role as a link in transmembrane signal transduction. Recently, Hulko et al. determined the solution NMR structure of the HAMP domain (PDB ID:2ASW) of an archaeal protein AF1503, a putative transmembrane receptor [Hulko, M., et al. (2006) *Cell 126*, 929-940]. Based on the NMR structure, we have modeled the relative orientation of transmembrane domains TM1 and TM2 using replica exchange molecular dynamics simulations (REXMD). We then performed MD simulations of the HAMP and TM complex in explicit lipid bilayers to investigate possible thermal motions as well as signal transduction mechanisms. In addition, as Hulko et al. suggested a signal transduction mechanism related to the canonical packing by rotation of the helices, we have investigated the helix rotation propensity through MD simulations. In this work, we will present the simulation results of the HAMP and TM complex as well as the HAMP domain alone.

3478-Pos Board B525

Redistribution Of The Human Mu-opioid Receptor (hMor) In HEK 293 Cell Membranes In Response To Agonists

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Opioid receptors play a role in a whole battery of physiological processes, including affective behaviour, neuroendocrine physiology and pain perception. The cell membrane, which is the physical environment of the receptor, is known to consist of domains with distinct lipid/protein composition and physical characteristics. Previous work (Moffet *et al.* (2000) *J. Biol. Chem.* 275: 2191-2198.) has shown that the G-protein signalling partners of hMOR localize to the ''so called'' detergent resistant membrane (DRM). Our previous studies using ligand-affinity atomic force microscopy (AFM) of hMOR-expressing Sf9 cell membranes indicated that hMOR localizes to mixed lipid domains, presumably corresponding to detergent soluble membranes (DSM). These results would indicate a physical separation of the receptor and its signalling partner prior to activation. However, the relationship between microdomains characterized using biochemical techniques (DSM/DRM) and membrane rafts defined by biophysical techniques remains unclear. Here we track active hMOR localization and lipid composition in detergent and detergent-free separated membranes. For each fraction, hMOR activity was assessed using a modified binding assay, and Western blot analysis was used to determine the relative amount of lipid raft marker, flotillin-1, and G-proteins. We show a redistribution of the receptor in response to its agonists, implying that hMOR distribution at the cell membrane helps to regulate it function.

3479-Pos Board B526

Evidence for precoupling of inactive M3 muscarinic receptors and G_q Protein

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Two models have been proposed to explain how G protein-coupled receptors (GPCRs) interact with heterotrimeric G proteins to transduce physiological signals. One model suggests that GPCRs and G proteins collide with each other randomly after receptor activation and that binding is transient. An alternative model suggests that GPCRs and G proteins are bound to each other (precoupled) before receptor activation. We have studied interactions between GPCRs and G proteins using fluorescence recovery after photobleaching (FRAP) and avidin-mediated crosslinking in HEK 293 cells. We have previously shown that immobile CFP-labeled α_{2A}-adrenoreceptors (C-α2ARs) do not decrease the mobility of the G proteins that they activate, consistent with a collision-coupling model. Here we show that immobile CFP-labeled M3 muscarinic receptors (C-M3Rs) decrease the lateral mobility of citrine-labeled $G\alpha_q$. C-M3Rs failed to decrease the mobility of venus-labeled $G\alpha_{oA}$. Conversely, the C-M4Rs (which activate $G_{i/o}$ proteins) failed to decrease the mobility of G_q-citrine. Slowing of $G\alpha_q$-citrine by immobile C-M3R was unaffected by an agonist (carbachol) or an inverse agonist (atropine), and thus did not depend on activation of the receptor. Slowing of $G\alpha_q$-citrine by immobile C-M3Rs was enhanced by carbachol when nucleotides were depleted, as predicted by the ternary complex model of G protein coupling. These results suggest that inactive M3Rs precouple with G_q proteins, and that different coupling models apply to different GPCR-G protein pairs.
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3480-Pos Board B527

Active α2A-receptors induce GTPγS release from activated Go proteins and subsequently sequestrate G proteins

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Recently, analysis of agonist-induced G protein activation and receptor/G protein interaction has become possible in single living cells by means of FRET. Using these methods, we aimed to investigate whether GTPγS-bound Go proteins interact with active α2A-receptors and whether bound-GTPγS can be released from Go proteins upon this interaction. In order to conduct these experiments, we had to gain control of the intracellular nucleotide composition by permeabilizing the cell membrane of transiently transfected HEK293 cells via short application of saponine.
Agonist-induced receptor/G protein interaction, monitored by means of FRET between tagged α2A-receptors and tagged Gγ, was significantly stronger in the presence of low concentration of GDP, GTP and GTPγS compared to corresponding 1000-fold higher concentrations of nucleotide. Superfusion of cells with GTPγS in the presence of agonist resulted in attenuation of FRET between G protein subunits corresponding to maximal activation of G proteins. When omitting GTPγS, the FRET signal recovered with kinetics accelerating with increasing concentration of agonist. This result suggested deactivation of G proteins due to receptor-induced dissociation of GTPγS from preactivated G proteins. As a second experiment, non-labeled GTPγS could displace radioactively labeled GTPγ35S in an agonist dependent manner. In the presence of saturating concentration of agonist, increased FRET between G protein subunits suggested a reduced activation of G proteins compared to non-saturating concentration of agonist. This effect was found for both intact cells and permeabilized cells in the presence of low concentration, but not in the presence of high concentration of GTPγS. These results suggested (I) interaction of active G proteins with active receptors (II) receptor-induced dissociation of GTP/ GTPγS from G proteins and (III) sequestration of inactive nucleotide-free G proteins at the active receptors.

3481-Pos Board B528

Rapid Binding of G$\beta\gamma$ Dimers to the c-terminus of GRK3

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One proposed function of macromolecular signaling complexes is acceleration of signal onset, as complexes would obviate the need for collision of randomly-diffusing molecules. Here we study the kinetics of heterotrimeric G protein signaling between molecules that are unlikely to be part of a complex. Binding of the GRK3 c-terminus (GRK3ct) to G$\beta\gamma$ dimers was detected using confocal microscopy and fluorescence or bioluminescence resonance energy transfer (FRET or BRET). Activation of pertussis toxin-insensitive G_{oA} heterotrimers led to translocation of GRK3ct-venus from the cytosol to the plasma membrane with a monoexponential time constant of 431 $\pm$ 35 ms (n=9) at room temperature. FRET between a membrane-associated GRK3ct-cerulean (masGRK3ct-C) and G$\beta\gamma$-venus (G$\beta\gamma$-V) increased with a time constant of 213 $\pm$ 32 ms (n=10) at 26.5 °C, and 67 $\pm$ 13 ms (n=13) at 37 °C. Fluorescence recovery after photobleaching suggested that masGRK3ct-V and heterotrimers were not part of a complex prior to activation. Agonist-induced BRET between masGRK3ct-Rluc8 and G$\beta\gamma$-V decreased as the relative expression of Gα increased. This is consistent with buffering of free G$\beta\gamma$ by excess inactive Gα subunits, suggesting that masGRK3ct-Rluc8 bound to free G$\beta\gamma$-V dimers rather than to rearranged heterotrimers. These results suggest that G protein signals that occur on a timescale of ~100 ms are not necessarily mediated by preassembled signaling complexes. *Supported by grants GM078319 from the NIH and MCB0620024 from the NSF.*

3482-Pos Board B529

Functional Evidence for Gi-Gq crosstalk through G protein-coupled Receptor Heterocomplexes

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We have studied signaling through a complex of two distinct G protein-coupled receptor (GPCR) types: a serotonin (Gq-coupled) and a metabotropic glutamate (Gi-coupled). We used two-electrode voltage clamp in Xenopus oocytes expressing the receptors and used endogenous calcium-activated chloride or heterologously expressed Kir channels to monitor the effects of GPCR signaling. Previous biochemical studies had shown Gi signaling by stimulating the serotonin receptor in the serotonin-glutamate receptor complex (González-Maeso et al., Nature 2008 452:93-97). Our work here focused on studying whether Gq signaling could also take place when stimulating the glutamate receptor.
Upon glutamate receptor activation, oocytes expressing both receptors elicited calcium-activated chloride currents, not observed in oocytes injected with the glutamate receptor alone. Furthermore, these currents could be blocked by a specific regulator of Gq-protein signaling (RGS2) and were accompanied by phosphatidylinositol (3,4)-bisphosphate (PIP2) hydrolysis in the membrane, results consistent with Gq-signaling. Finally, receptor chimeric studies further confirmed that the glutamate-serotonin complex needs to be formed in order to allow Gi-Gq cross activation.

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