fluorescence complementation (BiFC, Hu, Mol. Cell, 9, 789, 2002) and fluorescence correlation spectroscopy (FCS), we have specifically monitored the diffusion of homo-oligomers of three members of the adenosine receptor family of GPCRs (the A_1-, A_{2A}- and A_3-adenosine receptors (A_x-AR)) in microdomains of living cells. This approach has allowed us to directly investigate the membrane organisation of homo-oligomeric forms of these receptors.

FCS measurements were carried out as previously described (Briddon, PNAS, 101, 4673, 2004).on the upper cell membrane of CHO-K1 cells transiently expressing C-terminal fusions of each AR subtype with either wtYFP (representing total receptor population) or C-YFP and N-YFP (representing oligomeric receptors).

Homo-oligomers of all three subtypes were detected and showed a high degree of membrane localisation. For all three subtypes, receptors labelled with wtYFP (total receptor population) showed similar diffusion co-efficients (D=0.40, 0.51 and 0.43 $\mu m^2/s$ for A_1-, A_{2A}- and A_3-AR, respectively). The oligomeric A_3-AR (measured using BiFC) had a significantly faster diffusion co-efficient when compared to the A_3-AR total population (D=0.60 vs. 0.43 $\mu m^2/s$, $P<0.05$) suggesting that the homo-oligomeric A_3-AR represents a faster diffusing fraction of the total receptor population. This was not the case for the A_1- and A_{2A}-ARs. Further investigation into the extent of receptor dimerisation for each ARs subtype among the total population and their membrane mobilities was carried out using photon counting histogram (PCH) analysis and fluorescence recovery after photobleaching (FRAP). These data indicate important differences in the molecular organisation of the monomeric vs oligomeric forms of the A_3-AR, and also differences among receptor subtypes in their propensity for dimer formation.

3493-Pos Board B540

Live Cell Imaging Of The Kinetics Of Ligand Binding At The Human Adenosine A_3 Receptor

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The aim of this study was to investigate the association and dissociation kinetics of fluorescently labelled adenosine ligands at the human adenosine A_3 receptor at a single cell level. CHO cells stably expressing the human adenosine A_3 receptor were exposed to 100 nM of fluorescent ligand after which cells were washed with buffer alone or in the presence of an unlabelled adenosine ligand. Confocal fluorescence and phase images were obtained using a Zeiss 510 confocal microscope.

The association of ABA-X-BY630, a novel N^6-aminoalkyl derivative of adenosine which incorporates the BODIPY [630/650] fluorophore, was monophasic with an association rate constant, k_{on}, of 574700 $\pm$ 19000 $M^{-1}sec^{-1}$, n=7. ABA-X-BY630 dissociation was determined under conditions reflecting that of infinite dilution in the absence and presence of the selective adenosine A_3 antagonist, MRS 1220. Under both conditions, ABA-X-BY630 dissociation was monophasic, however the dissociation rate in the absence of antagonist (k_{off} = 0.019 $\pm$ 0.001 sec^{-1}, n=4) was significantly slower than that in the presence of 1 μM MRS 1220 (k_{off} = 0.080 $\pm$ 0.007 sec^{-1}, n=4).

In summary, confocal imaging has been used to directly measure, at single cell level, the binding kinetics of the fluorescent adenosine agonist, ABA-X-BY630. In addition, the perfusion system allows for the rapid removal of ligand and as such the comparison of ABA-X-BY630 dissociation in the absence and presence of antagonist. Under infinite dilution conditions, the dissociate rate of ABA-X-BY630 should be unaffected by the presence of a simple competitive antagonist. Therefore the ability of MRS 1220 to enhance the dissociation rate of ABA-X-BY630 suggests that there may be a negatively cooperative interaction occurring between the two ligands. Similar experiments have also been performed using additional fluorescently labelled adenosine ligands.

3494-Pos Board B541

Solid-State NMR Study of the Human Peripheral Cannabinoid Receptor CB2 in Lipid Bilayers

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Human peripheral cannabinoid receptor CB2 expressed in *E. coli.* has been purified, and successfully reconstituted in functional form into lipid bilayers composed of phosphatidylcholine, phosphatidylserine, and cholesteryl hemisuccinate (CHS). The reconstitution was carried out by detergent removal from the CB2-lipid-detergent mixed micelles on an adsorbent column, or by rapid dilution below the critical micelle concentration followed by washing on a concentrator. Proteoliposomes prepared at the CB2:phospholipid molar ratio of 1:600 showed the following basic physical properties: Free of detergents (as analyzed by high-resolution ^1H NMR), homogeneity of the CB2-to-lipid ratio over the proteoliposome particles (by sucrose gradient centrifugation), unilamellarity with a mean diameter of ~150-200 nm (by dynamic light scattering), and functional integrity (by G-protein activation assay). Using the proteoliposomes, CB2-lipid interactions were investigated by solid-state NMR. Order parameters of the phospholipid acyl chains measured by ^2H quadrupolar splittings indicated that CHS, a detergent-soluble analog of cholesterol, preferentially interacts with the 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phospholipids over CB2. By probing ^1H NMR saturation transfer, evidence for CB2-lipid interactions at the lipid acyl chains and less significant interactions at the glycerol backbone and the headgroups were observed. ^1H spin-lattice relaxation rates decreased notably at the acyl-chains upon CB2 incorporation, indicating reduced motion on the nanosecond timescale corresponding to the restriction of phospholipid wobbling about the bilayer normal. Structure-function relationships in view of the role of interactions between CB2 and anionic phosphatidylserine in activation of G-protein will also be discussed.

3495-Pos Board B542

Modern Molecular Models and Simulations of Opioid Receptor Dimers

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Like several other members of the G-protein coupled receptor (GPCR) family, opioid receptors interact among themselves at the plasma membrane to form dimers/oligomers. Despite recent compelling evidence for the involvement of transmembrane (TM) regions at the dimerization/oligomerization interface of GPCRs, the specific residues in contact are unknown for most receptors, including the opioid receptor subtypes. Based on prior inferences from correlated mutation analysis, we performed experimental testing of the interfaces of delta- (DOR) and mu- (MOR) opioid receptor oligomers by carrying out cross-linking studies on a series of substituted cysteines in TM1, 4 and 5. Strong cross-linking was observed by copper phenanthroline (CuP) (1:3mM) at position 4.58 in both DOR and MOR, and cross-linking, albeit less extensive, was also observed at 1.36 and 5.38, consistent with the involvement of these helices at inter-protomer interfaces in the dopamine D2 receptor. We used these experimental data to guide the construction of initial configurations of DOR and MOR homodimers in an explicit dipalmitoyl phosphatidyl choline (DPPC)-cholesterol-water environment. The TM regions of the individual protomers were built by homology modeling using the recent beta2 adrenergic receptor crystal structure as a template, while the loop regions were built using the fragment-based loop-modeling protocol of Rosetta. To explore the energetics and dynamics of the proposed homodimerization interfaces, we carried out metadynamics analyses of the DOR and MOR homodimers using collective variables that describe the relative position of the interacting protomers. The results provide new insights into the relative stability of opioid receptor dimers, and suggest specific residues and interactions that are responsible for the gain and/or loss in binding affinity. Given the robust bioluminescence resonance energy transfer (BRET) we observe in experiments with DOR and MOR homo- and heteromers, these predictions can be readily tested.

3496-Pos Board B543

FRAP Microscopy As A Tool To Analyze Beta-Adrenergic Receptor Di-/Oligomerization

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Many G protein coupled receptors have been described to assemble as dimers or higher order oligomers but their existence and functional relevance is still a matter of controversy. Based on current techniques stability, extent and size of complexes of these receptors is difficult to determine. Therefore, we took advantage of a novel generally applicable approach based on dual-color fluorescence recovery after photobleaching (FRAP) microscopy to analyze stability and extent of di- and oligomerization of membrane proteins. Extracellularly YFP-labeled receptors were immobilized with a polyclonal antibody directed against YFP. Then, changes in the lateral mobility of coexpressed intracellularly CFP-tagged receptors were studied and served as readout for receptor interactions. In order to allow for comparison with theoretically calculated effects relative expression ratios of intracellularly and extracellularly tagged receptors were carefully determined using a reference construct.

We established this approach with monomeric (CD86) and covalent dimeric (CD28) proteins, which have been previously characterized. CD86 was fully mobile indicating to exist as a monomeric entity. For CD28 we detected a restriction which was dependent on the relative expression ratio of receptors with an intracellular and an extracellular label. This restriction was in good agreement with theoretically calculated recoveries for dimers. Using this novel approach to investigate homo-interactions between beta-adrenergic receptors (beta-AR) we discovered previously unknown differences between beta1-

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.

3497-Pos Board B544

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.

3498-Pos Board B545

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.

3499-Pos Board B546

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.

Membrane Receptors & Signal Transduction II

3501-Pos Board B548

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