mutant (R108A) were performed to investigate the diffusion characteristics of these 'inactive' and 'active' states of the A_3-receptor. One slow moving complex was identified at the cell membrane of wild-type A_3-GFP transfected cells, with a diffusion co-efficent (0.087 $\mu m^2/s$) similar to that of τ_{D3} for the XAC-X-BY630; similar complexes were identified in the mutant A_3-receptor cell lines.We have subsequently used FCS in conjunction with fluorescent agonist and antagonist A_3-receptor ligands to compare the ligand binding and diffusion properties of these different activity states of the receptor at the subcellular level.

1514-Pos

Computational Insight into the Ligand-Induced Conformational Specificity of G-Protein Coupled Receptors

Davide Provasi, Juan Carlos Mobarec, Marta Camacho Artacho, Marta Filizola.
Mount Sinai School of Medicine, New York, NY, USA.

Several observations in the G-protein coupled receptor (GPCR) literature support the existence of ligand-specific intermediate conformational states that are likely to be involved in differential activation of signaling pathways. Fluorescence spectroscopy studies provide direct evidence for ligand-specific receptor conformations of the β2-adrenergic receptor, making this system an attractive target to test the ability of computational methodologies to predict different activated states of GPCRs. To this end, we designed a computational strategy that combines adiabatic biased molecular dynamics (ABMD) and metadynamics simulations. Firstly, ABMD is used to generate transition paths between the experimental inactive crystal structure of the β2-adrenergic receptor and a conformation containing established features of activated states of GPCRs (modeled using the opsin crystal structures). Secondly, metadynamics is applied to study how ligands with different efficacies affect the free-energy of different metastable states identified along these putative activation pathways. The calculated free-energy profiles of the different ligand-β2 adrenoceptor complexes help rationalize the published experimental results, including the different kinetics of catecholaminergic agonists such as epinephrine, norepinephrine, dopamine, and isoproterenol. Representative structures of the identified energy basins suggest specific residues and contacts that may help stabilize different activated states of the receptor. This information holds promise for the crystallization of different GPCR conformations.

1515-Pos

Structural and Kinetic Modeling of an Activating Helix Switch in the Rhodopsin-Transducin Interface

Peter W. Hildebrand.
Uni Berlin Charite, Berlin, Germany.

Extracellular signals prompt G protein-coupled receptors (GPCRs) to adopt an active conformation (R*) and to catalyze GDP/GTP exchange in the α-subunit of intracellular G proteins ($G\alpha\beta\gamma$). Kinetic analysis of transducin ($G_t\alpha\beta\gamma$) activation shows that an intermediary $R^*G_t\alpha\beta\gamma GDP$ complex is formed which precedes GDP release and formation of the nucleotide-free R*G protein complex. Based on this reaction sequence we explore the dynamic interface between the proteins during formation of these complexes. We start from the R* conformation stabilized by a $G_t\alpha$ C-terminal peptide (GαCT) obtained from crystal structures of the GPCR opsin. Molecular modeling allows reconstruction of the fully elongated C-terminal α-helix of $G_t\alpha$ (α5) and shows how α5 can be docked to the open binding site of R*. Two modes of interaction are found. One of them - termed stable or S-interaction - matches the position of the GαCT peptide in the crystal structure and reproduces the hydrogen bridge networks between the C-terminal reverse turn of GαCT and conserved E(D)RY and $NPxxY(x)_{5,6}F$ regions of the GPCR. The alternative fit - termed intermediary or I-interaction - is distinguished by a tilt (42°) and rotation (90°) of α5 relative to the S-interaction. It shows different α5 contacts with the $NPxxY(x)_{5,6}F$ region and the second cytoplasmic loop of R*. From the two α5 interactions, we derive a 'helix switch' mechanism for the transition of $R^*G_t\alpha\beta\gamma GDP$ to the nucleotide-free R*G protein complex. It illustrates how α5 might act as a transmission rod to propagate the conformational change from the receptor-G protein interface to the nucleotide binding site.

1516-Pos

Agonist-Specific Effects of TM V Serine Mutations on Dopamine D2S Receptor Voltage-Sensitivity

Kristoffer Sahlholm, Daniel Marcellino, Johanna Nilsson, Kjell Fuxe, Peter Århem.
Karolinska Institutet, Stockholm, Sweden.

Voltage-sensitivity has recently been demonstrated for agonist potency and affinity at certain G protein-coupled receptors. Using an electrophysiology assay in Xenopus oocytes, we have previously shown that the potency of dopamine in activating G protein-coupled potassium channels (GIRK) via the dopamine D2S receptor is reduced by depolarization from −80 to 0 mV. We recently investigated the voltage-sensitivities of a range of structurally related dopaminergic agonists at the D2S receptor.

The findings of this study led us to propose that a conformationally constrained interaction of the agonist with transmembrane segment (TM) VI of D2 is required for voltage-sensitivity. The hypothesis assumes that for the flexible phenethylamines, two hydroxyls (such as in dopamine) interacting with the conserved serines in TM V are necessary for voltage-sensitivity. Conversely, N,N-dipropyl-2-aminotetralin (DPAT) agonists do not require hydroxyls for voltage-sensitivity due to their inherently more rigid structure. To test this hypothesis, we mutated three conserved serines in TM V (S193A, S194A, and S197A) which have been shown to mediate binding to agonist hydroxyls. The voltage-sensitivity of non-hydroxylated DPAT was similar to that observed with the wild-type receptor at all of the three mutants, suggesting that the mutations did not allosterically alter the voltage-sensing properties of the receptor.

The S193A mutation drastically diminished voltage-sensitivity of dopamine, concomitantly with a marked reduction in potency. However, the S194A mutation which slightly decreased potency, did not appreciably affect the voltage-sensitivity of dopamine. At the S197A mutant, dopamine efficacy was decreased to such a degree that voltage-sensitivity could not be assessed.

In the literature, S193 has consistently been assigned a major role in dopamine binding. Our results suggest that this residue might also be important for voltage-sensitive interactions between dopamine and the D2S receptor.

1517-Pos

Influence of Membrane Composition on Function of Human Peripheral Cannabinoid Receptor CB2

Tomohiro Kimura, Alexei A. Yeliseev, Krishna Vukoti, Klaus Gawrisch.
NIH/NIAAA, Bethesda, MD, USA.

The human peripheral cannabinoid receptor CB2 is abundant in tissues of immune and hematopoietic systems. CB2 belongs to the class of heptahelical G-protein coupled receptors and regulates a wide range of physiological functions through binding of endogenous and exogenous cannabinoid ligands. We studied the influence of electrical surface potential of membranes and of hydrocarbon-chain order on rates of G-protein activation by CB2. The membrane surface potential was determined by a measurement of the electrophoretic mobility of proteoliposomes, while lipid hydrocarbon-chain order was quantified by a measurement of the order parameters using ^2H NMR. The receptor, expressed in *E. coli*, was purified and functionally reconstituted into lipid bilayers composed of phosphatidylcholine (PC), phosphatidylserine (PS), and cholesteryl hemisuccinate (CHS). CB2 was fully activated with the synthetic agonist CP-55,940. The rate of G-protein activation by the receptor increased about two-fold with increasing CHS content in the lipid matrix from 25 to 41 mol%. Similar effect was observed with increasing PS content. The increased activation rate correlated with a larger negative ζ-potential caused by the negatively charged headgroups. The increased order of lipid acyl chains due to interactions with the cholesteryl backbone of CHS had no significant effect on G-protein activation rates, as confirmed by addition of cholesterol instead of CHS. The results indicate the importance of anionic lipids for efficient coupling between the CB2 receptor and G-proteins.

1518-Pos

A Polybasic Region in the C-terminus of M3 Muscarinic Acetylcholine Receptors Mediates an Interaction with Gq Heterotrimers

Kou Qin, Sudha Kuravi, Nevin Lambert.
Medical College of Georgia, Augusta, GA, USA.

G protein-coupled receptors (GPCRs) form stable complexes with heterotrimeric G proteins when the former are activated and when the latter are not bound to guanine nucleotides. In addition to these active-state ternary (ligand-receptor-G protein) complexes some GPCRs have been suggested to form preassembled or precoupled complexes with G proteins prior to activation. We have previously reported that immobile M3 muscarinic receptors (M3Rs) decrease the mobility of heterotrimers that contain Gαq, consistent with an M3R-Gq complex. This interaction is unaffected by receptor ligands in intact cells, and is specific for M3Rs and Gq, as immobile M4Rs do not decrease the mobility of Gq heterotrimers, and immobile M3Rs do not decrease the mobility of GoA heterotrimers. In order to determine the structural basis of this interaction, we constructed a series of CFP-labeled M3R/M4R chimeras and tested their ability to decrease the mobility of venus-labeled Gq (Gq-V) using fluorescence recovery after photobleaching (FRAP). A chimera consisting of the M3R with the c-terminus of the M4R (M3M4ct) did not decrease Gq-V mobility. A polybasic region

(565KKKRRK570) immediately following helix 8 in the c-terminus of M3R was found to be necessary for the decrease in Gq-V mobility. We tested the hypothesis that an electrostatic interaction was involved in the interaction between M3Rs and Gq by repeating our FRAP experiments in permeabilized cells exposed to buffer solutions with high and low ionic strength. High ionic strength solutions inhibited the decrease in Gq mobility, whereas low ionic strength solutions enhanced this effect. These results suggest that an electrostatic interaction mediates an interaction between the c-terminus of M3Rs and Gq heterotrimers. The functional significance of this interaction is currently under study.

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1519-Pos

The Human Muscarinic Receptor Couples to GαI3 Via Catalytic Collision

Shai Berlin, Daniel Yakubovich, Tal Keren-Raifman, Nathan Dascal.
Tel Aviv University, Ramat Aviv, Tel Aviv, Israel.

Hundreds of G-protein coupled receptors (GPCR) are encoded in the human genome. All GPCRs react to a vast variety of ligands and initiate the G-protein activation cycle, by catalyzing the exchange of GDP by GTP on the Gα subunit. Classically, this mode of activation has been proposed to be of catalytic collision coupling nature, where a single receptor sequentially activates several G-proteins. However, recent biophysical and imaging studies challenged this concept and suggested that some GPCR and G-proteins form stable non-dissociating complexes prior to and after activation. We were interested in determining the mode of coupling between the human muscarinic 2 receptor (m2R) and Gαi3βγ. We used the G-protein activated K+ channel (GIRK) as a reporter for receptor activation and systematically quantified receptor's and channel's plasma membrane concentrations using fluorescent methods and radioligand assays. We found a decrease in activation time at high receptor density, with no change in channel concentration. However, maximal amplitude was attained at lower receptor density, suggesting an amplification process. No change in Gβγ concentration was observed, as judged by the unchanged Gβγ-dependent basal activity of GIRK. Additionally, increasing amounts of m2R did not increase Gαi concentration. Together, these results suggest a catalytic collision coupling mechanism. We constructed a model describing m2R's activation scheme and predicted that excessive Gα subunits should slow the activation process by occupying the activated receptor in ''dead-end'' interactions, not leading to channel activation. Increasing amounts of two fluorescent Gαi3 subunits were used to test the prediction. Indeed, both subunits slowed the evoked-current, without change in current amplitude. These results, together with our previous observations, suggest that the m2R activates Gαi3 via a catalytic collision coupling mechanism, where one receptor diffuses and activates several Gαβγ subunits, leading to the activation of GIRK.

1520-Pos

Structural Characterization of the N-terminal Region of the Saccharomyces Cerevisiae G-Protein Coupled Receptor, Ste2P

Stephanie Kendall[1], Chunhua Shi[2], Shelley Forgeron[3], Michele C. Loewen[1].
[1]University of Saskatchewan, Saskatoon, SK, Canada, [2]Carleton University, Ottawa, ON, Canada, [3]National Research Council of Canada, Saskatoon, SK, Canada.

Binding of α-factor pheromone to the G-protein coupled receptor, Ste2p, initiates signal transduction events that lead to mating of the yeast *Saccharomyces cerevisiae*. Recent indirect evidence also implicates the N-terminal region of Ste2p in modulating cell wall degradation and membrane fusion during later steps of mating. Toward deciphering mechanisms, structural studies have been initiated on the N-terminus of Ste2p. Initially, residues 1-71 of Ste2p were expressed as a fusion protein with HIS and KSI tags and affinity purified from *E.coli* in mg quantities. Subsequent cyanogens bromide cleavage at methionines yielded a hydrophobic peptide (Ste2p 2-54) that consistently disappeared upon HPLC enrichment. Similarly, a chemically synthesized fragment corresponding to Ste2p residues 14-43 could not be purified by HPLC. However, the addition of three lysines to both termini was found to decrease hydrophobicity sufficiently to enable HPLC purification. Circular dichroism studies of a chemically synthesized K^3-Ste2p-14-43-K^3 peptide indicated mostly random structure, with ~45% β-strand and a small percentage of α-helix in buffered water. The structure was found to be stable at temperatures up to 40°C. These results correlate with predicted 2o structure for the Ste2p N-terminal domain including: random chain with a β-strand-loop-β-strand fold followed by a C-terminal α-helix (Shi et al., J. Cell. Biochem. 107:630-38) and recent NMR evidence suggesting α-helix in a C-terminal overlapping region (residues 39-47; Neumoin *et al.,* 2009 Biophysical Journal 96: 3187-96). Preliminary ^{1}H-^{1}H NOESY and TOCSY NMR data for the K^3-Ste2p 14-43-K^3 peptide have been collected. As well, a recombinant version of K^3-Ste2p-2-43-K^3 is being produced to extend the N-terminal region to be analyzed and facilitate isotopic labeling for complete structural elucidation.

1521-Pos

Arrestin can Bind to a Single G-Protein Coupled Receptor

Hisao Tsukamoto, Abhinav Sinha, Mark DeWitt, **David L. Farrens**.
Oregon Health and Science University, Portland, OR, USA.

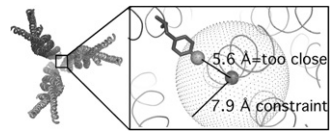
Termination of G protein-coupled receptor (GPCR) signaling typically involves phosphorylation of the receptor, followed by binding of a protein called arrestin. Here we tested the minimal stoichiometry required for this interaction, by determining if a single rhodopsin molecule can bind arrestin. To do this, we prepared nanoscale phospholipids particles, so-called nanodiscs, which contain only monomeric rhodopsin and measured their ability to bind visual arrestin. Our data clearly show that visual arrestin can bind to monomeric phosphorylated rhodopsin to stabilize its active form, called metarhodopsin II. Interestingly, we find beta-arrestin can also bind to monomeric rhodopsin in nanodiscs and stabilize metarhodopsin II. Together, these results suggest that in general, the minimal unit for arrestin binding is a monomeric GPCR.

1522-Pos

Solid-State NMR Demonstrates that Active Signaling Complexes of Bacterial Chemoreceptors Do Not Adopt the Proposed Trimer-Of-Dimers Structure

Daniel J. Fowler, Robert M. Weis, Lynmarie K. Thompson.
University of Massachusetts, Amherst, MA, USA.

The receptor dimers that mediate bacterial chemotaxis form signaling complexes with CheW and the kinase CheA. Based on the packing arrangement observed in two different crystal structures of two different receptor cytoplasmic fragments, two different models have been proposed for receptor signaling arrays: the trimers-of-dimers and hedgerow models. We have identified an inter-dimer distance predicted to be substantially different by the two models, labeled the atoms defining this distance through isotopic enrichment, and measured it with ^{19}F-^{13}C REDOR. This was done in two types of receptor samples: isolated bacterial membranes containing overexpressed, intact receptor, and soluble receptor fragments reconstituted into kinase-active signaling complexes. In both cases, the distance found was incompatible with *both* the trimers-of-dimers and the hedgerow models. Comparisons of simulated and observed REDOR dephasing were used to deduce a closest-approach distance at this interface, which provides a constraint for the possible arrangements of receptor assemblies in the kinase-active signaling state.



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1523-Pos

Site-Specific Fluorescent Labeling of Purified G-Protein-Coupled Receptors Using Genetically-Encoded Unnatural Amino Acids

Shixin Ye, Manija A. Kazmi, Terence Duarte, Saranga Naganathan, Thomas P. Sakmar, Thomas Huber.
www.sakmarlab.org, Rockefeller University, New York, NY, USA.

The introduction of unique chemical groups into proteins by means of site-directed mutagenesis with unnatural amino acids has numerous applications in protein engineering and functional studies. We first introduced *p*-acetyl-L-phenylalanine (Acp) or *p*-azido-L-phenylalanine (Azp) into the prototypical G protein-coupled receptor (GPCR) rhodopsin at specific sites. We employed an amber codon suppression system where the mutant opsin gene was co-expressed with the appropriate orthogonal pair of engineered tRNA and amino-acyl tRNA synthetase. We then used hydrazone (hydrazide) or Staudinger-Bertozzi (phosphine) ligation chemistry for the keto group (in Acp) or azido group (in Azp), respectively, to link a fluorophore at various solvent accessible sites in rhodopsin. In side-by-side comparisons of the two chemical ligation chemistries, which were carried out