(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.

Supported by grant GM078319 from the National Institutes of Health.

1519-Pos

The Human Muscarinic Receptor Couples to GαI3 Via Catalytic Collision

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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

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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

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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

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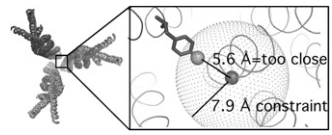
The receptor dimers that mediate bacterial chemotaxis form signaling complexes with CheW and the kinase CheA. Based on the packing arrangement observed in two different crystal structures of two different receptor cytoplasmic fragments, two different models have been proposed for receptor signaling arrays: 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.



This research was supported by U.S. Public Health Service Grant GM47601; DJF was partially supported by National Research Service Award T32 GM08515.

1523-Pos

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

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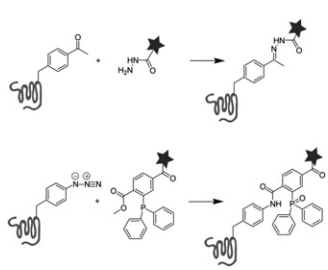
The introduction of unique chemical groups into proteins by means of site-directed mutagenesis with unnatural amino acids has numerous applications in protein engineering and functional studies. We first introduced *p*-acetyl-L-phenylalanine (Acp) or *p*-azido-L-phenylalanine (Azp) into the prototypical 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



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

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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

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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

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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

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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.