labeling of an azido side-chain in an engineered G protein-coupled receptor (GPCR) with the trifunctional reagent. A sequential affinity purification utilizing the epitope-tagged receptor and the handle on the trifunctional reagent allows removal of excess label and unlabeled receptor, respectively. We will show single-molecule fluorescence images of the receptors captured by immobilized avidin.

1407-Pos Board B317

Polymer-Supported Membranes for Probing Transmembrane Protein Diffusion and Interaction by Single-Molecule Techniques

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Polymer-supported membranes (PSM) are valuable models for cell membranes, since they lack the complexity of a cellular system and confine all membrane components in a two-dimensional space that can be investigated by a multitude of surface-sensitive spectroscopic techniques. However, functional reconstitution of transmembrane proteins into a PSM that show lateral diffusion in the membrane is challenging. Here, we present an approach for efficiently incorporating proteins into tethered PSMs. For this purpose, we have employed a hydrophilic, inert polymer cushion (polyethylene glycol) as a spacer between a glass surface and the membrane. The polymer was functionalized with lipophilic anchors for capturing of proteoliposomes to the surface. Incubation with a polyethylene glycol solution induced fusion of the bound vesicles into an extended bilayer. Efficient incorporation of single-spanning transmembrane proteins into these PSMs was achieved by their reconstitution into very small lipid vesicles, which were readily fused on the surface. Mobility of proteins and lipids in the membrane was demonstrated by fluorescence recovery after photobleaching that showed a mobile fraction of ~80 % for transmembrane proteins and full mobility of lipids. Single molecule tracking experiments confirmed free diffusion of lipids and reconstituted membrane proteins. For the transmembrane proteins, we obtained diffusion constants of ~0.6-0.8 $\mu m^2/s$ that are 5-fold lower than those obtained for lipids. Application of the system for functional analysis was shown by measuring the interaction of the transmembrane receptor IFNAR2 reconstituted into PSMs with its soluble ligand interferon-α2 by ensemble binding experiments and single-molecule co-diffusion analysis. Thus, we have established a versatile experimental platform to study mobility and interactions of transmembrane proteins in a defined membrane environment by ensemble and single-molecule techniques.

1408-Pos Board B318

Fluorescence Correlation Spectroscopic Examination of Insulin and IGF1 Binding to Receptors on 2H3 Rat Basophilic Leukemia Cells

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The insulin receptor (IR) and insulin-like growth factor 1 receptor (IGF1R) are tyrosine kinase receptors with significant structural similarity. Additionally, both IR and IGF1R have pleotropic but differential effects on cell growth and energy metabolism. The interactions of insulin and insulin-like growth factor (IGF1) with IR and IGF1R are well-characterized *in vitro.* Each ligand binds both classes of receptors with different apparent affinities. However, the kinetics of binding of these ligands at physiological concentrations *in vivo* remain elusive. We have examined, using fluorescence correlation spectroscopy, the binding of insulin as well as IGF1 to both IR and IGF1R on individual live 2H3 rat basophilic leukemia (RBL-2H3) cells. Ligand saturation experiments with fluorescein isothiocyanate-conjugated insulin (FITC-insulin) indicated the presence of two classes of low-capacity binding sites with surface densities of 49 sites/μm^2 and 110 sites/μm^2 and K_D's of 106pM and 75nM, respectively. Pre-labeling of cells with IGF1 showed that 81 sites/μm^2 of the FITC-insulin binding sites bind IGF1 with K_D of 82pM. Competitive binding experiments indicated that FITC-insulin and insulin bind with similar K_D of 106pM and 181pM and that IGF1 binds high insulin-affinity receptors with K_D of 22nM. These experiments also indicate that the relative affinities of insulin and IGF1 for these receptors remained constant when probed with 100pM to10nM FITC-insulin. Finally, we evaluated the off-rates (K_{off}) of IGF1 and FITC-insulin as $0.013min^{-1}$ and $0.016min^{-1}$ respectively. The on-rates K_{on} of insulin and IGF1 for both IR and IGF1R were then estimated using values determined for K_D and K_{off}. This project was supported in part by the NIH (RR023156), NSF (CHE-0628260) and American Heart Association (AHA0650081Z).

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Fluorogen Activating Peptides for Single Particle Tracking and Single Molecule Localization-Based Superresolution of FcRI Subunits

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Fluorogen activating peptides (FAPs) are genetically expressible tags that increase the fluorescence excitation cross-section of dye binding partners by up to four orders of magnitude. The binding of FAPs with corresponding fluorogen is characterized by nanomolar affinity, with bound lifetimes of up to ten seconds. The resulting long observation lifetimes for single peptides make the FAP system a convenient, expressible probe for single particle tracking on live cells. In addition, single FAP peptides repeatedly bind and activate new dye molecules. This fluorescence intermittency due to the equilibrium of dye binding and unbinding can be used for localization-based superresolution.

We apply this technology to study the localization and dynamics of the high affinity IgE receptor, FcRI, the primary multi-subunit (, , $_2$) receptor on mast cells and basophils. We have previously characterized the dynamics of the FcRI by tracking of quantum dot (QD)-labeled IgE bound to FcRI. To compare the behavior of the - and -subunits, we have generated an FAP-tagged FcRI -subunit that is expressed on the cell surface of rat basophilic leukemia cells and binds an exogenous fluorogen based on the malachite green dye. We use this probe in conjunction with Atto546 labeled IgE for two-color single particle tracking, to observe the relative spatial and temporal dynamics of the - and -subunits. We observe similar mobility for the two subunits in the resting state, and co-clustering and immobilization upon crosslinking of IgE. We also demonstrate the use of FAP fusion proteins for superresolution imaging to determine proximity of - and -subunits in the resting and activated states.

1410-Pos Board B320

Imaging Redistribution Dynamics of IgE Receptors and Signaling Partners at the Nanoscale with Storm

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In mast cells, crosslinking of IgE bound to the FcϵR1 receptor with multivalent antigen initiates cell activation and consequent inflammatory responses. Clustered IgE-FcϵR1 interacts with early signaling partners such as Lyn, Syk, and LAT, initiating a signaling cascade. Our previous scanning electron microscopy (SEM) studies quantified the nanoscale co-redistribution of FcRI with Lyn, Syk, and LAT within the plasma membrane upon antigen stimulation. In this study, we use stochastic optical reconstruction microscopy (STORM) to localize proteins on the cell surface with sub-diffraction (~20nm half width) resolution using a simple fluorescence microscope set up with TIRF illumination. We present, in snapshots, the time-dependence of FcRI reorganization after antigen addition in chemically fixed cells and quantify the extent of clustering using the pair autocorrelation function. The results of STORM measurements agree well with conclusions from SEM experiments. With multi-color STORM, we image the distributions of multiple protein species on the same cell and calculate the degree of protein co-clustering, before and after antigen stimulation, using the pair cross-correlation function. Ongoing experiments are aimed at quantifying the co-redistribution of FcRI with early signaling partners. We have also extended this technique to imaging living cells at room temperature to measure FcϵRI redistribution in real time and study the organization and dynamics of proteins undergoing stimulated responses. Our results confirm that STORM is an effective tool for quantitative imaging of cellular components at the nanoscale, and our ongoing studies are providing new information to clarify the physical basis for spatial assembly of specific proteins in the plasma membrane during early signaling events.

1411-Pos Board B321

Urocortin II Causes Phosphorylation of eNOS and Stimulation of NO Production in Cardiac Myocytes

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AIM: Urocortin II (UcnII) exerts beneficial effects in heart failure. In cardiac myocytes, UcnII exerts positive inotropic and lusitropic effects through a PKA-dependent pathway. We tested the hypothesis that, in addition, UcnII stimulates endothelial NO synthase (eNOS) and evaluated the underlying signaling pathways and mechanisms.

METHODS: UcnII-induced phosphorylation of Akt and eNOS was measured using phospho-specific antibodies. Isolated cardiac myocytes were loaded with 5x10-6M DAF-FM and UcnII-induced changes in NO production were assessed by changes in DAF-FM fluorescence in electrically paced myocytes (0.5 Hz, room temperature) by means of confocal microscopy.

RESULTS: In rabbit ventricular myocytes, UcnII caused increases in phosphorylation of Akt at Ser473 ($+89.4 \pm 21.4\%$) and Thr308 ($+60.4 \pm 39.7\%$) and phosphorylation of eNOS at Ser1177 ($+49.6 \pm 25.9\%$; n=6-11, all $P<0.05$ vs untreated controls). Wortmannin (300nM) and LY294002