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Periole, Xavier; Knepp, Adam M.; Sakmar, Tom P.; Marrink, Siewert-Jan; Huber, Thomas

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2624-Pos Board B394

Site-Directed Fluorescence Studies of Purified, Functional Cannabinoid Receptor Cb1: Agonist-Induced Conformational Changes in TM6 are Blocked by an Allosteric Modulator and Enhanced by a Novel CB1 Specific Antibody

Jonathan F. Fay, David L. Farrens.
Oregon Health and Science University, Portland, OR, USA.

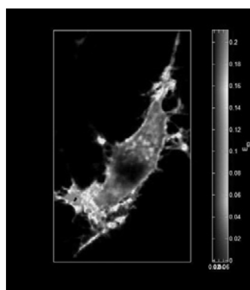
The human cannabinoid receptor CB1 is one of the most highly expressed GPCRs in the central nervous system, and a promising target for therapeutic applications. However, structural and biophysical information about this receptor have been limited due to difficulties in purifying the receptor in a functional form and working with its hydrophobic ligands. Here we report on our ability to purify and study a detergent-solubilized functional form of the CB1 receptor. Our site-directed fluorescence labeling studies show specific conformational changes in CB1 in response to different cannabinoid ligands. This work involved attaching a specific fluorescent label to transmembrane helix 6 (TM6) of CB1. We then studied this labeled sample using various fluorescence approaches. Our data shows specific spectral changes of the attached probe upon addition of various cannabinoid ligands, which we can clearly interpret to be due to drug-induced conformational changes in the receptor. We have subsequently used this labeled CB1 mutant to explore the effect of allosteric ligands and conformationally sensitive antibodies on TM6 movements in CB1.

2625-Pos Board B395

Model for the Oligomer Formation of Serotonin Receptors Based on Quantitative lux-FRET Measurements

Andre Zeug[1], Andrew Woehler[2], Erwin Neher[2], Evgeni Ponimaskin[1].
[1]Hannover Medical School, Hanover, Germany, [2]Max-Planck Institute for Biophysical Chemistry, Göttingen, Germany.

Förster Resonant Energy Transfer (FRET) is often used in experimental methods which aim at investigating the interaction of molecules at distances beyond diffraction limited resolution. Here we apply 'linear unmixing FRET' (lux-FRET) [1]. By using the lux-FRET we can obtain three important quantities, the FRET-efficiency multiplied by the fraction of donors and acceptors in FRET state as well as measures for the total donor and acceptor concentrations. In the present study we applied lux-FRET to analyse the oligomerisation behaviour of two serotonin receptors 5-HT_{1A} and 5-HT_7, which tend to form a dynamic system of homo- and hetero-dimers. From the modelling of measured lux-FRET data we conclude that the receptors have significantly different affinities to form oligomers with the dissociation constant order: $K_{5\text{-}HT1A\text{-}5\text{-}HT1A} > K_{5\text{-}HT7\text{-}5\text{-}HT1A} > K_{5\text{-}HT7\text{-}5\text{-}HT7}$. Quantitative FRET measurements on a custom tailored spinning disk system at single-cell level confirmed these results and also allowed to visualize distribution of mono- and dimers within the cell (Figure 1). Together with observation that the 5-HT_{1A} receptor-mediated signalling is significantly impaired in hetero-oligomers, our data suggest that receptor functions can be modulated by the dynamic interaction within oligomeric complexes.



REFERENCE:

[1] Wlodarczyk et al. Biophysical J, 94:986-1000. (2008)

2626-Pos Board B396

Understanding the Interaction Between Melanopsin and Arrestin using FRET

Devyani T. Ujla, Evan Cameron, Phyllis Robinson, Ph.D.
University of Maryland, Baltimore County, Baltimore, MD, USA.

A small subset of retinal ganglion cells known as the ipRGCs regulate several non-visual processes including pupillary light reflex, circadian rhythmicity, and sleep. These processes are mediated by the photopigment, melanopsin, expressed in the ipRGCs. Upon illumination, melanopsin initiates a signaling transduction cascade within the cell. This signal induces a depolarization resulting in the firing of action potentials that carry light information to higher order processing centers in the brain. Like most G-protein coupled receptors (GPCRs), melanopsin signaling is attenuated by G-protein coupled receptor kinase (GRK) phosphorylation. This phosphorylation is a cue for arrestin binding which terminates the signal. However, it is unknown if arrestin deactivates melanopsin. In mammals, three isoforms of arrestin are expressed: visual arrestin, βarrestin 1 and βarrestin 2. Studies have shown that βarrestin 1 and 2 are co-expressed with melanopsin, while visual arrestin localizes exclusively in the rods and cones. Therefore, we hypothesize that melanopsin is deactivated by either βarrestin 1 and/or 2. Using Forester Resonance Energy Transfer (FRET) we will determine the degree with which melanopsin and arrestin interact. To date, we have successfully constructed melanopsin-eGFP, βarrestin 1-eRFP, and βarrestin 2-eRFP expression vectors. We are currently attempting to express these constructs in HEK-293 cells and verify their expression and localization by Western blot assay and confocal microscopy.

2627-Pos Board B397

Mapping Interactions Between the Amino-Terminal Region of Secretin and its Receptor using Disulfide-Trapping

Maoqing Dong[1], Xiequn Xu[1], Alicja Ball[1], Joshua A. Makhoul[1], Polo P.C. Lam[2], Delia I. Pinon[1], Patrick M. Sexton[3], Ruben Abagyan[2], Laurence J. Miller[1].
[1]Mayo Clinic, Scottsdale, AZ, USA, [2]University of California, San Diego, CA, USA, [3]Monash University, Parkville, Victoria, Australia.

While NMR and crystal structures have defined the molecular basis for docking the carboxyl-terminal region of natural peptide ligands with the disulfide-bonded amino-terminal tail of class B GPCRs, the structural basis for docking the biologically critical amino-terminal regions of these ligands is much less clear. We previously utilized photoaffinity labeling to define spatial approximations between distinct positions within this region of secretin and residues within the core domain of its intact receptor. Now, we use a more powerful disulfide-trapping approach to systematically explore spatial proximities between cysteine residues incorporated into the amino terminus of secretin and into each of the extracellular loops (ECLs) of its receptor. This approach is less disruptive, due to the use of cysteines in place of the large photolabile moieties necessary for photolabeling, and the spatial information is more useful due to the shorter length of the disulfide bonds formed. Cysteines were incorporated into each of the six amino-terminal positions of secretin, with only positions 2 and 5 tolerated to yield reasonable binding affinities and biological activities. These two peptides were used to probe 61 ECL mutants in which cysteine residues were incorporated into the ECLs. The patterns of disulfide formation were quite distinct for the two probes. The position 2 probe predominantly labeled residues in the carboxyl-terminal region of ECL1 and amino-terminal regions of ECL2 and ECL3, while the position 5 probe labeled those within the carboxyl-terminal region of ECL2 and throughout ECL3. These data add substantial new insights for refining our understanding of secretin binding and activation of its receptor.

2628-Pos Board B398

Cell-Derived Vesicles as a Minimal Cell Prototype

Luigino Grasso, Romain Wyss, Joachim Piguet, Michael Werner, Pedro Pascoal, Ghérici Hassaïne, Ruud Hovius, Horst Vogel.
EPFL, Lausanne, Switzerland.

Cellular signaling reactions are classically investigated by measuring optical or electrical properties of individual living cells or suspensions of cells. Here we show how the binding of ligands to cell surface receptors and the subsequent activation of signaling reactions can be monitored in single, sub-micrometer sized native vesicles with single molecule sensitivity. The native vesicles are derived from live mammalian cells either by incubation with chemicals (e.g. cytochalasin) known to destabilize the interaction of the cytoskeleton with the plasma membrane, or by micromanipulation using optical tweezers. Such native vesicles comprise parts of the plasma membrane and the cytosol of the mother cell. They represent the smallest autonomous containers capable of performing cellular signaling reactions thus functioning like minimal cells. To demonstrate this, we measured in individual vesicles derived from individual cells with single molecule resolution the different steps of G protein-coupled receptor mediated signalling like ligand binding to receptors, subsequent G protein activation and finally receptor deactivation by interaction with arrestin. The observation of cellular signalling reactions in individual (sub)micrometer sized vesicles opens the door to downscale the analysis of cellular functions to the atto- and femtoliter range for multiplexing single cell analysis or investigating receptor mediated signalling in multiarray format.

2629-Pos Board B399

Probing the Receptor Dimer Interfaces of G-Protein Coupled Receptor in Model Membranes

Xavier Periole[1], Adam M. Knepp[2], Tom P. Sakmar[2], Siewert-Jan Marrink[1], Thomas Huber[2].
[1]University of Groningen, Groningen, Netherlands, [2]www.sakmarlab.org, Rockefeller University, New York, NY, USA.

How the components of the G protein-coupled receptor (GPCR) ''signalosome'' assemble and function in the membrane bilayer is not known. Using

a multidisciplinary approach combining computational and experiment work that leverages recent structural data, we present a model for the supramolecular structure of the rod cell disc membrane phototransduction machinery. We first show a comparison of multiple rhodopsin dimer interfaces in a model membrane using a coarse-grained molecular dynamics simulation approach accumulating more than a millisecond of simulation time. To characterize the preferred binding interface of a pair of rhodopsins, we determined the potentials of mean force as a function of the distance between two membrane-embedded receptors. The interfaces probed include helix 4 (H4), H4/H5, H5, H6 and H1/H8. The results show that the most stable rhodopsin dimer exists in a tail-to-tail conformation, with the interface comprising transmembrane H1 and H2 at the extracellular side and amphipathic H8 at the cytoplasmic one. The existence of the H1/H8 dimer was unambiguously corroborated by crosslinking experiments in which we identified CYS316 in H8 as the site of a chemical crosslink between two rhodopsins in native ROS disc membrane using proteolysis, CNBr cleavage, and high-resolution liquid-chromatography mass-spectroscopy (LC/MS). We then show how secondary interaction surfaces appear to stabilize extended ''lubricated'' rows of these dimers as seen earlier in atomic force microscopy studies. The synthesis of the new rhodopsin dimer orientation with the structures of the R*/Gt complex in two different orientations and 3-D densities of the complex obtained earlier by electron-microscopy argues for novel alternatives for the supramolecular organization of ROS membrane. The model we propose suggests the possibility for an efficient one-dimensional mechanism for Gt to search for active receptor (R*) even under low light conditions.

2630-Pos Board B400

Dynamic Assembly of the Receptor-G-Protein Signaling Complex

Thomas Huber[1], Xavier Periole[2], Adam M. Knepp[1], Siewert-Jan Marrink[2], Parag Mukhopadhyay[1], Thomas P. Sakmar[1].
[1]www.sakmarlab.org, Rockefeller University, New York, NY, USA,
[2]University of Groningen, Groningen, Netherlands.

The recent crystal structure[1] of the β_2 adrenergic receptor-Gs protein complex (β_2AR-Gs) marks an important milestone in a two decades long race to decipher one of the most important and widely utilized signal-transduction mechanisms in biology. However, it is unclear how the relatively drastic and unexpected conformational changes evolve from the initial encounter complex. The number of compositional and conformational microstates that evolve along the reaction pathway from the initial encounter of agonist-activated receptor with the GDP-bound heterotrimeric G-protein to the nucleotide-free agonist-receptor-G protein ternary complex is potentially very large.[2] Identification of these pathways is out of reach for crystallographic experiments, and we follow a complementary approach with large-scale molecular dynamics (MD) simulations of membrane-embedded, fully solvated metarhodopsin II alone and in complex with its cognate G-protein, transducin, in GDP-bound form. Here, we compare and contrast the relaxation of two different encounter complexes, one with an orientation similar to β_2AR-Gs, and the other 120° rotated. Interestingly, the two different pre-aligned encounter complexes yield induced-fit complexes that share a signature set of conformational changes involving highly conserved residues in the three allosterically coupled domains, the agonist-binding, G-protein-binding, and nucleotide-binding sites. How are these canonical and non-canonical binding modes possible for such a high fidelity signal-transduction system? Is it possible that Nature utilizes multiple, energetically similar states to relay the information encoded in the relatively small free energy of agonist binding across about ten nanometers distance to the nucleotide-binding pocket? It is tempting to speculate about the role of these energetically near-equivalent microstates when discussing the dominating entropic contribution to the binding free energy of agonists at the β adrenergic receptors.

[1]Rasmussen, et al. (2011) Nature 477, 549.
[2]Huber and Sakmar (2011) Trends. Pharmacol. Sci. 32, 410.

2631-Pos Board B401

Arrestin Allows All-Trans-Retinal to Enter the Ligand Binding Pocket of Phosphorylated Opsin

Martha E. Sommer, Klaus Peter Hofmann, Martin Heck.
Institut für Medizinische Physik und Biophysik, Berlin, Germany.

Within the rod cell of the retina, absorption of light by the G-protein coupled receptor rhodopsin leads to the formation of the agonist all-trans-retinal directly within the ligand binding pocket of opsin and the eventual formation of the active species Metarhodopsin II (Meta II). Meta II signaling is terminated by phosphorylation and binding of arrestin, and Meta II eventually decays to opsin and free retinal via the spontaneous hydrolysis of the covalent retinal Schiff-base linkage. It has been known for some time that arrestin binds photo-decayed phosphorylated rhodopsin, as well as phosphorylated opsin (OpsP) supplied with exogenous all-trans-retinal. We find that all-trans-retinal can enter OpsP and form a Schiff-base in an arrestin-dependent fashion, thus forming a Meta II-like species. The agonist, arrestin, and the receptor exist in coupled equilibria, such that arrestin stabilizes an activated form of OpsP that allows all-trans-retinal to enter the binding pocket. This is the first direct observation of the reversible formation of a Meta II-like species from opsin and all-trans-retinal in the native membrane environment. As we recently reported for arrestin binding to Meta II (Sommer ME, Hofmann KP and Heck M (2011) JBC 286: 7359-69), we now find that one arrestin binds for every two OpsP receptors in the presence of excess all-trans-retinal. Furthermore, arrestin can induce the formation of the Meta II-like species in only half of the OpsP population. Likewise, regeneration of half the arrestin-bound OpsP population with 11-cis-retinal is blocked, supposedly because the binding pocket is already occupied. Regeneration of the remaining half of OpsP requires release of arrestin via the removal of all-trans-retinal by retinal dehydrogenase. Together these results suggest that arrestin serves to both terminate Meta II signaling and to regulate retinoid flow through opsin.

2632-Pos Board B402

An Activating Helix Switch at the Rhodopsin-Transducin Interface

Peter W. Hildebrand, Alexander Rose, Klaus P. Hofmann.
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 (Gαβγ). Kinetic analysis of transducin ($G_t\alpha\beta\gamma$) activation has shown 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 and significant clockwise rotation 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 acts as a transmission rod to propagate the conformational change from the receptor-G protein interface to the nucleotide binding site. The results are discussed in light of the recent structure of the β_2 adrenergic receptor-Gs protein complex. A detailed mechanism of complex formation and GDP release is derived.

2633-Pos Board B403

Intermediates of the Channel Rhodopsin Photocycle

Eglof Ritter[1], Katja Stehfest[2], Patrick Piwowarski[1], André Berndt[2], Roman Kazmin[1], Peter Hegemann[2], Franz J. Bartl[1].
[1]Charité - Universitätsmedizin Berlin, Berlin, Germany, [2]Humboldt-Universität Berlin, Berlin, Germany.

Channelrhodopsins are sensory photoreceptors that mediate phototaxis in green algae (1, 2). Their function as ion channels renders them useful tools in the field of neuroscience. Due to the prolonged lifetime and light-sensitivity of the conducting state, mutantants of cysteine 128 are especially important. They can be switched on and off by light, and are therefore called step-function rhodopsins (3). Recent photocycle models have been proposed on the basis of visual and vibrational spectroscopy (4, 5), however, details of the photoreactions are still widely unknown. Here we present spectroscopical data on the slow cycling mutant C128T, with 200 fold extended lifetime of the conducting state. During the photocycle, a fraction of the proteins branches off into a sideway consisting of blue-shifted species which accumulate during prolonged illumination and play an important role in dark state regeneration (5). We will show that - depending on the illumination conditions - several structurally different dark states are regenerated. However, although such large structural alterations occur during the photocycle, they are not necessarily connected with on- and off-switching of the channel. A new model that connects chromophore isomerization and structural alterations of the protein will be discussed.

(1) Nagel, G. et al. (2002) Science 296, 2395-8.
(2) Nagel, G. et al. (2003) Proceedings of the National Academy of Sciences of the United States of America 100, 13940-5.