopy. Future experiments will use multicolor total internal reflection fluorescence (TIRF) microscopy and fluorescence cross-correlation spectroscopy (FCCS) to determine the kinetics of ligand binding and the stoichiometry of the signaling complex. These parameters will shed light on the mechanism of class A GPCR activation and could potentially inform future development of HIV entry blockers.

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Functional Signaling Changes Resulting from GPCR Heteromerization: Relevance to Psychosis

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Functional Signaling Changes Resulting from GPCR Heteromerization: Relevance to Psychosis

A broad spectrum of symptoms has been evidenced in psychotic disorders, while nearly 70 years of research has produced anti-psychotic drugs (APDs) that alleviate only certain ones of these symptoms and may cause the