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Dual-Color Fluorescent Labeling of G Protein-Coupled Receptors
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Single-molecule FRET (smFRET) has become a powerful tool for biophysical and biological research. Currently, progress is limited by the absence of general methods to incorporate two different fluorophores at specific sites in proteins. Thus it would be of great value to develop a labeling scheme that enables two different fluorophores to be conjugated to one receptor (protein) in a well-defined manner. To prepare the dual-color labeled receptor two requirements need to be satisfied: 1) the generation of two different reactive handles at specific positions in the receptor and 2) the appropriate bioorthogonal labeling chemistries to distinguish between them. Here we show that the combination of cysteine/maleimide reaction and azido/alkyne cycloaddition ('click' chemistry) meets these conditions. Maleimide chemistry specifically targets at the thiol group of cysteine, which can be easily generated or removed by mutagenesis. In contrast, the click chemistry requires the azido or alkynyl functionality, which are not naturally present in proteins. We utilized the amber codon suppression technique to incorporate unnatural amino acids carrying these reactive groups. The first fluorophore is then attached using a maleimide handle and the second by a click reaction. In a series of proof-of-concept experiments utilizing the wellcharacterized visual photoreceptor rhodopsin, we have successfully demonstrated site-specific double labeling of a GPCR. Moreover, we have optimized the maleimide labeling protocol for rhodopsin and are conducting a comparative study on the copper-catalyzed click chemistry and copper-free click chemistry. We will discuss the results in terms of background level, material cost, and labeling stoichiometry. We envision a series of interesting applications for the dual-color labeled receptors, e.g., to probe the conformational change involved in GPCR activation. We propose to generalize this approach to other proteins, thereby permiting study of GPCR signalosome.

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The Potential Role of (-)-Epigalocathine-3-Gallate in Protecting Cardiac Injury from Oxidation Stress through Lipid Rafts
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Emerging evidence indicates that green tea consumption is inversely linked to cardiovascular diseases. The present study was planned to determine the potential mechanism for cardioprotection of (-)-epigalocathine-3-gallate (EGCg) on hydrogen peroxide (H_2O_2)-induced oxidative stress in H9c2 rat cardiac myoblasts. H9c2 cells exposing to H_2O_2 suppress cell viability, while cells with EGCg pretreatment for 30 min prior to oxidative stress effectively improve cardiac cell survival. Measurement of intracellular reactive oxygen species (ROS) formation by dichlorofluorescein diacetate fluorescence showed that EGCg prevented ROS formation in H_2O_2-treated cells. Measuring fura-2 F340/F380 fluorescence ratio also indicated that EGCg could attenuate cytosolic Ca^{2+} overload in H_2O_2-treated cells. To identify the putative mechanisms underlying the EGCg signaling pathways, EGFP (enhanced green fluorescence protein) was ectopically expressed in H9c2 cells. This allowed us to monitor the fluorescence changes as a means to distinguish the effects of Triton X-100 dependent and independent compartments on the cell membrane. Results obtained indicated the involvement of Triton X-100 insoluble fraction on plasma membrane in transmission of the EGCg signals to mediate cardiac protection against oxidative stress. In addition, the cardiac proteomics study using a two-dimensional polyacrylamide gel electrophoresis was performed to identify the potential proteins for the EGCg-mediated cardioprotection. The adhesion molecules of β-catenin and N-cadherin, gap junction protein connexin 43, and glycogen synthase kinase are implicated in the end effectors involving cardiac protection from oxidative stress. Accordingly, information provided in the present study might aid to pharmaceutical invention on myocardial ischemic disorder.

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Subproteome of Thromboxane A2 Receptor in Transfected HEK293T Cells
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The thromboxane A2 receptor (TP) densities are significantly increased in atherosclerotic coronary arteries and aortae. A potential reason for the elevated TP protein levels in the diseased vasculature could be the rise in TP protein synthesis/traffic. To uncover TP synthesis/traffic mechanisms, we analyzed its subproteome in transfected HEK293T cells. TP immunoprecipitates were separated by 4-20% SDS-PAGE and bands at 37, 65 and 110 kDa were excised and trypsin digested. LC/MS/MS identified a group of endoplasmic reticulum (ER)-membrane spanning proteins in complex with TP in transfected cells (n=3) but not in non-transfected cells (n=3). As positive control, TP peptides were found in the 37 and 65 kDa bands that correspond to the size of its monomeric and dimeric forms. TP partners were: sarcoplasmic/ER calcium ATPase 2 (ATP2A2), phosphatidylinositide phosphatase SAC1 (SAC1), Dolichyl-diphosphooligosaccharide-protein glycosyltransferase subunit STT3A (STT3A) and Dolichyl-diphosphooligosaccharide-protein glycosyltransferase subunit 2 (RPN2). Twenty peptides of ATP2A2, 6 peptides of SAC1, 4 peptides of STT3A and 6 peptides of RPN2 were identified. STT3A and RPN2 are components of the oligosaccharyltransferase complex, which catalyzes co-translational N-glycosylation and mediates protein translocation across the ER membrane. N-glycosylation of either Asn^4 or Asn^{16} is required for TP expression predicting that STT3A and RPN2 might be involved in TP N-glycosylation. The dynamic localization of SAC1 in ER and the Golgi apparatus regulates protein secretion from the Golgi apparatus in response to proliferating signals. SAC1 might aid TP secretion and trafficking to the plasma membrane of cells undergoing proliferation. Since intracellular Ca^{2+} can regulate protein traffic it is possible that TP-ATP2A2 association serves as a feedback mechanism for TP expression. In summary, LC/MS/MS analysis identified ER membrane-spanning proteins that form macromolecular complexes with TP, which may be involved in the receptor increased synthesis/traffic in diseased/proliferating vasculature. Supported by NIH.

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Modular Mechanism of Wnt Signalling Inhibition by Wnt Inhibitory Factor 1
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Wnt morphogens control embryonic development and adult tissue homeostasis. In vertebrates the N-terminal WIF domain ($WIF\text{-}1_{WD}$) of six-domain Wnt inhibitory factor 1 (WIF-1) binds Wnts and inhibits signal transduction. Our human $WIF\text{-}1_{WD}$ crystal structure reveals a novel binding site for phospholipid; two acyl chains extend deep into the domain while the head group is surface exposed. Biophysical and cellular assays, combined with structure-guided mutagenesis, indicate a $WIF\text{-}1_{WD}$ Wnt-binding surface proximal to the lipid head group, but also implicate the five epidermal growth factor (EGF)-like domains (EGFs I-V) in Wnt binding. The crystal structure of six-domain WIF-1 reveals EGFs I-V wrapped-back to interface with $WIF\text{-}1_{WD}$ at EGF III. Binding studies locate a heparan sulfate proteoglycan (HSPG)-binding site in EGFs II-V, consistent with highly conserved positively charged residues on EGF IV. This combination of HSPG- and Wnt-binding properties suggests a modular model for WIF-1 localization, and signal inhibition, within morphogen gradients.

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Prolonged Stochastic Resonance in Single Ion Channel Recordings
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Stochastic resonance refers to the improved signal transduction through a system due to the addition of noise to that system. By *prolonged* stochastic resonance, we refer to the continued enhancement of signal transduction beyond the typical stochastic resonance maximum with increasing input noise. We present evidence of this phenomenon from single Alamethicin ion channels. Identification of the single channel in the presence of voltage noise was accomplished by extracting the conductance of the system by simultaneous voltage and current recording. We determined that, when applying low levels of voltage noise, no more than a single ion channel conducted at any given time. Further, we provide evidence that the prolongation of stochastic resonance in this system is due to the mechanotransductive effects of Alamethicin ion channels. It is well known that Alamethicin channels respond to mechanical tension in the lipid bilayer [1,2]. By extracting the capacitance of the system (again, via simultaneous voltage and current recordings), we record changes in membrane area with respect to applied voltage noise. We find that increasing voltage noise yields larger capacitances due to expansion of the lipid bilayer via the converse flexoelectric effect [3]. This areal expansion induces tension on the ion channel, increasing its conductance and enhancing signal transduction through the