correlation spectroscopy (Petersen et al., BJ 65:1135. 1993), with the improvement that the autocorrelation value g(0,0) was explicitly corrected for shot noise and CCD camera read noise.
Monovalent ligands in fluid membranes cause receptor clustering in T-cells and B-cells, in cytoskeleton-dependent processes. The high DNP concentration required for large-scale aggregation in RBLs suggests that degranulation signaling and large-scale aggregation may be two distinct cellular responses that depend differently on hapten concentration and presentation.
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3488-Pos Board B535
Single Molecule Studies of FcεRI Dynamics Link Mobile Receptors with Signaling
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The high affinity IgE receptor, FcεRI, is the principal multi-subunit immunoreceptor on the surface of mast cells and basophils. These receptors bind circulating IgE with high affinity and are activated when multivalent allergen crosslinks IgE-bound receptors. Crosslinking initiates a complex signaling pathway that ultimately leads to degranulation and release of key mediators of allergic inflammation. To study the dynamic events that induce FcεRI signaling, we generated two novel quantum dot (QD) probes for single particle tracking: monovalent QD-IgE that binds FcεRI without crosslinking and multivalent DNP-QD that mimics allergen by crosslinking DNP-specific IgE. Previously, we provided direct evidence that actin filaments ''corral'' resting receptor motion and are involved in receptor immobilization (Andrews et al, *Nature Cell Biology* 10:955). Simultaneous imaging of single QD-IgE-FcεRI complexes and GFP-tagged actin revealed that membrane-proximal actin bundles form a dynamic labyrinth that restricts receptor diffusion. Real-time imaging revealed that receptors become immobilized within seconds of crosslinking by high doses (1 μg/ml) of multivalent antigen and that immobilization is delayed when actin is disrupted. We have now investigated further the relationship between receptor immobilization and activation. We find that the kinetics of immobilization are dose dependent and receptors remain mobile at antigen doses corresponding to maximal degranulation (0.001-0.01 μg/ml). Using a novel hyperspectral microscope with ~1 nm spectral resolution, we tracked up to five spectrally distinct QDs simultaneously. Multi-color tracking of QD-IgE-FcεRI complexes revealed that small crosslinked clusters (2-4 receptors) remain mobile under activation conditions. In addition, DNP-QDs were shown to induce degranulation, yet DNP-QD-crosslinked receptors continue to diffuse. These results indicate that receptor immobilization is a feature of highly aggregated receptors, depends upon an intact actin cytoskeleton, and is more likely involved in signal termination than activation.

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Protein movement between membrane domains: the Epidermal Growth Factor Receptor (EGFR) signaling cascade
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We have developed a novel procedure to isolate membrane domains as they exist at 37°C and are using it to follow the redistribution of membrane proteins upon stimulation of EGFR. TACE (Tumor necrosis factor-α Converting Enzyme, also known as ADAM17) is responsible in ~80% of the physiological cases for initiating the signaling cascade, via trans-activation, by phosphorylating EGFR (to p-EGFR). Trans-activation did not affect the location of TACE. Instead, a significant amount of p-EGFR moved from a fraction rich in caveolin-1 (Cav-1) to TACE-containing fractions. PKC which participates in TACE signaling also became more concentrated in the TACE fractions. The ectodomain of TGF-α, cleaved by TACE, is a physiologically important agonist for EGFR. Upon stimulation, TGF-α moved to the TACE fraction. We conclude that stimulation of the EGFR signaling cascade leads to colocalization of EGFR, TACE, and TGF-α along with PKC. By also measuring lipid compositions, we have arrived at our current mechanistic picture of protein redistribution upon EGFR stimulation: At rest, most of the EGFR is in caveolae. But 5-20% of the non-phosphorylated EGFR is in domains rich in sphingomyelin-rich domains concentrated in TACE and flottilin-2. EGFR continuously cycles between caveolae and the TACE domains. Upon stimulation, TGF-α moves into TACE domains and is thereby cleaved. The released ectodomain activates the EGFR that is in these domains. Independent of whether this initial hypothesis proves correct, our data shows that multiple signaling proteins are recruited to the same domains. These domains, containing proteins shared by separate cascades, may provide a means to functionally connect cascades to create integrated cellular processing.
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Investigating the Role of Receptor Clustering and Dynamics in Transmembrane Signaling by Functional Arrays of Bacterial Chemoreceptors
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Both clusters and conformational changes are thought to be important in the transmembrane signaling mechanism of bacterial chemotaxis receptors. Full signaling activity of these receptors requires assembly of a ternary complex with two other proteins, CheA and CheW. Weis & coworkers have recently developed an approach for assembling active arrays of the receptor cytoplasmic domain complexes at vesicle surfaces, and have demonstrated that the surface density modulates the activity of the receptor: high density increases the kinase activity and low density increases the methylation activity (Besschetnova et al, Proc. Natl. Acad. Sci. USA 105, 12289). In order to determine how ligand binding modulates this density-dependent equilibrium, we have reconstituted the intact receptor into membrane vesicles at a range of lipid:protein ratios. Preliminary results indicate that the kinase activity of the intact receptor exhibits a density dependence similar to that of the cytoplasmic fragment arrays: the fully methylated receptor shows little activity change with density but the demethylated receptor requires high density for full activity. Thus methylation state modulates the density-dependent equilibrium of the intact receptor and experiments are in progress to determine whether attractant ligand modulates it in an opposing manner. We are also assembling vesicle surface-associated functional arrays of the receptor cytoplasmic fragment for further biophysical studies: (1) solid-state NMR distance measurements to determine the array geometry, and (2) mass spectrometry measurements to determine whether receptor dynamics change with signaling state.
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3491-Pos Board B538
Multi-color Single Quantum Dot Tracking To Characterize Membrane Receptor Interactions On Living Cells
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Single particle tracking (SPT) provides the necessary spatial and temporal resolution to acquire information on the diffusional and interaction dynamics of individual proteins. Quantum Dots (QDs) are bright and photostable probes that make long-term SPT possible and their large Stokes shifts facilitate multi-color SPT through single wavelength excitation. We developed two-color single QD tracking methods and analyses to characterize receptor dynamics as well as transient protein-protein interactions down to the ~10 nm scale. The analyses of receptor trajectories provide quantification of dynamic parameters that cannot be obtain from traditional biochemical techniques. Specifically, we developed a Hidden Markov Model (HMM) approach to identify and extract the lifetime and kinetic rates for dimerization and map regions of the plasma membrane explored by monomeric and dimeric receptors. We apply this technique to study the interplay between transmembrane receptor tyrosine kinases, erbB1 and erbB2, using QD-conjugated ligands, variable fragments of heavy-chain only antibodies (Nanobodies), and reduced monoclonal antibody fragments. Single-color QD-SPT is used to characterize the receptor diffusional dynamics in the presence or absence of ligands or inhibitors. The diffusion coefficients we measured by single QD tracking are consistent with published values obtained from tracking probes labeled with organic fluorophores. Using spectrally distinct QDs and simultaneous two-color imaging, we directly observe transient homo- and hetero-dimers even in the absence of ligand. Addition of EGF, the ligand for erbB1, prolonged dimer interactions. Current efforts seek to extract dimerization parameters from these two-color data sets using the HMM analysis.

3492-Pos Board B539
An Investigation Into the Membrane Diffusion and Organisation of Adenosine Receptor Homo-oligomers
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Oligomerisation of G-protein coupled receptors (GPCRs) is now a widely accepted phenomenon, although its effects on receptor signalling, pharmacology and organisation are still unclear. Using a combination of bimolecular

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.

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.

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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-