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RNAi screening has identified a requirement for Stim and Orai and their mammalian homologs to produce store-operated Ca^{2+} entry (SOCE) and CRAC channel activity. Here, we evaluate the three human homologs of Orai expressed together with STIM1 separately and in combinations in HEK cells. Promiscuous heteromultimerization within Orai members was found by co-immunoprecipitation. Coexpressed with STIM1, Orai1 induced a large inwardly rectifying Ca^{2+} current with a form of Ca^{2+}-induced slow inactivation. A point mutation of Orai1 (E106D) drastically altered the ion selectivity of the induced CRAC current and eliminated slow inactivation while retaining an inwardly rectifying I–V characteristic. Using the amplified current model of co-expressed Orai1 + STIM1, we show that CRAC current can be recorded in cell-attached and inside-out patches. A C-terminal portion of Stim lacking the putative Ca^{2+}-binding motif and the transmembrane segment interacted with Orai by co-immunoprecipitation and effectively activated Ca^{2+} influx in S2 cells in the absence of store depletion. Expression of the corresponding STIM1 C-terminus, coexpressed with Orai1, was sufficient to generate CRAC current without store depletion. Furthermore, 50 μM 2-APB is able to sensitize/activate exogenous Orai3 channel activity without store depletion. The structural determinants responsible for the different Orai channel activities are being revealed by analyzing a series of Orai chimeras and point mutants.

2937-Pos Differential Pharmacology of 2-aminoethoxydiphenyl borate (2-APB) on CRAC Channels

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STIM1 in the endoplasmic reticulum (ER) and CRACM1 (or Orai1) in the plasma membrane are essential molecular components for controlling store-operated Ca2+ entry (SOCE) via CRAC channels. Combined over-expression of both proteins reconstitutes amplified CRAC currents and all three mammalian CRAC channel homologs (CRACM1, CRACM2 and CRACM3) represent functional store-operated channels with distinctive properties in terms of kinetics of activation, selectivity for Ca2+, Ba2+, and different feedback regulation by intracellular Ca2+. The dissection of the various molecular components of SOCE is complicated and only few pharmacological tools are available to address this problem. Although 2-aminoethoxydiphenyl borate (2-APB) cannot be considered a SOCE-specific compound, it has previously been found to affect native CRAC channels by potentiating CRAC currents at low concentrations (< 5 μM) and inhibiting them at high concentrations (> 10 μM). We have previously reported that 50 μM 2-APB completely blocks CRACM1, reduces CRACM2 by ~50% and fails to suppress CRACM3, but enhances it ~7-8 fold. We now have qualitatively and quantitatively assessed the effects of 2-APB on both store-operated and store-independent activation of CRAC channels and reveal that the three homologs exhibit strikingly different pharmacological responses to different concentrations of 2-APB that are mediated by differential effects on the CRAC channel itself as well as through its interaction with STIM1.

2938-Pos ADAR-mediated RNA Editing of Orai1 is A Key to Different Selectivity of Store-operated Channels

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Store-operated Ca^{2+} entry (SOCE) is known to be mediated by store operated channels (SOCs) that may have different Ca^{2+} selectivity. While Orai1 is thought to encode Ca^{2+}-selective SOC (CRAC) in nonexcitable cells, the molecular nature and the reasons for a poor cation selectivity of SOCs in other cell types remain a mystery. Here we present first evidence that Orai1 may encode different types of SOC, and the variations in their selectivity may result from post-translational modification of Orai1, with ADAR-mediated RNA editing being a key to this process. Patch-clamp, Ca^{2+} imaging and molecular approaches were used to study Ca^{2+}-selective CRAC in RBL-2H3 cells and cation-selective SOC (cat-SOC) in SMC. We discovered that: 1) siRNA knock down of Orai1 impairs SOCE and whole-cell SOC currents in both, RBL and SMC cells; 2) ADAR1 expression in RBL cells is significantly lower than in SMC; 3) overexpression of functional ADAR1 in RBL results in transformation of inwardly rectifying I_{CRAC} into a linear current, and appearance of significant Mn^{2+} influx through SOCE pathway; 4) siRNA knock down of endogenous ADAR1 in SMC results in disappearance of significant Mn^{2+} influx, and appearance of inwardly rectifying I_{CRAC} component of the whole-cell current. Thus, expression and functional activity of ADAR1 may change the selectivity of SOC channels. We propose that while the original (un-edited) Orai1 molecule forms Ca^{2+}-SOC(CRAC), ADAR-mediated RNA editing of specific residues may create post-transcriptional modified variants of Orai1 that form cat-SOCs with different cation selectivity. These important findings resolve the long lasting controversy about the relationship between SOC channels with different cation selectivity, and demonstrate how one gene (Orai1) may encode different SOCs, with ADAR-dependent RNA editing adjusting their selectivity to the specific needs of different cell types.

Membrane Receptors & Signal Transduction - I

2939-Pos Endothelial Cells Increase Tumor Cell Invasion

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Endothelial cells cultured as a monolayer on gels or membranes are known to form a barrier against leukocyte and fibroblast cell invasion into the underlying extracellular matrix. We investigated whether endothelial cells can also promote tumor cell transmigration and enhance invasion. We developed a 3D collagen gel assay to analyze the invasion depth and number of invaded cells in the presence or absence of closed endothelial monolayers. We tested 51 tumor cell lines derived from carcinoma, melanoma, adenoma, rat A3 mesenchymal sarcoma, human K562 erythroleukemia cells and mouse embryonic fibroblasts (MEF). Macrovascular endothelial cells were isolated from human umbilical veins and human microvascular pulmonary endothelial cells were used from 1st to 3rd passage. Tumor cells and fibroblasts were stained with carboxyfluorescein diacetate and DNA-intercalating dye Hoechst 33342 to distinguish them from endothelial cells. Transmigration and invasion of tumor cells (seeded at 200 cells/sqmm) were analyzed after 3 days. We quantified invasiveness for each cell line in the presence and absence of endothelial monolayers as average invasion depth (in mm) multiplied by the number of invasive cells per sqmm field of view. Seventeen tumor cell lines, mesenchymal sarcoma cells, K562 cells and fibroblasts showed dramatically increased invasion in the presence of endothelial cells. These results demonstrate that micro- and macrovascular endothelial cells are unable to form a barrier against seventeen tumor cells, A3 sarcoma cells, and K562 leukemia cells as well as MEFs independent of their tissue origin. These findings suggest a new role for endothelial cells as "enhancer" for cell invasion and tumor cell metastasis.

2940-Pos CD24 Expression Increased Cytoskeletal Dynamics Determining Tumor Cell Transmigration And Invasion

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CD24 is a small, heavily glycosylated cell-surface protein, linked with a glycosyl-phosphatidylinositol anchor in lipid rafts. We tested the hypothesis that CD24 expression increased tumor cell invasion through enhancing cytoskeletal dynamics. Human lung carcinoma cells (CD24 negative cells) were stably transfected with CD24 and sorted for high and low CD24 expression. The invasiveness of the CD24 high and low transfectants was determined by the number-density multiplied by the average cell invasion depth in a 3D-collagen gel after a 3 day incubation period. Invasiveness was 2-fold increased in CD24-high cells compared to CD24-low cells and 3-fold increased compared to CD24 negative cells. Cytoskeletal dynamics was determined from the creep response of individual cells measured with high-force magnetic tweezers. For creep measurements, step forces from 0.5–10 nN were applied to fibronectin-coated beads. The bead displacement vs. time followed a power-law. The power-law exponent was taken as a measure of cytoskeletal dynamics, with low values corresponding to a solid-like, static behavior, and high values corresponding to a more liquid-like, dynamic behavior. CD24-high cells had a signifcantly higher exponent (0.44 ± 0.01, n=29) compared to CD24-low cells (0.36 ± 0.02, n=36). In summary, the ability to rapidly remodel their cytoskeleton seems to be a key factor for the ability of tumor cells to invade a connective tissue matrix and to metastasize.

2941-Pos Effect of Receptor Dynamics on the Cell Surface of Bone Stem Cells on Cell Differentiation Studied in Genetic Mouse Models Displaying Alterations in Peak Bone Density

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Bone density is regulated by a complex signaling system of growth factors that regulate bone stem cell differentiation. In order to maintain healthy bone equilibrium between osteoblasts and osteoclasts must be maintained.

BMPs are key mediators in osteoblast differentiation and also in the pathogenesis of common clinical disorders of the skeleton and other tissue. They elucidate their signal through serine threonine kinase receptors that shuttle on the plasma membrane between different membrane domains. Therefore understanding the dynamics of these receptors on the plasma membrane and the organization of the membrane is key to control osteoblast differentiation and bone formation.

We utilized two congenic mouse strains, B6.C3H-6T (6T) and B6.C3H-1-12 (1–12), that differ from C57BL/6 (B6) controls by either reduced or enhanced peak bone density. These mice are valuable translational models for regulation of bone formation and resorption. Using the Family of Image Correlation Spectroscopy (FICS), Atomic Force Microscopy (AFM) combined with molecular biological methods we determined the response to BMP signaling on the receptor dynamics and signaling of the bone stem cells in these mice. Our data indicate that each mouse type displays significant differences in membrane receptor shuttling and signalling in response to BMP stimulation. These data suggest that membrane domain dynamics and BMP receptor localization are key pathways for osteoblast differentiation and indicate that BMP signaling affects peak bone acquisition and may affect the outcome of bone diseases.

2942-Pos ICAM Dynamics During Formation of the Immunological Synapse

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Board B245

Formation of the immunological synapse is an important step in the activation of CD4+ T-cells and the beginning of an immune response. Surface proteins organize into a micron-scale "bulls-eye" pattern at the junction between a T-cell and an Antigen Presenting Cell (APC), however the mechanism by which this sorting occurs remains unclear.

Here we investigate the biomechanics underlying synapse formation. Using supported lipid bilayers as synthetic APC surface mimics provides an ideal experimental platform to monitor the dynamics of synapse assembly in real time using fluorescence microscopy. By incorporating a recombinant ICAM-YFP fusion protein, we are able to quantitatively map protein density throughout the synapse with unprecedented resolution. We show that formation of the ring-like pattern of ICAM along the synapse periphery precedes the clustering of TCR-MHC complexes in the synapse center. This strongly suggests that localization of ICAM (and its receptor on the T-cell, LFA-1) at the synapse periphery is not mediated via height- or density-based exclusion by TCR-MHC complexes, as has been proposed in the past.

Ultimately, we seek to understand the biophysical basis of protein sorting in the immunological synapse, with specific emphasis on the ring-like pattern formed by ICAM-LFA-1 complexes. We believe that understanding the mechanism by which the synapse assembles will reveal clues regarding its function in T-cell activation.

2943-Pos Single Quantum Dot Tracking Of IgE Receptors Reveals Actin Corralling And Crosslinker-induced Immobilization Kinetics

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The high affinity IgE receptor, FcεRI, is the principal multi-subunit immunoreceptor on the surface of human 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 concomitant release of key mediators of allergic inflammation. Crosslinking also leads to receptor immobilization. However the details of immobilization and its role in signal initiation are not completely understood.

To study the dynamic events that induce FcεRI signaling, we generated monovalent quantum dot (QD)-IgE that binds FcεRI without crosslinking. Multi-color QD tracking revealed that, in the resting condition, multiple QD-IgE-FcεRI complexes can be co-confined within the same small region (up to 2 μm in size) for extended times. However, rigorous analysis of these trajectories found no evidence of homotypic interactions. Simultaneous imaging of QD-IgE-FcεRI and GFP-tagged actin provided direct evidence of actin "corralling" receptor motion that could explain the observed co-confinement. As receptor immobilization has been shown to be a consequence of crosslink-induced activation, we developed a real-time assay for this process. Employing this assay, we found that immobilization occurs within seconds of crosslinking (tave=12.6 s). Disruption of the actin cytoskeleton by latrunculin treatment increased the diffusion coefficient of both resting and crosslinked receptors by two fold, consistent with the removal of actin corrals. Inhibition of FcεRI phosphorylation did not alter receptor immobilization kinetics. Furthermore, we showed that direct crosslinking by multivalent antigen is required for immobilization since crosslinking of a subset of Dansyl-specific-IgE bound receptors does not induce immobilization of DNP-specific QD-IgE-FcεRI complexes. These results indicate that receptor immobilization lies upstream of FcεRI phosphorylation, and, as such may serve to initiate the signaling cascade.

2944-Pos Conformational dynamics of the Rhodopsin / Transducin complex: Insights from all atom Molecular Dynamics simulations

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G-protein coupled receptors (GPCRs) are a major class of cell membrane proteins that serve as the primary sensors of eukaryotic cells. As implied by their name, GPCRs signal through their interaction with their intracellular partners, the G-proteins. Their pharmacological significance is illustrated by the fact that although they represent less than 1% of open reading frames in the human genome, more that half of known drugs target of GPCR signaling. However, their study is hampered by the lack of high-resolution structural data, whereas the only known structure is that of rhodopsin, that has been used extensively as a modeling template. Mutagenesis and other biochemical studies have shown that the GPCR - G-protein association involves complex interactions distributed among a network of several residues, over an interface of the order of 1000 square Angstroms, however a detailed structural model of the complex is yet to be established. Motivated by the recent availability of results from molecular docking between rhodopsin in its dark state and heterotrimeric transducin, and the development of sophisticated parallel load-balancing techniques implemented in the NAMD platform, we are using 100ns-long Molecular Dynamics (MD) simulation trajectories to investigate the dynamics of the ternary complex in a DOPC membrane/water environment. We characterize the conformational changes that occur on the interacting partners as a consequence of complex formation and the driving forces of protein-protein interactions. Intermolecular contact maps reveal the interacting sites in the sequence of rhodopsin. Our results

suggest novel experiments that can be used to test the stability of the complex in the dark state.

2945-Pos IP3 Receptor Subtype-dependent Activation Of The Store-operated Calcium Influx, ICRAC

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The inositol 1,4,5-trisphosphat (IP3)-sensitive subcompartment of the intracellular calcium store (ER) is a heterogeneous compartment. Previous studies have shown that a specialized (IP3)-sensitive substore within the ER functionally couples to calcium-release activated calcium (ICRAC) activation upon receptor-stimulated IP3 production. It was found that a significant dissociation exists between IP3-induced calcium release mediated by the IP3 receptor (IP3R) and ICRAC activation. While IP3 causes considerable calcium release in the nanomolar range, ICRAC channel recruitment occurs only at micromolar IP3 concentrations, arguing in favor of a functionally separate ICRAC store characterized by reduced sensitivity to intracellular IP3. Such differing response thresholds of calcium stores to IP3 can be linked to the differential localization and activity of enzymes involved in IP3 metabolism. Store heterogeneity is also critically influenced by subcellular localization of calcium release channels within the ER. Three different genes encode IP3-sensitive receptors in vertebrates, represented by IP3R type I, II or III. The question therefore arises whether IP3R subtype composition contributes to the formation of a distinct ICRAC store. We investigated the ability of IP3R type I, II and III to mediate and modulate ICRAC in DT40 chicken B lymphocytes. Respective DT40 clones expressed none, all three, or one distinct type of IP3R. By analyzing the ICRAC signal upon IP3 mediated store depletion in these cells, we demonstrate that IP3R type II and type III participate in IP3-induced recruitment of ICRAC, but IP3R type I does not. IP3R type II and type III modulate the IP3 induced ICRAC signal differently in respects to affinity and cooperativeness. Our data suggest that the expression pattern of IP3R contributes to the formation of specialized ICRAC stores in B cells.

2946-Pos Distribution of BMP Wild Type II Receptor and The Mutants Underlying Primary Pulmonary Hypertension (PPH) on Cell Membrane

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PPH is a progressive and fatal disease with a median survival from diagnosis of 2.8 years. Heterozygous mutations within the BMP type II receptor (BMPR-II) gene have been identified in familial and sporadic PPH (1). Current research shows that three mutants of BMPRII, site mutation in kinase domain (D485G) and in cytoplasmic tail (N515K), and the truncating mutation in the cytoplasmic tail (R899X), are expressed on the cell surface, but alter the known BMP signaling pathways (2). In order to design more specific therapeutics for treatment of PPH, the dynamics of these mutants should be explored. We studied the distribution of the wild-type BMPRII and three mutants by image correlation spectroscopy (3). Our results showed that the aggregation and clustering of BMPRII and the three mutants on the membrane differed on both human pulmonary artery smooth muscle cells (PASMC) and c2c12 cells. Specifically, BMPRII formed highly aggregated clusters on the cell surface that, after BMP2 ligand stimulation, aggregated further. However, all the three mutants were highly dispersed on the cell membrane, possibly to the monomeric level in some cases. Stimulation by BMP2 led to significant increases in aggregation, but they were always more dispersed than the unstimulated wild-type receptors. These results indicated that the aggregation of BMP receptors may be affected in PPH. We hypothesize that the dispersion and probably mislocalization of the mutant receptors contribute to different BMP signaling observed in PPH. New therapeutics that can rescue the clustering of the receptors may provide new effective treatments for PPH.

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2947-Pos Dimerization Is Not Required For Nuclear Translocation Of Activated Herk1

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Many cellular functions such as gene expression and cell division/death are regulated by signal transduction involving the mitogen-activated protein kinase (MAPK) pathway. Upon activation (by MEK), ERK (a member of the MAPK family) translocates from the cytoplasm to the nucleus, where it activates transcription factors. Thus, nuclear translocation is critical for the successful relay of signals and evocation of cellular responses. However, the molecular states and mechanism(s) underlying the redistribution of ERK remain unclear.

It has been proposed that dimerization of ERK is a requirement for nuclear translocation. We have examined hERK1 (human ERK1) localization, cytoplasmic-nuclear translocation, and dimerization state in live cells using real-time fluorescence microscopy and fluorescence correlation spectroscopy (FCS). Specific fluorescent tagging in vivo of hERK1 and mutant hERK1-Δ4, a purported dimerization-deficient mutant, was achieved by fusion with EGFP. Comparison of the nuclear accumulation dynamics of the wild type and mutant proteins revealed that both accumulated in the nucleus to the same level, indicating that dimerization of hERK1 is not required for nuclear entry. This result was consistent with real-time homoFRET and FCS measurements, which did not detect dimerization of wild type GFP-hERK1 upon activation. However, the rates of nuclear accumulation and phosphorylation were lower in the case of GFP-hERK1-Δ4. Mathematical modeling of the Ras-Raf-ERK pathway using a two-compartment model and systematic sensitivity analysis was employed to determine whether a defect in phosphorylation could result in the observed differences between wild type and hERK1-Δ4. The model predicts that the behavior of the mutant can be explained by decreased phosphorylation of ERK by pMEK and increased stability of the MEK-ERK complex.

2948-Pos Preferential Binding of Ligands to Active and Inactive Conformational States of G Protein Coupled Receptors

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Board B251

G protein coupled receptors are the largest family of cell surface receptors responding to diverse ligands initiating signal transduction cascades. Because of their important physiological roles they represent the currently largest family of drug targets. Ligands have different pharmacological effects, suppressing or activating signal transduction. It is not known what determines these differential effects. Intuitively, ligands with activating or suppressing roles would be expected to differentially stabilize active and inactive conformations of the receptors, respectively. To quantitatively test this hypothesis, we have generated computational models of transmembrane regions of different G protein coupled receptors in two different conformations. The inactive conformation was modeled using the only crystal structure of any G protein coupled receptor available, that of the inactive, dark state of rhodopsin [1] as the template. The active conformation was created based on a recent model of the light-activated state of rhodopsin [2]. Ligands for which the natures of their pharmacological effects on the receptors are experimentally known were docked to the modeled receptor structures. We find that ligand binding pockets of different receptors overlap with the retinal ligand binding pocket in rhodopsin, and that ligands have strong preferences for active and inactive states depending on their modulatory nature. This suggests that the pharmacological profile of a ligand can be predicted based on its preference for different receptor conformations.

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2949-Pos Physico-chemical Characterization of Biologically Active Bacterial Lipopetides: Molecular Basis for Innate Immune Recognition

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Amphiphilic compounds of the cell wall of bacteria are potent inducers of an innate immune response. However, the physicochemical basis of immune-recogniton of these molecules by proteins and receptors of the immune system is not very well characterized. Since the importance of lipoproteins from the cell wall of Gram-positive and Gram-negative bacteria for the initiation of an innate immune defence is being increasingly recognized, we have investigated a selection of synthetic lipopetides with respect to their aggregate-structure, molecular conformation, fluidity of the acyl chains and their interaction with lipids of biological membranes.

We present experimental data on the biophysical mechanisms underlying lipopeptide bioactivity. Investigation of selected synthetic diacylated and triacylated lipopeptides revealed, that the geometry of these molecules (i.e. the molecular conformations and supramolecular aggregate structures) and the preference for membrane intercalation provide an explanation for the biological activities of the different lipopeptides. This refers in particular to the agonistic or antagonistic activity (i.e., their ability to induce cytokines in mononuclear cells or to block this activity, respectively).

The analytical data show that our concept of 'endotoxic conformation', originally developed for bacterial lipopolysaccahrides (LPS), can be applied also to the investigated lipopeptides, and suggest that the molecular mechanisms of cell activation by amphiphilic molecules are governed by a general principle.

2950-Pos Cell Surface ATP Synthase-Mediated Shear-Stress Mechanotransduction in Vascular Endothelial Cells

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Board B253

Vascular endothelial cells (ECs) recognize changes in shear stress, the frictional force exerted by flowing blood, and transmit signals to

the interior of the cell, which leads to cell responses that involve changes in cell morphology, cell function, and gene expression. The precise mechanism of shear-stress mechanotransduction, however, are not completely understood. Our previous studies demonstrated that Ca^{2+} signaling plays an important role in shear-stress mechanotransduction. When cultured human pulmonary artery ECs (HPAECs) were exposed to flow, the intracellular Ca^{2+} concentration increased in a shear-stress-dependent manner. The flow-induced Ca^{2+} response occurred in the form of an influx of extracellular Ca^{2+} via an ATP-operated cation channel, P2X4. We recently found that shear-stress-induced activation of P2X4 requires ATP, which is supplied in the form of endogenous ATP released by HPAECs. HPAECs released ATP in response to shear stress, and the ATP release was markedly suppressed both by a membrane-impermeable ATP synthase inhibitor, angiostatin, and by an anti-ATP synthase antibody, which suggests the involvement of cell-surface ATP synthase in shear stress-induced ATP release. Immunofluorescence staining of HPAECs showed that cell-surface ATP synthase is distributed in lipid rafts and co-localized with caveolin-1, a marker protein of caveolae. Depletion of plasma membrane cholesterol with methyl-β cyclodextrin disrupted the lipid rafts and abolished the co-localization of ATP synthase with caveolin-1, which resulted in a marked reduction in shear-stress-induced ATP release. Down-regulation of caveolin-1 expression by transfection with caveolin-1 siRNA also markedly suppressed ATP-releasing responses to shear stress. These results suggest that the localization and targeting of ATP synthase to caveolae/lipid rafts is critical for shear stress-induced ATP release. Although how shear stress activates cell-surface ATP synthase remains unknown, cell-surface ATP synthase seems to be involved in shear-stress mechanotransduction through activation of purinoceptors, such as P2X4.

2951-Pos Spatial Approximation between Secretin and the Third Extracellular Loop of its Receptor Provides Refined Insights into the Structure of the Docked Ligand-Receptor Complex

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The amino terminus of Family B G protein-coupled receptors is known to play a critical role in ligand binding and receptor activation, however a detailed molecular understanding of the ligand-receptor complex does not yet exist. Using photoaffinity labeling, we have previously shown that residues spread throughout secretin, in positions 6, 12, 13, 14, 18, 21, 22, 23, and 26, each covalently labeled distinct residues only within the amino-terminal domain of the receptor. To date, only probes with photolabile residues at the amino terminus of secretin have covalently labeled the receptor core domain. To better understand the precise mode of docking of the natural ligand to this receptor, the relationship of this domain and the receptor core region, and the molecular mechanism of receptor activation, we have utilized intrinsic photoaffinity labeling to identify a critical spatial approximation between residue five of secretin and a residue within the proposed third extracellular loop of the secretin receptor. This was achieved by purification, deglycosylation, cyanogen bromide cleavage and sequencing of the labeled wild type and mutant secretin receptors. This constraint has been used to refine our evolving model of the natural ligand-occupied secretin receptor. For the first time, we have refined the helical bundle and loop regions of this model. This model incorporates insights coming from the recently reported NMR and crystal structures of the amino-terminal domains of other Family B G protein-coupled receptors. This model is fully compatible with all experimentally-derived constraints currently available.

2952-Pos A Computational Study on GPCR Activation: Ligand Stabilized Conformational States of Rhodopsin and Beta$_2$ Adrenergic Receptor

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Board B255

Recent experimental studies on G protein coupled receptors (GPCRs) indicate that structurally different ligands with varied efficacies stabilize distinct receptor conformations thus exhibiting functional selectivity. This raises the possibility of designing GPCR drugs with functional selectivity for a particular signaling pathway. It is therefore critical to understand the conformational changes that the ligand induces on the receptor. We have developed a computational method to study the Ligand Induced Transmembrane Conformational changes (LITiCon) in GPCRs. This method involves a systematic spanning of the conformational subspace of the transmembrane (TM) helices, for predicting the conformational changes due to helical rotations and hinge bending. We have first validated this approach by applying it to bovine rhodopsin, where information regarding the active conformation is available from experiments (e. g. NMR and cysteine cross-linking). During the MD simulation, we observe an increased separation between the cytoplasmic ends of TMs 3 and 6, and a modulation of the proline kink in TM6, both of which are in agreement with fluorescence quenching experiments. Next we have applied the LITiCon method to study the activation of the β_2 Adrenergic receptor (β_2AR). We have studied the ligand stabilized receptor conformations of five different β_2AR ligands, a full agonist norepinephrine, partial agonists salbutamol, dopamine and catechol and an inverse agonist, ICI-118551. In agreement with the tryptophanbimane quenching experiments, norepinephrine and dopamine break the ionic lock and engage the rotamer toggle switch, whereas salbutamol only breaks the ionic lock and catechol only engages the rotamer toggle switch. The inverse agonist ICI-118551 does not engage any of the molecular switches, but still induces helical movements. These results along with virtual ligand screening results on the ligand stabilized conformations will be discussed.

2953-Pos Multiscale Modelling of CheA

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The histidine kinase CheA plays an important role in the regulation of bacterial chemotaxis. It operates through its trans autophosphorylation leading to activation of response regulators CheY and CheB, as part of a two component switch which alters tumbling frequency and leads to sensory adaption respectively. It exists in the cell as a dimer, with individual monomers containing two separate structured domains connected by long unstructured regions, and a central structured region with kinase, regulatory and dimerisation domains. Atomistically detailed molecular dynamics simulations of each of these domains offers insights into the individual properties of the domains, including the hydrogen bonding patterns maintaining the phosphorylated histidine's pKa, cleft opening motions and domain dynamics. However, the computational demands of atomistic simulations limit their applications to smaller proteins and/or single domains. The development of simplified (i.e. more abstract) models of protein structure enables us to describe the physical restraints imposed by the linker regions on the conformational dynamics of the complete structure. We demonstrate that such multiscale modelling can offer novel insights into structure-function relationships, in the absence of detailed structural data.

2954-Pos Osmo- and Chill Sensing by Bacterial Membrane Sensor Proteins

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We study the secondary, Na+ coupled betaine uptake system BetP and the two-component sensor kinase MtrB of Corynebacterium glutamicum. These proteins perceive a number of different stimuli related to osmotic stress as well as to other physical changes in their surrounding. The carrier protein BetP consists of a catalytic domain (12 transmembrane segments), and two regulatory domains, which are located at the N- and C-terminal parts of the protein, respectively, both exposed to the cytoplasm. The function of BetP is uptake of glycine betaine in response to osmotic stress. The sensor kinase MtrB consists of two transmembrane domains, a cytoplasmic loop, and a C-terminal cytoplasmic domain. MtrB transmits its signal to the cognate response regulator MtrA, which in turn leads to transcription regulation of several target genes, including betB. We are investigating possible physical parameters (e.g. solute concentration on both sides, temperature, membrane strain, physical state of the surrounding phospholipids etc.) with respect to their significance as stimuli of these osmoreactive sensor proteins. We have identified particular domains in the two proteins as being responsible for signal input and transduction, by application of mutant analysis using in vitro systems (proteoliposomes). BetP and MtrB were shown to respond to both signals originating from the hydrophilic (internal and external surrounding) as well as from the hydrophobic phase (membrane surrounding). Moreover, the regulatory response of BetP was shown to critically depend on the surrounding phospholipid membrane, both with respect to headgroup and fatty acid composition.

2955-Pos Stochastic Simulation Reveals The Mechanisms Of Immunological Synapse Formation In B Cells

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Board B258

B cell receptors have been shown to cluster at the intercellular junction between a B cell and an antigen-presenting cell (APC) in the form of a segregated pattern of B cell receptor/antigen (BCR/Ag) complexes known as an immunological synapse. We use a Monte Carlo model to investigate the mechanisms of B cell synapse formation. We find that differences in affinity and bond stiffness between BCR/Ag and LFA-1/ICAM-1 are sufficient to drive synapse formation in the absence of membrane deformation. When significant membrane deformation occurs as a result of receptor-ligand binding, our model predicts the affinity-dependent mechanism needs to be complemented by an additional, signaling-driven mechanism in order for synapses to form. Our results also show that B cell synapse formation is optimal for a limited range of receptor-ligand complex diffusion coefficient values, typically one-to-two orders of magnitude lower than the diffusion coefficient of free receptors. We have found a nonlinear relationship between the mean-square displacement and the mobility of BCR/Antigen complexes, which can be used to deduce the order of magnitude of their diffusion coefficient. Our analysis shows how such antigen movement data at the single molecule level can provide insight into the B cell synapse formation mechanism.

2956-Pos Voltage-Sensitivity of Dopamine D_2 and Histamine H_3 and H_4 Receptors

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Board B259

Dopamine D_2 receptors play a critical role in activity-dependent synaptic plasticity in the striatum, and regulate the transitions between different states of electrical activity, whereas histamine receptors regulate striatal dopamine release. The D_2 receptor is the main target for anti-psychotics, and its affinity towards dopamine has been shown to be increased in psychotic patients. Recently, voltage sensitivity has been reported for the ligand binding proper-

ties of a few neurotransmitter receptors, raising the question whether dopamine and histamine receptors are also regulated by voltage.

Our present electrophysiology data from *Xenopus* oocytes indicate that the D_2 receptor is indeed voltage-sensitive. Comparing concentration-response relationships for the activation of GIRK channels via D_2 receptor stimulation by quinpirole or dopamine at −80 and at +40 mV revealed rightward shifts of about tenfold upon depolarisation for both agonists. D_2 receptor activation did not alter GIRK conductance-voltage relationships, nor did application of agonist to oocytes expressing the D_2 receptor alone elicit any current. These results suggest that the voltage-induced shifts in agonist potency are attributable to a mechanism residing within the D2 receptor which likely bears relevance to its function in gating of synaptic input and regulating plasticity.

Similar experiments with histamine receptors suggest not only that these receptors are also sensitive to voltage, but that voltage-sensitivity can be agonist-dependent. The histamine H_4 receptor concentration-response relationship for histamine shows a rightward shift upon depolarization, whereas the potency of R-α-methylhistamine (a low-affinity agonist at the H_4 receptor) is not affected by transmembrane voltage. The histamine H_3 receptor exhibits voltage-sensitivity for both (R)-α-methylhistamine (a high-affinity agonist at the H_3 receptor) and histamine. These ligand-selective effects could have important implications for the actions of drugs targeting G protein-coupled receptors and for understanding the functional basis of high- and low-affinity states.

2957-Pos Deconstructing Ras/SOS Signaling Using Model Membranes

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Board B260

The protein-protein interactions between Ras, a signaling GTPase, and SOS, its activator, are critically important in determining cell fate across many species. We have used model membrane systems to quantitatively characterize Ras/SOS interactions in an environment similar to the inner leaflet of the plasma membrane, both with supported lipid bilayers and small vesicles. The recapitulation of this system uses a synthetic linking strategy to attach Ras to the membrane. Conversely, SOS is actively recruited to the membrane via two Ras binding sites, one catalytic and one allosteric. These investigations have uncovered multiple layers of SOS regulation linked to the presence and competence of the allosteric Ras binding pocket. The layers of regulation require that the protein-protein interactions be localized to a membrane, and are themselves a function of protein construct, protein surface density, and membrane composition. These model membrane systems offer a useful way to interrogate important signaling interactions and reveal effects that standard solution-based assays are unable to probe. New work using the supported membrane configuration to observe SOS-catalyzed nucleotide exchange on a single molecule basis will be discussed.

2958-Pos Measurement of Molecule Recruitment by Ratiometric Fluorescence Imaging Shows Coordination of Signaling during Phagocytosis

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Board B261

Fcγ receptors (FcγR) are the most extensively studied of the various receptors responsible for phagocytosis. The zipper model of FcγR-mediated phagocytosis states that the ligands and receptors interact in an ordered progression as the phagocytic cup advance over the particle, with each receptor generating downstream signals autonomously. However, recent studies suggest that receptors may not work autonomously; rather, signaling during phagocytosis may be coordinated over the forming phagosome. To test whether signaling for phagocytosis is a linear function of the stimulus, we measured the relationship between the number of ligated receptors and the magnitude of downstream signals. We developed a ratiometric fluorescence imaging method to measure the quantity of signaling molecules recruited to forming phagosomes in macrophages. Time-lapse widefield fluorescence images of cells expressing both yellow fluorescent protein (YFP) chimeras of signal molecules and cyan fluorescent protein (CFP) were taken. We generated a 'path length' image, which was the product of CFP image times the average ratio of YFP intensity to CFP intensity. The intensity difference between the actual YFP image and the calculated 'path length' image is proportional to the magnitude of signal molecule recruitment. Using this method, we measured recruitment of Syk-YFP, a kinase that binds to activated receptors, and YFP-Tapp1PH domain, which detects PI(3,4)P2 production, on phagosomes ingesting beads with different ligand (IgG) densities. Beads with high ligand density recruited more Syk-YFP during phagocytosis than beads with low ligand density. In contrast, YFP-Tapp1PH domain was recruited to a similar magnitude to beads of high and low ligand density. This indicates that early stages of signaling during phagocytosis are linearly related to ligand density and later stages of lipid signaling are nonlinear.

2959-Pos Molecular Bases of Neuroimmune Interaction in a Coculture Approach Observed with Confocal Laser Scanning Microscopy

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Board B262

During last two decades, there has been exponential increase in data illustrating that the nervous and immune systems are not disparate entities. The nervous system including the brain and the peripheral neurons can stimulate or inhibit activities of the innate and adaptive

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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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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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.