immune systems through the hormonal and neuronal pathway. In turn, the immune system can influence nervous system activity through the release of immune mediators and cytokines. In this situation, we have developed a unique technique for studying neuro-immune interaction with con-focal laser scanning fluorescence microscopy. It relies on guiding immune and nerve cell interaction by creating an adhesive environment using a matrigel-coated culture dishes. With this technique, we are able to study details of the mechanism of how nerve cells communicate with immune cells (mast cells and T lymphocytes) and vice versa. We showed that nerve-mast cell communication can occur in the absence of an intermediary transducing cell and that the neuropeptide substance P, operating via NK-1 receptors, is a mediator of this communication. In addition, recently, we found that ATP released from activated mast cells mediates the activation of nerve cells. Further, with the technique, we were able to study details of the molecular mechanism of nerve-mast cell interaction and we found that N-cadherin and SynCAM predominantly mediate attachment and promote the communication between mast cells and nerve cells. This is a fundamentally important study for the neuro-immune interaction. It would lead to new therapeutic modalities for diseases based on neuro-immune interaction such as neurogenic inflammation, intestinal bowel diseases, asthma, and autoimmune disorders.

2960-Pos Disruption of a PDZ Binding Motif within the Endothelin A Receptor Eliminates Heterodimerization and Sustained Calcium Signaling

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Board B263

Evidence suggests that endothelin A (ET_A) and B (ET_B) can form dimers that influence receptor function, but mechanisms underlying dimerization are unclear. Here we used the CFP/FlAsH fluorescent resonance energy transfer (FRET) pair to investigate ET_A and ET_B dimers. Full-length human ET_A and ET_B were C-terminally tagged with a tetracysteine motif (C4), which binds the FRET acceptor FlAsH. HEK293 cells stably expressing one of these constructs (ET_A-C4 or ET_B-C4) were transfected with ET_A or ET_B C-terminally tagged with CFP (ET_A-CFP, ET_B-CFP). FRET efficiencies of 27.4±3.5%, 14.5±2.8% and 22.0±1.7% were observed for ET_A:ET_B, ET_A:ET_A and ET_B:ET_B, respectively, indicative of robust receptor dimerization. Next we investigated an ET_A C-terminal PDZ binding motif for its role in dimer formation. Both a PDZ truncated mutant and a double-point mutant showed complete loss of FRET for ET_A:ET_B and ET_A:ET_A, including a reduced ability to co-immunoprecipitate the PDZ mutants, linking this motif to dimer formation. ET-1 stimulation of HEK293 cells expressing ET_A:ET_A or ET_B:ET_B produced a transient elevation in intracellular calcium that was blocked by the appropriate ET_A or ET_B selective antagonist. In contrast, ET_A:ET_B demonstrated a sustained calcium rise over 10 minutes that was blocked only by inclusion of both antagonists. In addition, ET_A:ET_A and ET_B:ET_B internalized upon ET-1 stimulation as monitored by CFP fluorescence, whereas ET_A:ET_B did not. Heterodimers containing PDZ mutations reverted to a transient calcium response and also internalized in response to ET-1. The results suggest that ET_A receptors form functional homo- and heterodimers in part through a C-terminal PDZ binding motif. Moreover, heterodimers appear to function distinctly from homodimers or monomers through delayed internalization and a sustained calcium response to ET-1 that required both an ET_A and ET_B antagonist for pharmacological inhibition.

Membrane Receptors & Signal Transduction - II

2961-Pos Investigation of the Diffusion Dynamics of the Two Tumor Necrosis Factor Receptors by Fluorescence Correlation Spectroscopy

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Board B264

The inflammatory cytokine tumor necrosis factor (TNF) is a product of activated immune cells, like macrophages and T lymphocytes, and acts as a general activator of the innate immune system. TNF binds to two cell surface receptors, TNFR1 and TNFR2. TNF signal initiation is induced by binding of up to three receptors per TNF. TNFR1 leads to the activation of the transcription factor NF-κB (nuclear factor kappa B) but also to the initiation of programmed cell death. TNFR2 interact with TRAF (TNF receptor associated factor) molecules, leading also to the activation of NF-κB.

In this work, fluorescence correlation spectroscopy was used to unravel the dynamics of both receptors. Although structurally similar in their extracellular and transmembrane domain, the dynamics of these receptors in the plasma membrane are markedly different. Upon binding of the ligand TNF the diffusion constant of TNFR2 is reduced from 3.3×10^{-9} to 0.8×10^{-9} cm^2/s within 30 min. This can not be explained by the sole formation of higher ordered receptor clusters, since the fluorescence intensity of TNF treated receptors revealed the presence of two to five receptor molecules only. Studies with TNFR2 lacking the cytoplasmic signaling domain indicate that interactions of the receptor with the cytoskeleton are unlikely to be responsible for the reduction in receptor diffusion. Rather, it is proposed that the ligand activated TNFR2 is recruited to slowly diffusing membrane microdomains. In contrast to TNFR2, unstimulated TNFR1 shows a diffusion constant of 1.2×10^{-9} cm^2/s and a significantly broader distribution of diffusion times. Furthermore and in contrast to TNFR2, the diffusion velocity of TNFR1 can be enhanced by cholesterol depletion of the cellular membrane. Hence, both TNFR1 and TNFR2 show differential affinities to slowly diffusing membrane structures of distinct nature.

2962-Pos Regulation of Collagen Fibrillogenesis by Kinase-Dead DDRs

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Board B265

The assembly of the collagen fibers, the major component of the extracellular matrix (ECM) governs a variety of physiological processes. Collagen fiber assembly is a tightly controlled process in which several factors including cell-surface receptors and secreted proteins play a crucial role. The aim of this study was to elucidate how the widely expressed cell surface receptors, namely Discoidin Domain Receptors (DDR1 and DDR2) affect collagen fibrillogenesis at the cellular level. In our earlier work, we established that the soluble extracellular domain (ECD) of DDRs can bind to collagen and regulate fibrillogenesis in-vitro. In this study we generated expression plasmids that encoded kinase-dead membrane anchored or soluble form of DDR1 and DDR2. These constructs were transfected into mouse osteoblast cells and DDR1 and DDR2 stable expressing cell lines were selected. These cell lines were subsequently treated with ascorbate (25 μg/ml) and the resulting ECM was analyzed using transmission electron microscopy. Alternately, fluorescently labeled collagen was added to cells transiently expressing kinase dead-DDRs and the resulting collagen morphology was assessed using fluorescence microscopy. Collagen content was quantified by a hydroxyproline assay for normal and the stably transfected cells. Our results elucidate how DDR expression not only reduces the amount of collagen deposition but also induces significant morphological changes in the resulting fibers. These results signify the importance of kinase-dead isoforms of DDRs, which are naturally present in several cell lines and overexpressed in a number of malignancies.

2963-Pos Cellular Stimulation by Means of Designed Surfaces Investigated by Fluorescence Imaging Techniques

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Board B266

Biological cells communicate cues form the external environment through plasma membrane receptors. Ligand-receptor binding alters the ligand mobility, receptor orientation, lateral distributions, and topological densities; these are phenomena which will subsequently trigger biochemical events involved in signal transduction cascades. The major goal of this research is to explore ligand-receptor binding effects, signal transduction and cellular responses to stimulation by biophysical measurements. Functional biomolecules presented on 2D surface will be examined for their biological activity towards stimulation of immune cells in terms of Jurkat (T cells) and RBL (mast cells) by total internal reflection fluorescence microscopy (TIRFM) imaging. Particle tracking and fluorescence recovery after photobleaching (FRAP) techniques are applied to characterize dynamic membrane protein (re-) distributions, which will give molecular insight into cellular protein/protein interactions, local adhesion, and signaling complex formation involved in membrane signal transduction.

2964-Pos Microenvironment Dependent Signalling and Crosstalk in Stress Exposed Human Mononuclear Cell Populations

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Board B267

Intercellular communication through contact and non-contact interactions plays crucial role in cells proper function in response to stimuli, survival, proliferation, differentiation, and programmed death. However many details of the involved signalling pathways in various cells in various conditions are yet far from being fully elucidated. The aim of this study was to supply new data concerning crosstalk of adherent and non-adherent human mononuclear cells in stress conditions. We cultured peripheral blood derived lympocytes and monocytes/monocyte-derived dendritic cells in presence/absence of growth factors/cytokines, virtually alone and in coculture. Various mononuclear cell populations were exposed to different stress factors (energy/nutrient deprivation, hypoxia/hyperoxia, reactive oxygen species) or therapeutic agents (low level near-infrared / far-red laser irradiation, natural antioxidants) separately and/or in coculture,and were further cultured virtually alone and/or in coculture. Using appropriate molecular reporters changes occurring in various cell populations in the mitochondrial reticulum state, cells redox state, survival/proliferation rates, and death-style choices were followed up. The data obtained by phase contrast, fluorescence, and confocal microscopy, electrophoresis/immunoblotting and flow cytometry, document significant photobiomodulation of natural antioxidant effects, and microenvironment dependent cellular crosstalk, more important in stress conditions.

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2965-Pos Expression of G Protein-Coupled Receptors in *E. coli* and Yeast Systems

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Board B268

We tested several expression systems to enhance the yield of human GPCRs in fermentative processes for subsequent structural studies by NMR spectroscopy. To this end, different media and carbon sources were tested. As an expression host for the Y1-, Y2- and CCKA-receptor we choose *Escherichia coli*, which has the advantage of producing large amounts of protein. However, the protein is accumulated in inclusion bodies, which requires cumbersome refolding of the denatured protein. The composition of the medium plays an important role with regard to the strategies of isotopic labelling of the protein. Complex media, which lead to large amounts of biomass, are not suitable for specific ^{13}C enrichment of the receptor. Therefore, we optimized a defined mineral salt medium to increase the biomass yield. Our research resulted in an optical density of ~ 40 and in an increase of protein expression of up to 400 μg/g wet weight. Fully functional GPCRs can be expressed in yeast (*Saccharomyces cerevisiae*). Here, we prepared the muscarinic acetylcholine receptor M3Δ3. Because the G_q-signal transduction of the GPCR is coupled to the metabolism of this yeast strain, the receptor is functionally expressed. The preparation of the functional protein was performed in a detergent solubilization buffer with subsequent purification and reconstitution in liposomes. Analysis of the receptor was conducted by an ELISA test confirming the expression of the receptor. For a first functional characterization, fluorescence quenching was used to measure binding of the ligand to its receptor, yielding a K_D value of ~100 nM.

2966-Pos Location, Structure, and Dynamics of a Synthetic Cannabinoid Ligand CP 55,940 in Lipid Bilayers: A Solid-state NMR Study

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Board B269

CP55,940 is a widely-used specific ligand of cannabinoid receptors CB1 and CB2. The compound has low solubility in water and accumulates in biological membranes as measured by using tritium-labeled CP55,940. It has been speculated that such hydrophobic ligands approach the receptor from the lipid matrix. To gain basic knowledge for elucidating the receptor-binding mechanism, following topics on CP55,940-lipid interactions were investigated by solid-state NMR:

1. Location of the ligand phenol group in the bilayers,
2. orientation of the phenol with respect to the bilayer normal,
3. order parameters of the ligand nonyl tail attached to the phenol,
4. perturbation on order parameters of lipid acyl chains upon ligand-binding.

1-Palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) bilayers were used. Ligand-lipid ^1H NMR cross-relaxation rates measured in two-dimensional MAS NOESY, and ring-current induced chemical-shift perturbation on the lipid ^1H MAS NMR revealed that the location of the phenol group is centered about the ester carbonyl groups. ^1H-^{13}C dipolar couplings in the phenol determined by DIPSHIFT experiments showed that the σ-bond connecting the phenol and hydroxycyclohexane groups is perpendicular to the bilayer normal, pointing the hydroxyl groups towards the water phase. Order parameters of the nonyl tail was studied by ^2H NMR on specifically deuterated CP55,940. The order parameters are larger only by 0.01-0.02 than that of the lipid terminal methyl group at ambient temperature. Hydrophobic anchoring of the nonyl tail thus accompanies high conformational flexibility. ^2H NMR on *sn*-1 chain deuterated POPC monitored the bilayer phase-state as well as perturbation of lipid packing due to the presence of CP55,940. Marked disorder induced by the ligand in the last half (C9-16) of palmitoyl chain in contrast to the first half (C2-8) agrees with the primarily interfacial location of the ligand.

2967-Pos Single-molecule Imaging Analysis of Interactions between Signaling Molecules Ras and Raf in Living Cells

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Board B270

Molecular recognition between H-Ras (Ras) and Raf1 (Raf) is an essential process to switch on intracellular Ras-MAPK signaling system. However, molecular mechanism for regulation of the switching has remained to be unknown. Here, we used single-molecule imaging analysis to study kinetics of interactions between individual Raf and Ras molecules and conformational changes of Raf during interaction with Ras in living cells.

Dissociation between Raf and an inactive form of Ras (RasGDP) was a first-order reaction, while, the dissociation between Raf and an active form of Ras (RasGTP) was a consecutive reaction in which dissociation from the initial binding state was negligible. These results suggest that the initial binding state of Raf with RasGTP is different from that with RasGDP.

To confirm this suggestion, we constructed an intra-molecular FRET probe by fusing GFP and YFP at the amino and carboxyl termini of Raf (G-Raf-Y), respectively, and detected conformational changes of individual Raf molecules during interaction with Ras. FRET signal was high for G-Raf-Y both in the cytoplasm and bound to RasGDP in resting cells, however, FRET signal was low for G-Raf-Y bound to RasGTP even at the start point of association. These results show that the initial binding state of Raf with RasGTP is different from that with RasGDP and that the conformational change of Raf from closed form to open form was induced instantaneously (less than several ten msec.) after binding to RasGTP.

The study suggests that the active form of Ras is not merely a binding site on the plasma membrane for Raf but an inducer of conformational change of Raf.

2968-Pos The Influence of Fluorescent Label on the Diffusion Co-efficient of G-protein Coupled Receptors as Determined by Fluorescence Correlation Spectroscopy

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Board B271

G-protein coupled receptors (GPCRs) are a large family of cell surface proteins which bind ligands ranging from photons to large peptides. Much of the information about the membrane organisation of GPCRs has come from receptors fluorescently labelled with auto-fluorescent proteins such as GFP. Here, using fluorescence correlation spectroscopy, we have compared the diffusion of GPCRs labelled with GFP to those labelled with YFP and the YFP variant, Topaz (Tpz), to investigate whether the choice of label affects the membrane diffusion co-efficient, D. Constructs of the human β_2- and β_3- adrenoceptors and A_1-adenosine receptor (β_2R, β_3R and A_1-AR) were prepared with the intracellular C-terminal fused to GFP, YFP or Tpz. We performed FCS measurements on the upper membrane of CHO cells transiently expressing the receptor fusions (see Briddon *et al.*, PNAS, *101*, 4673, 2004). The β_3R-YFP fusion showed two components with diffusion coefficients of D_1=1.35x10^{-6} and D_2=3.29x10^{-9} cm^2/s. Similar results were obtained for the β_3-Tpz (D_1=1.69x10^{-6}, D_2=3.44x10^{-9} cm^2/s). However, for the β_3R-GFP fusion both components showed significantly slower diffusion (D_1=2.26x10^{-7}, D_2=7.56x10^{-10} cm^2/s). Interestingly, for both the β_2R and A_1-AR fusions, the GFP-tagged receptor also showed slower diffusion than that of the YFP- or Tpz-fusions. Possible explanations for this difference involve differences in the photophysics of the label or in the linkers present between the receptor protein and the label, which have arisen as a consequence of the construction of the vector. We investigated these possibilities by using differing linkers and also using fluorescent receptor ligands and N-terminal labelling strategies as alternative methods for determining receptor diffusion co-efficients. Our studies show that the choice of fluorescent label can significantly influence the membrane diffusion of GPCRs in cell membranes.

2969-Pos Functional evidence for G protein-coupled receptor heterocomplexes

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Board B272

We have studied signaling of two unrelated G protein-coupled receptor (GPCR) types: a serotonin (Gq-coupled) and a metabotropic glutamate (Gi-coupled) receptor to a G protein-gated K+ channel (Kir3), using two-electrode voltage clamp in Xenopus oocytes expressing these molecules. We have compared the properties of specific agonist receptor-stimulated currents for each of the GPCRs expressed alone or together with each other.

Perfusion of a glutamate receptor agonist yielded a classic Gi-coupled monophasic Kir3 current activation, typical of Gi-mediated responses. Co-expression of the serotonin receptor changed the response to the glutamate receptor agonist from monophasic to biphasic. This biphasic response is typical of mixed simultaneous Gi- and Gq-mediated responses. Additionally, co-expression of the serotonin receptor yielded a significant reduction in the amount of activation, further supporting the idea of a novel complex between the two GPCRs signaling differently than the independent receptors.

These preliminary studies suggest that along with homomeric complexes between some GPCRs that belong to the same class, heterocomplexes are also possible, enabling simultaneous signaling of distinct signaling pathways in response to a single agonist.

2970-Pos FRET Reveals Kinetic Steps of Receptor-Mediated Modulation of Kv7 K^+ Current and PIP_2

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Board B273

We measured the kinetics of muscarinic suppression of M current and seek a kinetic model of the signaling cascade. Activation of M_1 muscarinic receptors (M_1R) couple through $G\alpha_q$ to stimulate phospholipase C (PLC), cleave phosphatidylinositol 4,5-bisphosphate (PIP_2), and close PIP_2-requiring Kv7.2/7.3 potassium channels. M current is maximally suppressed by 10 μM oxotremorine-M with time constants τ_{on} = 6.1 s and τ_{off} = 181 s.

We investigated the kinetics of steps intermediate to Kv7 channel closure by Fluorescence Resonance Energy Transfer (FRET) using photometric observations of single cells transfected with fluorescently labeled effectors. FRET between M_1R-CFP and $G\beta_1$-YFP increased with agonist (τ_{on} = 320 ms, τ_{off} = 3.7 s). Interaction between $G\alpha_q$-CFP and $PLC\beta_1$-YFP occurred with similar kinetics (τ_{on} = 1.4 s, τ_{off} = 5.8 s), suggesting that receptor, G proteins, and PLC are in close proximity prior to agonist exposure when PLC is over-expressed. FRET between labeled pleckstrin homology domains, PH($PLC\delta_1$)-CFP and -YFP, reported PIP_2 depletion (τ_{on} = 6.9 s, τ_{off} = 81 s), similar to M current kinetics. The time lag between PLC activation and PIP_2 depletion suggests that PLC availability limits the rate of M current suppression. In addition, PLC (but not G protein) over-expression increased the rate of M current suppression 3-fold (τ_{on} = 1.6 s). Since this follows the kinetics of $G\alpha_q$/PLC interaction closely, PIP_2 hydrolysis, release from channels, and channel closure must occur rapidly when PLC is abundant.

Evidently neither activation of receptor and G proteins, nor channel release of PIP_2 and closure, are rate-limiting during mus-

carinic suppression of M current. We are working to distinguish between a collision-coupled model in which G protein diffusion is rate-limiting and a pre-coupled model in which PIP_2 hydrolysis is rate-limiting.

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2971-Pos Identification of Residues Critical for Oligomerization of the G Protein-coupled Secretin Receptor by Cysteine-scanning Mutagenesis and BRET

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Board B274

While G protein-coupled receptors are reported to form oligomeric complexes that can have functional importance, the molecular basis for this remains unclear. We have previously demonstrated that the Family B secretin receptor forms homo-oligomers through interactions with transmembrane (TM) segment IV, with roles contributed by lipid-facing residues, Gly^{243} and Ile^{247}. Here, we use cysteine-scanning mutagenesis and bioluminescence resonance energy transfer (BRET) to map other residues within this TM segment that could also contribute to the oligomerization interface. We systematically mutated 14 residues in TM IV to cysteines, and evaluated their ability to oligomerize when expressed in COS cells. Mutations of three helix-facing residues, Gly^{241}, Trp^{242}, and Pro^{245}, interfered with normal cell surface expression. All other mutants tagged at the carboxyl terminus with *Renilla* luciferase or with yellow fluorescence protein bound secretin and signaled normally. Cells expressing the complementary-tagged receptors were treated with thiol-specific crosslinker cuprous phenanthroline to establish a covalent bond between spatially-approximated cysteine residues. BRET was measured and was repeated after competitive disruption of oligomers with synthetic TM IV peptide to distinguish covalent from non-covalent association. Non-disruptable BRET signals were observed for the mutants replacing residues Phe^{240}, Gly^{243}, Ala^{246}, Ile^{247}, and Ala^{250}, suggesting involvement in the dimerization interface. In contrast, residues Ala^{239}, Ser^{244}, Phe^{248}, and Val^{249}, as well as two lipid-facing residues low in the helix (Gln^{235} and Gly^{236}), had BRET signals disrupted by the peptide, suggesting distance and/or geometry incompatible with disulfide bond formation. We built a 3D molecular model of the secretin receptor dimerization complex that is consistent with these data.

2972-Pos The Stability of GPCR Dimers in the Plasma Membrane of Living Cells

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Board B275

G protein-coupled receptors (GPCRs) are thought to exist as dimers or higher order oligomers in the plasma membrane. Much has been learned about the structural arrangement of GPCR protomers within GPCR oligomers, but less is known about the stability of protomer-protomer interactions. Although some GPCRs are known to dimerize via the formation of disulfide bonds, the possibility that functional dimers may dissociate has not been explicitly tested. To address this question we coexpressed dopamine D2 receptors that were fused to either CFP at their n-terminus (C-D2R) or venus at their c-terminus (D2R-V) in HEK 293 cells. C-D2R and D2R-V were equally mobile within the plane of the plasma membrane, as indicated by fluorescence recovery after photobleaching (FRAP). Antibody crosslinking of the extracellular CFP moiety of C-D2R essentially immobilized this receptor, whereas the mobility of D2R-V in the same cells was inhibited to a lesser extent. D2R protomers can be covalently linked by oxidative crosslinking at cysteine168 near the extracellular end of the fourth transmembrane domain (Guo *et al.*, *JBC*, ***278***:4385, 2003). Oxidative crosslinking produced a further decrease in D2R-V mobility in the presence of antibody-crosslinked C-D2R. This effect was not observed in cells expressing C-D2R and D2R-V C168S, which bears a serine instead of a cysteine at position 168. These results suggest that D2R homodimers may dissociate in the plasma membrane of living cells.

2973-Pos Quantitative Characterization of C-reactive Protein Interaction with Fc-gamma Receptors by Ultrasensitive Confocal Fluorescence Imaging of Living Cells

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Board B276

C-reactive protein (CRP) is a prototype acute phase protein that may be intimately involved in human disease. Its cellular receptors are still under debate; the main candidates are Fc receptors for immunoglobulin G (FcγRs), as CRP was shown to bind specifically to FcγRI and FcγRIIa.

Using ultrasensitive confocal live-cell imaging with subsequent quantitative analysis of membrane fluorescence on FcγR-transfected COS-7 cells, we have shown that FcγRIIa binds fluorescently labeled CRP with lower avidity than antibody-antigen complexes. In a follow-up study we have demonstrated CRP binding to FcγRI with avidity similar to that of FcγRIIa. CRP binding to FcγRI in the presence of the γ-chain, investigated by transfection of COS-7 cells with a vector containing both FcγRI and the γ-chain, was enhanced by ~ 30-fold.

Recently, we have studied CRP binding to Fcγ receptors (FcγRI and II) naturally expressed at high levels on the plasma membranes of macrophage-like cells from a human leukemia cell line - Mono Mac 6. We have detected prominent binding of fluorescently labeled

CRP on the cell surface with avidity in the micromolar range, which was similar to the values obtained earlier for the transfected COS-7 cells. This CRP binding could be inhibited by pre-incubation with human, but not mouse IgGs and was thus FcγR-specific. Blocking of FcγRI by an FcγRI-specific antibody abolished CRP binding essentially completely, whereas application of antibodies against FcγRII did not have a noticeable effect. In the fluorescence images of Mono Mac 6 cells, the intensity patterns of bound CRP correlated well with those of fluorescently labeled FcγRI, but not FcγRII. These results provide clear evidence of specific interactions between CRP and Fcγ receptors (predominantly FcγRI) naturally expressed on the surface of a macrophage-like cell line.

2974-Pos Muscarinic M_2 Receptor (M_2Rs) Signalling in Airway Smooth Muscle:. A Novel Mechanism Involving BK_{Ca} Channels

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Board B277

This study investigates the mechanism by which stimulation of M_2Rs inhibits large conductance calcium-activated K^+ channels (BK_{Ca} channels). In HEK293 cells stably over-expressing the M_2R and transiently transfected with the α-subunit of the BK_{Ca} channel cloned from bovine trachea, carbachol (CCh; 10 μM) inhibited the whole-cell currents by 53% (n = 22). This inhibitory effect was abolished after pre-treatment of the cells with 500 ng/ml PTX or by over- expression of the G_ißγ scavenger transducin α (TDα). The PLC inhibitor U73122 (2.5 μM) and the PKC inhibitor Goe6976 (100 nM) partially reversed the CCh effect. In cell-attached recordings, CCh induced a transient increase in the channel open probability (*NP*o). This transient effect was prevented by pre-treatment of the cells with PTX, U73122, or by over-expression of TDα. In inside-out patches, 100 nM TDßγ decreased *NP*o by 55% (n = 15). After additional application of 300 nM TDα, *NP*o returned to baseline. Co-immunoprecipitation experiments demonstrated a direct interaction between BK_{Ca} channels and Gßγ. In HEK293 cells stably over-expressing the α_{2A} adrenoceptor (α_{2A}-AR) and transiently transfected with the BK_{Ca} α-subunit, 10 μM noradrenaline produced no effect. However, in HEK293 cells stably over-expressing M_2Rs or α_{2A}-ARs and transiently transfected with GIRK1/4 channels, both CCh and noradrenaline induced inward rectifier currents. In isolated smooth muscle cells from mouse trachea, CCh induced a PTX-sensitive inhibition of BK_{Ca} currents (by ~50%) which was partially reversed by the PKC inhibitor. The results demonstrate that Gßγ subunits released from PTX-sensitive G_i proteins by activated M_2Rs, stimulate intracellular calcium release via PLC-IP_3 and receptor-specifically inhibit BK_{Ca} channel activity through a direct effect and through activation of PKC.

2975-Pos Estrogen-induced Upregulation of 5-HT_{2A} and 5-HT_{2B} Receptor Transcript Levels in Rat Myometrium

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Board B278

We have recently reported that there is an increased sensitivity to serotonin-induced contraction in myometrium of late pregnant rats. The increase in serotonin sensitivity was associated with more abundant transcript and protein levels of 5-HT_{2A} receptors in late pregnant myometrium. Earlier work showed that the contractile response to 5-HT of ovariectomized rat uterus can be increased by E2 treatment and it was postulated that the increase in the contractile response to 5-HT was due to changes in the number of 5-HT receptors. However, to date, there is no information on the nature of the 5-HT receptors regulated by estrogen in myometrium. Here, we investigated the nature of the 5-HT2 receptor(s) (5-HT_{2A}, 5-HT_{2B} and 5-HT_{2C}) and whether estrogen treatment could contribute to alter the expression of these receptors at the level of their mRNAs. Ovariectomized rats were treated with E2 pellets (0.17 mg/pellet, for 10 days) to produce E2 levels comparable to late pregnancy or with placebo and transcript levels were measured by quantitative Real Time PCR in myometrium. Our results demonstrated that 5-HT_{2A} and 5-HT_{2B} receptors transcript levels are upregulated approximately 3 fold in estrogen treated (n=4) as compared to sham (n=3) animals. Interestingly we also found that 5-HT_{2C} receptor is also present in myometrium (n=3) and it is only slightly upregulated by E2 treatment. Transcript levels of the house keeping gene RPL32 remained unmodified. The estrogen-induced increase in mRNA levels of 5-HT_{2A} and 5-HT_{2B} receptors could result either from changes in the turnover of mRNA or from the upregulation of gene transcriptional activity. Analysis of the promoter region of the rat 5-HT_{2A} receptor showed sequences that might be regulated by estrogen supporting in this case the role of estrogen in the upregulation of 5-HT_{2A} gene transcriptional activity.

2976-Pos Deposition of Complement Protein C3b on the Erythrocyte Membrane Promotes Formation of a C3b-Glycophorin A-Band 3-Membrane Skeleton Complex

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Board B279

Complement activation results in opsonization of targets for phagocytosis. Decay-accelerating factor (DAF, CD55) protects normal cells from complement-mediated damage by inhibiting C3 activation on autologous membranes. We used single particle tracking and tether-pulling experiments to measure DAF lateral diffusion, lateral confinement, and membrane-skeletal association in human erythrocyte membranes. In native membranes, 78% of DAF molecules exhibited Brownian lateral diffusion. Fluid-phase complement activation caused C3b deposition onto erythrocyte glycophorin A (GPA). DAF, C3b, GPA, and band 3 were laterally immobilized in membranes of complement-treated cells, and GPA became physically associated with the membrane skeleton. Proteomic analysis showed that band 3, α-spectrin, β-spectrin, and ankyrin were present in a complex with C3b in complement-treated cells. C3b deposition was also associated with a significant increase in erythrocyte membrane stiffness and/or viscosity. We suggest that complement activation stimulates formation of membrane skeleton-linked DAF-C3b-GPA-band 3 complexes on the erythrocyte surface. These complexes could promote senescent erythrocyte removal from the circulation.

2977-Pos Functional Coupling of the A_3 Adenosine Receptor to the Sarcolemmal ATP-sensitive Potassium Channel in Murine Ventricular Cardiomyocytes

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Board B280

Activation of the A_1 adenosine receptor (AR) provides protection against ischemia/reperfusion injury most likely by facilitating opening of the cardiac sarcolemmal K_{ATP} ($sarcK_{ATP}$) channel. Recently, administration of A_3AR agonists has also been reported to protect the myocardium against ischemia/reperfusion injury. Unlike the A_1AR, activation of A_3AR does not negatively affect systemic hemodynamics including heart rate and blood pressure. However, the underlying cardioprotective mechanism of A_3AR activation remains unknown. In the present study, the functional coupling between the A_3AR and $sarcK_{ATP}$ channel was investigated. The effects of the specific A_3AR agonist, CP-532,903 (1 μM), on the $sarcK_{ATP}$ channel current (I_{KATP}) was monitored using the whole-cell configuration of the patch clamp technique. I_{KATP} was recorded from ventricular myocytes enzymatically isolated from hearts obtained from wild-type (WT) and A_3AR gene knock-out (A_3KO) mice. In all studies, potential input from A_1, A_{2A}, and A_{2B}ARs was blocked by the extracellular application of CPX (500nM) and ZM 241385 (500nM). In WT myocytes, CP-532,903 elicited an outward current with a density of 3.8 ± 1.6 pA/pF (mean ± SEM, n=12). This current was blocked by glibenclamide (1 μM), and thus, was identified as I_{KATP}. In myocytes obtained from A_3KO mice, the ability of CP-532,903 to elicit I_{KATP} was markedly attenuated with a resultant current density of 0.36 ± 0.31 pA/pF (n=4). This is the first study to provide evidence for functional coupling between the A_3AR and the $sarcK_{ATP}$ channel in cardiac myocytes. Combined with our previous results showing that cardioprotection provided by CP-532,903 is absent in Kir6.2 KO mice lacking the pore-forming subunit of the $sarcK_{ATP}$ channel, these findings suggest that $sarcK_{ATP}$ channel opening is a critical step underlying cardioprotection elicited by A_3AR activation.

2978-Pos Negative Concentration Dependency of the Association between Epidermal Growth Factor Receptor and an Adaptor Protein Grb2

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Board B281

Negative Concentration Dependency of the Association between Epidermal Growth Factor Receptor and an Adaptor Protein Grb2
Epidermal growth factor receptor (EGFR) is a plasma membrane protein which is responsible for cell proliferation. Phosphorylation of the cytoplasmic tyrosine residues of EGFR upon binding of EGF induces recognition of various intracellular signaling molecules, including an adaptor protein Grb2. Here, the reaction kinetics between EGFR and Grb2 was analyzed by visualizing single molecules of Grb2 conjugated to the fluorophore Cy3 at the N-terminus (Cy3-Grb2). The plasma membrane fraction was purified from cells expressing EGFR after stimulation with EGF and attached to cover slips. Unitary events of association and dissociation of Cy3-Grb2 on the EGFR in the membrane fraction were observed at different concentrations of Grb2 (0.1–100 nM). The dissociation kinetics could be explained using a multiple-exponential function with a major (>90%) dissociation rate of 8 s^{-1} and a few minor components suggesting the presence of multiple bound states. In contrast, the association kinetics for the wild type could be described by a stretched exponential function, suggesting the presence of multiple reaction channels from many unbound substates. Transitions between the unbound substates were also suggested. Unexpectedly, the rate of association was not proportional to the Grb2 concentration: an increase in Cy3-Grb2 concentration by a factor of ten induced an increase in the reaction frequency approximately by a factor ofbout three. This effect can compensate fluctuation of the signal transduction from EGFR to Grb2 caused by variations in the expression level of Grb2 in living cells. Measurements using Y1068F mutant of EGFR, which lacks one of the major association site of Grb2, suggested that the concentration dependency was caused by dynamic interactions between EGFR and Grb2.

2979-Pos Depolarization Induces a Conformational Change in the m2 Muscarinic Receptor

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Board B282

G-protein coupled receptors (GPCRs) are activated by binding of agonists. Unexpectedly, it was recently shown that the agonist binding affinity of several GPCRs depends on membrane potential. Two GPCRs, the m2 and m1 muscarinic receptors (m2R and m1R respectively) were shown to display depolarization induced charge movement associated currents, analogues to "gating currents" of voltage gated channels. The gating charge-voltage relationship of the m2R was shown to correlate with its affinity-voltage relationship. Furthermore, mutation of the m2R which abolished the gating currents concomitantly abolished the voltage dependency of the agonist binding. Motivated by these results, we examine here whether the depolarization induced charge movement causes a conformational change in the binding site of the m2R which in turn affects the agonist binding affinity of the GPCR. To this aim we combined gating currents measurements with fluorescence measurements using tetramethylrhodamine maleimide (TMRM) conjugated to cysteines on the m2R receptor expressed in Xenopus oocytes. Voltage changes applied to a TMRM-labeled wild type m2R resulted in mainly a very fast fluorescence change with electrochromic characteristics. However, when removing one of the two endogenous cysteines of the third extracellular loop, we detected a slower component of the fluorescence signal, which correlates with the time course of the gating currents.

Site-specific fluorescence labeling of residues known to be part of the binding site produced a large fluorescence change upon voltage change, indicating that membrane depolarization induces conformational changes in the agonist binding site cleft. These results are compatible with the notion that depolarization induces a conformational change in the binding site of the m2R.

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2980-Pos Effects of hT1R3's R813 on Sweet Receptor Function

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Board B283

Functional importance of intermolecular conformational changes involving transmembrane domains (TMDs) in sweet receptor activation has been suggested. hT1R3, one of two monomers of the Class C human heterodimeric sweet receptor, exhibits sequence-conserved microdomains shown to be functionally important in Class A. The focus of these molecular and computational studies is on the contribution of one such motif, xP+xY (where + is R or K) in Helix VII, to the sweet receptor function. Based on rhodopsin TMD structure, we created hT1R3 comparative homology model. Interaction energy analysis was conducted, aiding in choosing candidate residues for site-directed mutagenesis studies. Briefly, we calculated a pair-wise residue interaction energy matrix by averaging the corresponding non-bonded energy components over a MD trajectory starting from the initial homology structure. This matrix was then decomposed into eigenvalues and associated eigenvectors, allowing detection of functionally significant residues regulating receptor activity. As a result, R813 of xP+xY motif in hT1R3 was pinpointed as a 'hot residue'. Further local energy calculations identified L806, A807, and F809 backbone interactions with the side-chain of R813 as the strongest in this region. The R813N mutant showed much weaker interactions with L806, A807, and F809 computationally than the wild-type R813. *In vitro* R813N mutant correlated with its energy calculations, showing a significant decrease in receptor function in response to a panel of sweeteners. Similar computational analysis for R813K mutant demonstrated an intermediate strength of interactions of this mutant compared to wild-type and R813N. This predicted graded response is being characterized through ongoing functional studies. These studies reveal that the R in the xP**R**xY motif of hT1R3 has a Pro-kink stabilizing function. Since the positively charged residue is conserved in Class C members, we suggest that this mechanism may be shared by other Class C receptors.

2981-Pos Nano-scale Mapping And Quantification Of VEGFR2 Expressed With Respect To Fibronectin On Substrates

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Board B284

Cellular systems involve a great number of intra- and intercellular activities that are mediated by molecules related to signal transduction. The activation of one transmembrane molecule with chemical or mechanical stimuli triggers a subsequent expression of another molecule via intracellular mediators. Understanding, modeling, and predicting receptor-mediated cell functions are enhanced by measurement of receptor distribution and single molecule visualization with nano-scale resolution. Here we demonstrate a close relationship between fibronectin-activated integrin and vascular endothelial growth factor receptor 2 (VEGFR2) via functionalized force imaging, enabled by scanning probe microscopy and molecular force spectroscopy of intact cells with biomolecule-conjugated probes. We determined the number, distribution, and cytoskeletal association of VEGFR2 expressed as a function of fibronectin concentration on substrates on which human umbilical vein endothelial cells (HUVECs) were seeded. The spatial distribution and concentration of fibronectin were tailored through an array of Au nanoparticles with controlled spacing in order to determine the effect of fibronectin concentration on both VEGFR2 expression and association with cytoskeleton. In addition to demonstrating the powerful capability

of functionalized force imaging, our result illustrates that activation of the integrin-fibronectin system is closely related to the VEGFR2 system via common mediators involved in signal transduction.

2982-Pos The hydroelastic curvature mechanism of Venus flytrap closing

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Board B285

The Venus flytrap captures insects with one of the most rapid movements in the plant kingdom. Here we present detailed experimental investigation of the trap closure by mechanical and electrical stimuli and the model of this mechanism, which provides accurate description of experimental data. The leaf is assumed to comprise two hydraulic layers at its inner and outer surfaces of the leaf, where pressure difference can be maintained. The minimum elastic energy of the leaf, including mean and Gaussian curvature, corresponds to the closed state. The open state is the energized configuration created by pressure difference. The opening of channels between two hydraulic reservoirs triggers the trap closing. Resulting flux of water removes the pressure holding the trap ajar and the system relaxes to its closed state with minimum energy. Trap closing by electrical stimulus obeys the all-or-none law: there is no reaction for under-threshold stimulus and the speed of closing does not depend on stimulus strength above threshold. We used uncouplers, blockers of ion channels and aquaporins to interrogate mechanisms of different phases of closing. The novel non-invasive charge-injection method together with physiologically active agents gives insight into different steps of signal transmission and responses in plant kingdom.

2983-Pos Electrical Response of Higher Plants to Induced Heat Stress

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Board B286

Action potentials in higher plants are theorized as the information carriers in intercellular and intracellular communication in the presence of environmental stressors [1,2]. Among the most common stressors is heat stress. The response reactions of plant tissues and organs can be local or transmitted over long distances. Heat shock proteins, found in plant and animal cells, are partly responsible for the rapid response of plants to stress and the repair of plant tissue that has been damaged by stress through the activation of various pathways. In this work, the speeds of propagation of thermally induced action potentials in green plants are discussed. The speeds were found to be comparable to those occurring in various mammalian species. These rapid action potentials in green plants were recorded in real time using modern data acquisition techniques. According to our measurements, a single application of localized heat stress induces fast action potentials in *Aloe vera* (67 m/s). Electrical signals propagated along all leaves of the *Aloe vera* plants were studied. Possible pathways for electrical signal propagation in vascular plants are also discussed.

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Cardiac Electrophysiology

2984-Pos Remodeling of KCNE2 Subcellular Localization in Decompensated Heart Failure

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Board B287

The molecular correlates of $I_{to,f}$ currents, Kv4.3, Kv4.2 and KChIP2 transcripts are downregulated in heart failure. KCNE2, a modulatory β-subunit, can associate in expression systems with Kv4.2/Kv4.3 channels potentiating current amplitude. We speculated that KCNE2 transcripts and/or expression might be modulated in pathological heart hypertrophy induced by pressure overload. The transaortic constriction (TAC) procedure was used to create pressure overload and heart failure (TAC-HF) in male mice. Real-time PCR showed that transcript levels of Kv4.3 (1±0.07 to 0.53±0.07), Kv4.2 (1±0.13 to 0.34±0.01) and KChIP2 (1±0.1 to 0.6±0.06) were downregulated in TAC-HF. On the other hand, KCNE2 transcripts were not significantly different between TAC-HF and CTRL. To gain insight on the potential KCNE2 association with Kv4.2/Kv4.3 channels and how this association may change in TAC-HF, cardiomyocytes from CTRL and TAC-HF were labeled with anti-KCNE2, -Kv4.2 and -Kv4.3 antibodies. Both Kv4.3 and Kv4.2 localizes mainly along the T-tubules with almost no labeling at the surface membrane, whereas KCNE2 was distributed both at the surface and tubular membranes. Kv4.3 and Kv4.2 subcellular localization did not change in TAC-HF, whereas KCNE2 completely disappeared from the T-tubules and only was distributed at the surface membrane. The fact that Kv4.3 and Kv4.2 distribution was similar in both TAC-HF and CTRL animals supports the view that the remodeling of KCNE2 in decompensated TAC is not the result of a disruption of the T-tubular system but it is a specific KCNE2 remodeling. We speculate that the reduction of Kv4.3 and Kv4.2