Our results indicate that along with complexes between GPCRs that are coupled to the same class of G-protein, heterocomplexes are also possible, enabling simultaneous signaling of distinct signaling pathways in response to a single agonist.

3483-Pos Board B530

Cellular Association and N-glycosylation of Glucagon and Related Receptors

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Increasing evidence suggests that oligomerization and N- glycosylation influence cell surface expression of G-Protein Coupled Receptors (GPCRs). However, the importance or occurrence of these post-translational events remain controversial or unknown for many GPCRs. For the GPCRs in the glucagon family, which play important roles in regulating cardiac activity, insulin secretion and glucose metabolism, an understanding of the post-translational mechanisms that regulate their association and cell surface expression is particularly limited. Therefore, we initiated studies on glucagon (Gluc), glucagon-like peptide 1 (GLP-1), and glucose-dependent insulinotropic polypeptide (GIP) receptors to determine if they self-associate preferentially and if N-glycosylation plays a role in their function or cell surface expression. These three families of GPCRs are similar in primary sequence, suggesting they may both homo- and heterodimerize, and contain between 2 and 4 N-glycosylation sequons in predicted extracellular regions. Using transient expression in Chinese Hamster Ovary cells and Bioluminescence Resonance Energy Transfer (BRET), we show high levels of GFP emissions of GIP, GLP-1 and Gluc receptors with themselves, but lower levels when they are co-expressed. Mutation of putative N-glycosylation Asn residues to Gln produces shifts in mobility consistent with the removal of N-glycans, and raises perinuclear levels of intracellular receptors as determined by immunocytochemistry and structured illumination microscopy. Furthermore, preliminary data from cAMP assays suggest that the Gluc receptor is not functional after glucagon stimulation when all putative N- glycosylation sites are mutated. These data suggest that each of these three related families of GPCRs associate preferentially with themselves, and that they are N-glycosylated in CHO cells, which augments their cell surface targeting.

3484-Pos Board B531

Strong cAMP Response to a GPCR Agonist Challenge Despite Apparent Inactivation

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A large variety of neurotransmitters, hormones, paracrine agents, and odorants exert their effects through G protein coupled receptors (GPCRs). These receptors activate intracellular G proteins that in turn modulate the activity of different effector proteins. Based largely on the study of the light-activated receptor rhodopsin and the beta-adrenergic receptor, the vast majority of GPCRs are assumed to undergo a process of inactivation or silencing involving receptor phosphorylation and capping by one of the arrestins. The advent of improved cAMP sensors in living cells and enhanced molecular information about phosphodiesterases has led to an increased appreciation of the role that these enzymes play in the shaping and termination of cAMP signals. We report here a high-resolution measurement of vasoactive intestinal peptide (VIP)-triggered cAMP signals near the surface membrane in pituitary GH4C1 cells, using modified cyclic nucleotide-gated ion channels. The signals are transient and the falling phase is due solely to an increase in phosphodiesterase activity. There is no detectable receptor inactivation on this timescale. Surprisingly, this system is highly responsive to subsequent increases in VIP levels. We show that such responsiveness can arise in a system in which receptors remain active and phosphodiesterase activity is just slightly higher than adenylyl cyclase activity, but not in a system that exhibits classical receptor inactivation. The upregulation of phosphodiesterase activity represents a type of inactivation or desensitization that causes a decline in the response to the initial agonist dose and limits the intracellular spread of cAMP. However, this mechanism has the unique feature of allowing the cell to respond to subsequent agonist challenges.

3485-Pos Board B532

Interaction Between Cationic Lipids and Endotoxin Receptors

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It has been recently demonstrated that diC14-amidine, a cationic lipid, activate cytokine secretion (TNF-alpha, IL-12, IFN-gamma, IP-10) in dendritic cells through a Toll-like Receptor-4-dependent mechanism [Tanaka et al., 2008]. This receptor is involved in the recognition of the bacterial endotoxins (lipopolysaccharides, LPS) and activation of innate immune system. DiC14-amidine could activate this cascade by different mechanism [Lonez et al., 2008].Our first hypothesis is that diC14-amidine interacts with the co-receptor of TLR4, MD-2. DiC14-amidine has 2 acyl chains that mimic LPS acyl chains. Molecular dynamics simulations of the insertion of diC14-amidine in the MD-2 cavity revealed that two amidine molecules do occupy a volume identical to that of one tetra-acylated lipid A molecule, an antagonist of TLR4, in the cavity. A non-exclusive alternative could consist in a modification of the membrane environment of TLR4, upon insertion of cationic lipids in the membrane bilayer. The ability of diC14-amidine liposomes to fuse with cell membranes was demonstrated by confocal microscopy or (FRET) Fluorescence Resonance Energy Transfer measurements. Fusion (lipid mixing) with the cell membrane would be a way to insert cationic lipid in the lipid bilayer of the plasma membrane and to modify the lipid-protein interactions involved in the function of membrane proteins. Biophysical studies (AFM, X-ray scattering) revealed that amidine molecules adopts an interdigitated structure and does not require additional lipids (PE) to be fusogenic. This raises also the question of the role of interdigitated structures in the fusion (lipid mixing) mechanism.

References
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3486-Pos Board B533

Dynamic Changes In The Monomer-Dimer Equilibrium Of The Intercellular Adhesion Molecule CEACAM1 Revealed By Live Cell TIRF-Based FRET Microscopy

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The carcinoembryonic-antigen-related cell-adhesion molecule (CEACAM) family of proteins has been implicated in various intercellular-adhesion and intracellular signaling-mediated effects that govern the growth and differentiation of normal and cancerous cells. Recent studies also show that members of the CEACAM family play an important role in modulating the immune responses associated with infection, inflammation and cancer. At the epithelial cell surface, CEACAM1 (also known as BGP, C-CAM and CD66a) is believed to exist as a cis-homodimer. The dimeric state is thought to regulate the ability of the receptor to recruit signaling molecules, including SRC-family kinases and the tyrosine phosphatase SRC homology 2 (SH2)-domain-containing protein tyrosine phosphatase 1 (SHP1). To characterize the dynamics and monomer-dimer equilibrium of CEACAM1, we have applied a live-cell combinatorial microscopy imaging approach. Time-lapse total internal reflection fluorescence (TIRF) imaging of HELA cells overexpressing cyan fluorescent protein (CFP)- and yellow fluorescent protein (YFP)-CEACAM1 constructs revealed a heterogeneous distribution of bright, micron-sized jagged structures that rapidly changed shape and diffused across the cell surface. Homo- and hetero-Forster resonance energy transfer (FRET) imaging techniques (acceptor photobleaching, sensitized emission, and polarization anisotropy) were then used in combination with TIRF microscopy to determine that these jagged structures are likely aggregates of monomeric CEACAM1 proteins while the rest of the membrane contains lower concentrations of dimeric CEACAM1. This combinatorial microscopy strategy not only compliments traditional biochemical assays, but also provides new quantitative insights into spatial-temporal dynamics of this important membrane protein. We are exploiting these insights to further understand the various immune responses associated with the CEACAM family.

3487-Pos Board B534

Mobile Haptens in Lipid Bilayers Cause Large-Scale Clustering of IgE Receptors

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Rat basophilic leukemia (RBL) mast cells express IgE receptors on the membrane that, when aggregated, initiate biochemical events that lead to the exocytosis of inflammatory mediators (degranulation). Low concentrations of monovalent haptens in fluid supported lipid bilayers have been found to cause degranulation, but microscopically visible aggregation has not been previously observed. To investigate possible receptor aggregation at higher hapten concentration, RBL cells were loaded with a fluorescent anti-DNP IgE and then deposited onto fluid supported lipid bilayers containing DNP-lipids at up to 50 mole %. Total internal reflection fluorescence microscopy was used to image the cells; large clusters were observed at DNP concentrations $\geq$ 10 mol %. The characteristic cluster sizes and densities were analyzed with image

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.

3489-Pos Board B536
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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3490-Pos Board B537
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