and beta2-AR: beta1-AR exhibited a transient mode of interaction whereas in contrast the vast majority of beta2-AR formed stable higher order complexes.

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The Effect of Detergent on Human Mu-opioid Receptor (hMOR) Localization as a Function of Pretreatment with Agonist and Antagonist

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Like many other seven transmembrane G-protein coupled receptors (GPCRs), the human mu-opioid receptor (hMOR) interacts with multiple members of the pertussis toxin-sensitive Gi and Go protein families, to regulate adenylyl cyclase, Ca2+ and K+ channels. Notably, opioid agonists represent the most powerful analgesic drugs for the clinical management of pain, through binding opioid receptors. However, not all agonists exert the same level of effect.

Plasma membranes are organized into specialized micro-domains differing in composition, biological function and physical properties. In recent years, detergent resistant membranes (DRM) are thought to serve as molecular sorting platforms to concentrate signalling molecules (e.g. opioid receptors) based on membrane fractionation and cholesterol depletion experiments. It remains unclear whether membrane organization with detergent has an effect on hMOR localization.

Here we track active hMOR and lipid composition in isopycnic membrane fractions in the presence and absence of CHAPS detergent. hMOR activity was assessed using a modified binding assay. The relative amount of lipid raft marker (flotillin-1), actin and G-proteins were assessed by Western blot analysis. The data show the effects of detergent on receptor distribution. Relocation of the hMOR receptor in the membrane indicates an additional level regulation at the cell membrane.

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The Effect of Agonist Activation and Homodimerisation on the Membrane Diffusion of the Human Histamine H_1 Receptor

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There is considerable evidence to support the existence of dimers of G protein-coupled receptors, including the histamine H_1 receptor (H_1R), although their functional significance remains unclear. We have used bimolecular fluorescence complementation (BiFC) in combination with fluorescence correlation spectroscopy (FCS) to selectively monitor histamine-mediated changes in the diffusion characteristics of the dimeric H_1R (using BiFC), as well as those of the total H_1R population (using labelling with YFP).

cDNAs encoding YFP or the C-terminal and N-terminal YFP fragments were cloned into pcDNA3.1 to produce fusions to the C-terminus of the H_1R. CHO-K1 cells were transiently transfected with the relevant cDNAs and FCS measurements were performed on the upper cell membrane and analysed as previously described (Briddon, *et al.* (2004) PNAS, 101, 4673-4678).

Translational diffusion of the H_1R in the cell membrane, measured as the average diffusion time through the FCS detection volume, was significantly faster for oligomeric H_1R (14.1 ± 1.1ms) than the total receptor population (17.3 ± 1.1ms). Following stimulation with 0.1mM histamine, there was a significant increase in both the diffusion time (17.3 ± 1.1ms vs. 21.6 ± 1.0ms) and particle number (1.02 ± 0.08 vs. 1.28 ± 0.07) of the total receptor population after 10 minutes. This returned to control values after 20 and 40 minutes. For the dimeric receptor population, however, there was no significant change in either translational receptor diffusion or particle number following histamine exposure.

Since FCS only detects the diffusion of mobile particles, the increase in particle number for H_1YFP following 10 minutes agonist stimulation may reflect mobilisation of previously immobile receptors. The increased diffusion time could indicate association with larger protein complexes involved in receptor signalling, desensitisation or internalisation. The absence of such changes for dimeric receptors suggests fundamental functional differences between monomeric and oligomeric receptor populations.

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Monitoring The Activation Of Rhodopsin By The Transient Fluorescence Of Fluorescently Labeled Helix 8

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The transient changes of the fluorescence of bovine rhodopsin in ROS membranes selectively labeled with Alexa594 at cysteine 316 in helix 8 were followed in time from 1 μs to 10 s after flash excitation of the photoreceptor. A large light-induced transient fluorescence increase was observed with time constants in the ms- range at pH6. Using transient absorption spectroscopy the kinetics of this structural change at the cytoplasmic surface was compared to the formation of the signaling state MII (360 nm) and to the kinetics of proton uptake as measured with the pH indicator dye bromocresol purple (605 nm). The fluorescence kinetics lags behind the deprotonation of the Schiff base. The proton uptake is even further delayed. These observations show that in ROS membranes (at pH 6), the sequence of events is: Schiff base deprotonation, structural change, proton uptake. From the temperature dependence of the kinetics we conclude that the Schiff base deprotonation and the transient fluorescence have comparable activation energies, whereas that of proton uptake is much smaller.

3500-Pos Board B547

Structural And Dynamic Effects Of Cholesterol At Preferred Sites Of Interaction With Rhodopsin Identified From Microsecond Length Molecular Dynamics Simulations

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A key unresolved question about GPCR function is the role of membrane components in receptor stability and activation. In particular, cholesterol is known to affect the function of membrane proteins, but the details of its effect on GPCRs are still elusive. Here, we describe how cholesterol modulates the behavior of TM1-TM2-TM7-helix 8(H8) functional network, that comprises the highly conserved NPxxY(x)5,6F motif, through specific interactions with the receptor. The inferences are based on the analysis of microsecond molecular dynamics (MD) simulations of Rhodopsin in an explicit membrane environment. We found that cholesterol primarily affects specific local perturbations of the TM domains such as the helical kink parameters in TM1, TM2 and TM7, and that these local distortions, in turn, relate to rigid-body motions of the TMs in the TM1-TM2-TM7-H8 bundle. The specificity of the effects stems from the non-uniform distribution of cholesterol around the protein. We find three regions that exhibit the highest cholesterol density throughout the trajectory. In one of these regions, cholesterol interacts with Pro7.38 in TM7 and with nearby residues in the extracellular (EC) loop 3, a location that resembles the high-density sterol area from the electron microscopy data (Ruprecht et al., 2004, EMBO J;23:3609-3620). A second cholesterol concentration region is in agreement with the recent X-ray crystallography data on beta2-adrenergic GPCR (Cherezov et al.,2007,Science,318:1258-1265), near residues Val1.58, Tyr2.41 and Ile4.43. In the third region, we find cholesterol interacting strongly with Tyr2.63 in TM2 and proximal residues Phe3.30, Leu3.27, Thr3.23 and Phe3.20 on the EC side of TM3. Through correlation analysis we connect local effects of cholesterol on structural perturbations with a regulatory role of cholesterol in signaling.

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NMR Structure of the "Finger" Loop of Rod Arrestin Induced by Meta-II Rhodopsin Binding

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An unstructured ''finger'' loop connects β-strands V and VI in x-ray crystal structures of arrestin. Several recent studies identified this region as a possible major interaction site for specific binding of arrestin to photo-activated and/or phosphorylated meta-II rhodopsin. We studied binding of a peptide representing the amino acid sequence of the loop around residue 75 of visual arrestin to light-activated bovine rhodopsin. At low millimolar concentrations the peptide enhances formation of the photointermediate meta-II rhodopsin after receptor activation. The extra meta-II assay reveals competition of the peptide with transducin and arrestin for binding to activated (Rh*) and activated phosphorylated rhodopsin (P-Rh*), respectively. The high resolution structure of the meta-II rhodopsin-bound arrestin peptide was derived from the difference of two-dimensional liquid state NMR spectra recorded in the presence of either dark-adapted or light-activated rhodopsin-rich disc membranes. The peptide remains unstructured upon addition of dark-adapted rhodopsin. However, receptor activation causes dramatic changes in 2D NOESY spectra of the peptide. Specifically, the ligand is in rapid exchange between a free unstructured and a conformationally well defined meta-II-bound form. Efficient cross-relaxation of proton spins in the bound state causes strong NOESY cross-peaks reflecting the bound peptide structure. The specific pattern of binding-induced NOESY peaks indicates a helical conformation of the bound peptide. Restrained molecular dynamics calculations confirmed an α-helical peptide structure. A

structural model of the interface between meta-II rhodopsin and the arrestin loop region is proposed based on molecular docking.

3502-Pos Board B549

Exploring the Thermodynamics of Activation Pathways of Bovine Rhodopsin with Fast Molecular Dynamics Simulations

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Recent progress made in the understanding of the structure and function of G-protein coupled receptors (GPCRs) points to compelling biophysical and crystallographic evidence for distinct conformational changes of the prototypical family member bovine rhodopsin/opsin upon illumination. To investigate possible activation pathways of bovine rhodopsin from an early inactive intermediate (lumirhodopsin) produced by the cis-trans photoisomerization of its retinal chromophore to an activated ligand-bound opsin-like conformation of the protein, we carried out several independent biased molecular dynamics simulations of the receptor in an explicit dipalmitoylphosphatidylcholine (DPPC) membrane bilayer. The simulations revealed significantly different activation pathways between inactive and opsin-like activated forms of bovine rhodopsin, whose thermodynamic behavior was evaluated by metadynamics using as reaction coordinates both the position along the identified representative pathways, and the distance from them. The results point to three common metastable states that are likely to correspond to a photoactivated deprotonated Meta I-380/Meta IIa, and two different Meta IIb-like intermediates of bovine rhodopsin. Inferences from these molecular models are expected to shed new light into mechanisms of allosteric modulation of rhodopsin function.

3503-Pos Board B550

Molecular Mechanism of Rhodopsin Photoactivation

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Rhodopsin is a highly specialized GPCR that is activated by the rapid photochemical isomerization of its covalently bound 11-cis retinal chromophore. Using two-dimensional solid-state NMR spectroscopy, we define the position of the retinal in the active metarhodopsin II intermediate and the protein conformational changes that couple retinal isomerization to breaking of the ''ionic lock'' between transmembrane (TM) helices H3 and H6. Retinal isomerization leads to steric strain within the retinal binding site between the β-ionone ring and helix H5, and between the C19/C20 methyl groups and EL2. These interactions trigger the displacement of EL2, deprotonation of the Schiff base nitrogen and protonation of Glu113. Motion of the β-ionone ring leads to rearrangement of the hydrogen bonding network centered on H5, while interactions of the C19 and C20 methyl groups are involved in rearrangement of the EL2. Motion of the β-ionone ring is also coupled to the motion of Trp265, which triggers the shift of helices H6 and H7 into active conformations and the rearrangement of the hydrogen bonding network centered on the conserved NPxxY sequence. Motion of helices H5, H6 and H7, in turn, is coupled to the rearrangement of electrostatic interactions involving the conserved ERY sequence at the cytoplasmic end of H3, exposing the G protein binding site on the cytoplasmic surface of the protein. The location of the retinal and reorganization of the protein upon receptor activation provides a structural basis for understanding the action of agonists and antagonists in the large family of class A GPCRs.

3504-Pos Board B551

Rhodopsin Activation in Membranes: Thermodynamic Model of the Two Protonation Switches

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Activation of the G protein-coupled receptor (GPCR) rhodopsin is initiated by light-induced isomerization of the retinal ligand, which triggers two protonation switches in the conformational transition to the active receptor state. Disruption of an interhelical salt bridge occurs by deprotonation of the retinal protonated Schiff base (PSB) together with uptake of a proton from the solvent by Glu134 of the conserved cytoplasmic E(D)RY motif. Using a combination of UV-visible and Fourier-transform infrared (FTIR) spectroscopy of rhodopsin, we access deprotonation of the PSB and activating conformational changes of the helix bundle separately, and study the pH-dependent activation mechanism of rhodopsin in different membrane environments. The data are analyzed using a thermodynamic framework based on the Hofmann-Hubbell scheme derived for rhodopsin in detergent, distinguishing between PSB deprotonation, activating conformational changes, and cytoplasmic proton uptake in the transitions from Meta I to Meta II_a, Meta II_b, and Meta II_bH^+, respectively. While a classical Henderson-Hasselbalch-like equilibrium between Meta I and Meta II_bH^+ is observed at 10 °C, more complex titration curves with non-zero alkaline endpoints are found above 20 °C. These reflect partial population of an entropy-stabilized Meta II_b state, in which the PSB salt bridge is broken and activating helix movements have taken place, but where Glu134 remains unprotonated. This partial activation is converted to full activation only by coupling to the pH-dependent protonation of Glu134 from the solvent, which enthalpically stabilizes the active receptor conformation. In a membrane environment, protonation of Glu134 is therefore a thermodynamic rather than a structural requirement for activating helix movements. In light of conservation of the E(D)RY motif in rhodopsin-like GPCRs, protonation of this carboxylate may serve a similar function in signal transduction of other members of this important receptor family.

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Real-Time Dynamics Changes of Helix 8 upon Phosphorylation of Rhodopsin and Arrestin Binding

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The G-protein coupled receptor (GPCR) rhodopsin transduces light signals in photoreceptor cells. For a rapid recovery of the receptor, efficient signal termination is mandatory. On the molecular level, this termination process starts with the phosphorylation of serines and/or threonines in the C-terminus of rhodopsin by rhodopsin kinase. Subsequently, the phosphorylated photoexcited rhodopsin is recognized by visual arrestin, which binds to the cytoplasmic loops of the receptor.

In this work we focus on the real-time dynamics of helix 8, a putative cytoplasmic surface switch region in class-I GPCRs. Using multidimensional time-correlated single photon counting [1] and site-specific fluorescence labelling [2], we measured the time-resolved anisotropy changes of phosphorylated and photoactivated phosphorylated rhodopsin upon interaction with arrestin. Distinct real-time dynamics changes of helix 8 were observed after phosphosphorylation and interaction with arrestin. From real-time dynamics studies of helix 8 in rod outer segment membranes we concluded that a reduced mobility of helix 8 is a prerequisite for rhodopsin activation [2]. FTIR-Fluorescence cross-correlation measurements indicate further that changes in the environment of helix 8, as measured with a fluorescent reporter group, may correlate with a partial loss of secondary structure upon Metarhodopsin II formation [3]. The observed mobility changes after phosphorylation highlight the importance of helix 8 as a surface element, which is involved in activation, molecular recognition, and deactivation of the receptor.

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pH Dependent Interaction of Rhodopsin with Anthocyanin Plant Pigments

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Anthocyanins are a class of natural compounds common in flowers and vegetables. Because of the increasing preference of consumers for food containing natural colorants and the demonstrated beneficial effects of anthocyanins on human health, it is important to decipher the molecular mechanisms of their action. Previous studies indicated that the anthocyanin cyanidin-3-glucoside (C3G) modulates the function of the photoreceptor rhodopsin. Here, we show using selective excitation ^{1}H NMR spectroscopy that C3G binds to rhodopsin. Ligand resonances broaden upon rhodopsin addition and rhodopsin resonances exhibit chemical shift changes as well as broadening effects in specific resonances, in an activation-state dependent manner. Molecular docking studies also support this conclusion. Thermal denaturation and stability studies using circular dichroism (CD), fluorescence and UV/Visible absorbance spectroscopy show that C3G exerts a destabilizing effect on rhodopsin structure while it did not significantly alter G-protein activation and the rates at which the light-activated Metarhodopsin II state decays. These results indicate that the mechanism of C3G enhanced regeneration may be based on changes in rhodopsin structure promoting access to the retinal binding pocket. The results provide new insights into anthocyanin-protein interactions and may have relevance for the enhancement of night vision by this class of compounds. This work is also the first report of the study of ligand binding to a full-length membrane receptor in detergent-micelles by ^{1}H NMR spectroscopy. Such studies were previously hampered by the presence