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.

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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.

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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.

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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.

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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-