under physiological conditions where receptor function is maintained, we conclude that the azido group serves as a more satisfactory chemical handle than the keto moiety. The fully-functional fluorescently-labeled receptor should prove useful for kinetic studies of ligand-receptor interaction.

1524-Pos

Probing the Binding Sites and Transmembrane Prolines of GPCRs Using Unnatural Amino Acids

Ethan B. Van Arnam, Kristina N. McCleary, Michael M. Torrice, Kiowa S. Bower, Elizabeth H. Jensen, Henry A. Lester, Dennis A. Dougherty.
California Institute of Technology, Pasadena, CA, USA.

We describe a general application of the nonsense suppression methodology for unnatural amino acid incorporation in functional G protein-coupled receptors (GPCRs). We have evaluated key aromatic residues in the binding sites of the M2 muscarinic acetylcholine receptor and the D2 and D4 dopamine receptors. In addition, highly conserved transmembrane proline residues of the D2 dopamine receptor have been probed with proline analogues and α-hydroxy acids. Receptors were expressed in *Xenopus* oocytes, and activation of a G protein-coupled, inward-rectifying K^+ channel (GIRK) provided, after optimization of conditions, a quantitative readout of receptor function. Incorporation of a series of fluorinated tryptophan derivatives at W6.48 of the D2 receptor establishes a cation-π interaction between the agonist dopamine and W6.48, suggesting a reorientation of W6.48 on agonist binding, consistent with proposed ''rotamer switch'' models. Interestingly, no comparable cation-π interaction was found at the aligning residue in the M2 receptor. Incorporation of α-hydroxy acids at transmembrane proline sites 4.59, 5.50, 6.50, and 7.50 yielded D2 receptors with EC_{50} values similar to wild-type, whereas natural amino acids other than proline proved detrimental to receptor function. We propose that lack of hydrogen bond donor ability, common to both proline and α-hydroxy acids, is key to the functional role of proline within GPCR transmembrane helices.

1525-Pos

Anodic Aluminum Oxide Nanopores as Substrate for Functional and Structural Studies on G Protein-Coupled Membrane Receptors

Olivier Soubias[1], Tomohiro Kimura[1], Holly C. Gaede[2], Alexei A. Yeliseev[1], **Klaus Gawrisch[1]**.
[1]SNMR, LMBB, NIAAA, NIH, Bethesda, MD, USA, [2]Dept. Chem. Texas A&M Univ., College Station, TX, USA.

Functional and structural studies on GPCR are ideally conducted on single, protein-carrying bilayers with unrestricted access for ligands and G-proteins. It is desirable to have a large accessible surface area and protection from a solid support. Reconstitution of GPCR into bilayers supported by porous anodic aluminum oxide (AAO) nanopores meets those requirements. Extrusion of proteoliposomes through the nanopores resulted in formation of tubules of a single lipid bilayer that covers the inner surface of pores. We successfully reconstituted the GPCRs rhodopsin and recombinant peripheral cannabinoid receptor CB2 at functional conditions and high concentration into the cylindrical AAO nanopores with a diameter of 200 nm and a length of 60 μm. One square centimeter of AAO filter yielded 500 cm^2 of membrane surface. The lipid tubules are open at both ends such that buffer passes easily through the pores. Detergents used for protein reconstitution are flushed out within minutes. By ^2H NMR we demonstrated that neither lipid headgroups nor hydrocarbon chains of fluid bilayers are perturbed by the solid support. Photoactivation of rhodopsin in the pores, monitored by UV-vis spectrophotometry, was indistinguishable from rhodopsin in unsupported liposomes. Metarhodopsin-II in the tubules activated G-protein that was delivered through the pore openings. By NMR diffusion experiments we determined that tubular bilayers are assembled as short pieces with a length of a micrometer or less that adhere to the surface by their edges. The tubules possess undulation with a radius of curvature of 100-400 nm. We have evidence for a layer of water with an average thickness of 3 nm between the bilayers and the pore surface. It explains why neither protein function nor fluid bilayer properties are perturbed by the solid support.

1526-Pos

Novel Technology to Study Chemokine Receptor Signaling Complexes

Amy Grunbeck, Thomas Huber, Shixin Ye, Sourabh Banerjee, Thomas P. Sakmar.
Rockefeller University, New York, NY, USA.

C-C chemokine receptor 5 (CCR5) is a G protein-coupled receptor (GPCR) involved in immune responses and it is the primary co-receptor required for HIV-1 cellular entry. To obtain functional heterologous over-expression of engineered GPCRs is one of the major hurdles in GPCR research aimed at elucidating structure-activity relationships. It can be especially difficult to obtain structural information about GPCRs in signaling complexes with other cellular proteins that form a so-called ''signalosome.'' We have developed two new technologies for investigating the structure and function of GPCRs, which we have now applied to CCR5. First, we have established a membrane nanoparticle system called NABBs (nanoscale apolipoprotein bound bilayers), which are self-assembling discs that maintain receptors in a native-like membrane environment outside of the cell. We have incorporated functional CCR5 into NABBs and plan to use this platform to reconstitute the ternary complex with chemokine ligand and G protein from purified components. In addition, we have adapted unnatural amino acid mutagenesis for use with GPCRs. This is a method to incorporate amino acids with unique side chains at specific sites in the receptor. We introduced p-benzoyl-L-phenylalanine, into CCR5 at various positions on both the extracellular and intracellular surface of the protein using the amber suppression technology. Since the benzoyl moiety is a photo-activatable crosslinker, these mutants can now be used to map the specific sites of interaction between ligand, receptor, and G protein as predicted from molecular modeling and molecular dynamics simulations. Our methods will form the basis of a new experimental paradigm in the structural biology of signaling complexes on a mesoscopic level. Ultimately, these methods will be useful for developing a chemically-precise model for how an extracellular ligand stimulates a GPCR to activate a cytosolic G protein.

1527-Pos

Single-Cell Biochemical Assays for the Molecular Targets of Disease

Christopher Sims, Nancy Allbritton, Dechen Jiang, Shan Yang, Angie Proctor, Ryan Phillips.
University of North Carolina, Chapel Hill, NC, USA.

Molecularly targeted therapies are at the forefront of clinical science, and are expected to lead to personalization of medical treatments for each patient. Most such therapies are directed at inhibiting specific signal transduction enzymes or pathways, thus creating a critical need for assays capable of measuring the activities of these proteins in disease models and in patient samples. The ability to measure relevant enzyme activity in primary cell samples at baseline and/or after treatment would provide the ability to tailor patient therapy based on aberrant signal transduction, validate mechanisms of resistance in patients, and would offer an invaluable pharmacodynamic tool to assess whether resistance is associated with inadequate target inhibition. Here we report our current efforts to create the analytical and chemical tools needed to directly measure the enzymatic activities of therapeutic targets including protein kinases, lipid modifying enzymes and the proteasome. Fluorescent reagents are under development that report the activity of these various enzymes in model cells lines and primary cells. The basic design incorporates enzyme substrates that are modified to create compounds which can be loaded into cells where they are modified by the enzyme of interest. Work has included modification of peptides to confer membrane permeability and to achieve long intracellular lifetimes. Microelectrophoretic separations combined with low-level fluorescence detection enable the quantitative analysis of these compounds from single mammalian cells. This capability addresses three major issues currently faced in the biochemical analysis of clinical samples: the need for direct measurement of the enzymatic activity of target proteins; sample size requirements that are feasible for clinical implementation; and sample heterogeneity that can mask pertinent aspects related to therapeutic response.