and a putative model for the transition state is proposed. This information can now be used to rationalize the energetic and conformational effects of oncogenic mutations and the binding of antibodies.

1504-Pos

Oligomerization of Membrane Receptors: FRET Analysis Using Coiled-Coil Tag-Probe Labeling and Spectral Imaging

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Oligomerization of membrane receptors in living cell membranes plays an important role in regulation of receptor activity and trafficking. Förster resonance energy transfer (FRET) techniques are often employed to detect receptor oligomerization using receptors fused with fluorescent/luminescent proteins. However, the large size of fluorescent proteins often interferes localization and function of target receptors. Furthermore, controlling the labeling ratio (donor/acceptor), which is important for analysis of FRET, is usually difficult when two fusion receptors with different colors are co-expressed. Posttranslational labeling methods using a short tag peptide and a fluorescent probe that specifically binds to the tag enable a smaller size of label and easy control of labeling ratio in multicolor labeling [1,2]. We recently developed a quick (< 1 min) and surface-specific tag-probe method using a high-affinity heterodimeric coiled-coil formation between the E3 tag (EIAALEK)3 attached to the target receptor and the Kn probes (KIAALKE)n (n = 3 or 4) labeled with a fluorophore [3]. Here we examine oligomerization of the metabotropic glutamate receptor (mGluR) using this novel technique. The receptors were labeled with Rhodamine Green (donor) or Tetramethylrhodamine (acceptor) fluorophores. A constant FRET signal was observed for mGluRs transiently expressed in CHO cells, indicating constitutive oligomer formation. Spectral imaging and demixing of emission spectra abolish crosstalk between channels that is inevitable in conventional filter detection therefore simplify quantification of FRET efficiency from sensitized acceptor emission, allowing analysis of stoichiometry of the oligomerization.

[1] Murel et al. Nat. Mathods (2008) 5, 561-567.
[2] Meyer et al. PNAS (2006) 103, 2138-2143.
[3] Yano et al. ACS Chem. Biol. (2008) 3, 341-345

1505-Pos

How does the State of Aggregation of Rhodopsin in Retinal Discs Influence the Variability of its Activated Life Time?

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Single photon responses (SPRs) in the retinal rod are less variable than expected assuming that deactivation of the receptor rhodopsin (R*) occurs in a single memoryless step. It has been suggested that SPRs reproducibility can be explained by a sequential increase of affinity of R* to the protein involved in its deactivation arrestin (Arr) which leads to a reduction in the life-time variability of R*. This increase in affinity is promoted by a serial phosphorylation of rhodopsin catalyzed by rhodopsin- kinase (RK).

This deactivation mechanism has been tested successfully by means of stochastic simulation assuming rapid diffusion of all signaling molecules. However, evidence suggests that, in native rod discs, rhodopsin is found forming dimers organized in paracrystalline arrays covering about half of the membrane surface.

In this work, we test the hypothesis that packing induced crowding effects, in conjunction with the competitive interactions between R* an the other proteins involved in the signaling cascade (G protein (G), RK, and Arr) will influence the variability of the half-time of R*. In particular, we explore whether the local decrease of inactivated G around R* (as it becomes activated by R*) facilitates interactions of the receptor with Arr and RK, increasing the probability of R* deactivation. This would then lead to a reduction in its trial to trial variation.

In order to explore these issues, we implement a mesoscopic Monte Carlo simulation in a two-dimensional grid representing the membrane, and follow the stochastic encounters and reactions between the species involved in the signal cascade. We perform the simulations and present data on the variability of the half life of R* under two scenarios: rapid diffusion of all proteins, and immobile paracrystalline arrays of rhodopsin.

1506-Pos

Comparative Interaction of Tricyclic Antidepressants and Mecamlyamine with the Human α4β2 Nicotinic Acetylcholine Receptor

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We compared the interaction of tricyclic antidepressants (TCAs) with that for the noncompetitive antagonist mecamylamine with the human (h) α4β2 nicotinic acetylcholine receptor (AChR) in different conformational states, by using functional and structural methods. The results established that: **(a)** TCAsinhibit (±)-epibatidine-induced Ca^{2+} influx in HEK293-hα4β2 cells with potencies that are in the same concentration range (IC_{50} = 2.2-6.8 μM) as that for mecamylamine ($IC_{50} = 3.0 \pm 0.7$ μM), **(b)** [^{3}H]imipramine binds to a single binding site located in the hα4β2 AChR ion channel with relatively high affinity ($K_d = 0.83 \pm 0.08$ μM), **(c)** TCAsinhibit [^{3}H]imipramine binding to hα4β2 AChRs with affinities (K_i = 1.0-2.1 μM) higher than that for mecamylamine ($K_i = 143 \pm 31$ μM), **(d)** imipramine and mecamylamine do not differentiate between desensitized and resting AChRs, **(e)** imipramine interacts with the desensitized AChR mainly by an entropy-driven process, whereas the interactions with the resting AChR are mediated by a combination of enthalpic and entropic components, and **(f)** neutral imipramine and mecamylamine interact with a domain formed between the leucine (position 9') and valine (position 13') rings by van der Waals contacts, whereas protonated mecamylamine interacts electrostatically with the outer ring (position 20'). Our data indicate that although TCAs interact with a binding domain located between the leucine and valine rings, and mecamylamine predominantly acts at the outer ring and by intercalating between two M2 segments, both drugs may efficiently inhibit the ion channel.

1507-Pos

Common Dynamic Behavior of Inactive G-Protein Coupled Receptor Structures for Diffusible Ligands

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The crystal structures of three different family A G-protein coupled receptors (GPCRs) for diffusible ligands, i.e., engineered forms of human β2-adrenergic (B2AR) and adenosine A2A receptors (A2AR), as well as a turkey β1-adrenergic receptor (B1AR) mutant, have recently become available in the literature. Although the overall helical-bundle topology is conserved among these three inactive GPCR structures, several differences emerge from their comparison, particularly at TM1, the extracellular region, the cytoplasmic side of helices TM5-TM7, the ligand-binding pocket, and the long loop regions. Although one cannot exclude the possibility that crystallographic artifacts may be causing some of these structural differences, it remains to be addressed whether these different GPCR structures would share a common dynamic behavior during molecular dynamics simulations in an explicit lipid-cholesterol-water environment. The results of nanosecond-scale simulations of ligand-free inactive crystallographic forms of B2AR, B1AR, and A2AR were analyzed in terms of inter-residue contact variability over time. Contacts that remained in place during most of the simulations were recognized as stable contacts. Among them, the most stable contacts were found to be common among the three GPCR structures, and to involve residues that are conserved among family A GPCRs. We propose that these stable contacts define a common dynamic behavior of inactive GPCR structures for diffusible ligands, and are therefore important for keeping the receptors in an inactive state.

1508-Pos

Protonation Switches in GPCR Activation: Physiologically Significant Rhodopsin Photointermediates

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Rhodopsin is a paradigm for GPCRs, yet unlike other class A members, its bound chromophore's UV/vis absorbance provides excellent time-resolved information about GPCR activation steps. At least three species equilibrate on the millisecond time scale after rhodopsin photoexcitation in a membrane lipid environment. The first equilibrium is pH-independent and involves Meta I_{480}, the visible absorbing, protonated Schiff base (PSB) species, and Meta IIa, the UV absorbing, deprotonated SB species. The second equilibrium, involving spectrally silent proton uptake by Meta IIa to produce Meta IIb, accounts for the fact that low pH causes anomalous disappearance of the protonated SB species, Meta I_{480}. The equilibria affect production of the G protein-activating species R* and are of great interest. However they must be studied promptly because inactivation steps follow, and long illumination increases secondary photolysis of photoproducts. We used time-resolved absorbance measurements of bovine rhodopsin on the microsecond-to-hundred millisecond

time scale to study kinetics of lumirhodopsin decay and the effect of membrane environment on the first equilibrium constant, K_1, and on the pK_a of the second equilibrium. Reconstituted membranes of rhodopsin with POPC, DOPC, or a mixture of DOPC and DOPE were studied at 30°C. We also extended previous 20°C studies of the pH dependence of the equilibria in the native disk membranes, to determine how increased temperature affects lumirhodopsin decay through the purely transient 380 nm absorbing species, Meta I_{380}, into the final equilibrium mixture. Meta I_{380} has recently attracted substantial interest, since time-resolved circular dichroism measurements on the microsecond timescale suggest the chromophore has a different conformation than in later 380-nm photointermediates. Our results suggest SB deprotonation precedes other activating changes in the protein. Significant details are now emerging that give new insights into rhodopsin activation and complement FTIR and spin-label approaches.

1509-Pos

Multi-Scale Dynamics of Rhodopsin Activation as a Paradigm for GPCR Function

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G-protein coupled receptors (GPCRs) are membrane proteins that act as signaling cascade initiators responsible for a myriad of cellular processes. Ligand binding causes GPCR conformational changes that allow the receptor to interact with its cognate G-protein. Several techniques have structurally characterized rhodopsin photointermediates, but none have directly revealed the protein dynamics.Here we report a model whereby ps-ns ligand dynamics are coupled to the μs-ms protein motions during activation. These protein motions represent activated conformational substates on a hierarchy of time scales. Previous models propose that rhodopsin activation is a simple switch whereby retinal isomerizes from an 11-*cis* to an *all-trans* conformer, transforming from an inverse agonist to an agonist. In contrast, our model is motivated by FTIR and UV-visible results showing thermodynamic coupling to several substates in rhodopsin activation (metaI, metaII$_a$, and metaII$_b$) [1]. Furthermore, new ^{2}H NMR data from selectively labeled retinal ligands bound to rhodopsin are able to show that each retinylidene methyl group, especially the C9-methyl, acts as a dynamical hotspot in the activation pathway [2]. Relaxation times are fitted to three-fold jump and continuous diffusion models and correlated to methyl rotation rates in the different rhodopsin activation states, revealing distinct site-specific characteristics for each photointermediate. Recent solid-state NMR [3] and EPR [4] studies showed appreciable protein movements in the photointermediate pathway, further supporting our data. An activation mechanism emerges whereby conformational substates depend on a multivariate energy landscape encompassing retinal and protein dynamics as well as lipid bilayer interactions. [1] M. Mahlingam *et al*. (2008) *PNAS***105**, 17795-17800. [2] M.F. Brown *et al.* (2009) *BBA*, in press. [3] S. Ahuja *et al*. (2009) *J. Biol. Chem.***284**, 10190-10201. [4] C. Altenbach *et al*. (2008) *PNAS***105**, 7439-7444.

1510-Pos

Consequences of Fast, Stochastic Rhodopsin Shutoff for a Model of Phototransduction in Rods

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Rod photoreceptors signal the number and timing of photon absorption, a property that requires that each single photon response be of similar amplitude from trial to trial. How such reproducibility is achieved has been the subject of much experimental and theoretical work, which has demonstrated the importance of multiple steps in rhodopsin deactivation and diffusion of second messengers (Mendez et al., 2000; Bisegna et al., 2008). So far, all previous models have assumed that rhodopsin lifetime is significantly longer than recent measurements indicate (Krispel et al., 2006; Burns and Pugh, 2009). Additionally, recent biochemical studies have provided new details about the dependence of rhodopsin deactivation on phosphorylation level (Vishnivetskiy et al., 2007) that should inform a complete model of light response kinetics and reproducibility. We have implemented a spatio-temporal model of phototransduction in which the rhodopsin deactivation scheme is a stochastic multi-step process lasting no more than 50 ms. The parameters of this model were constrained using an extensive data set obtained from a variety of transgenic mouse lines, each developed to perturb rhodopsin activity, PDE deactivation, or Ca^{2+} feedback. Our simulations demonstrate the relative contributions of stochastic rhodopsin deactivation, Ca^{2+} feedback to guanylate cyclase, and second messenger diffusion to single photon response variability under biologically relevant constraints.

1511-Pos

Functional Structures of Photo-Activated Rhodopsin Disk Membranes Using Single Particle Tracking

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Heterotrimeric G-proteins interact with their G-protein coupled receptors (GPCRs) via key binding elements comprising the receptor-specific C-terminal segment of the alpha-subunit and the lipid anchors at the alpha-subunit N-terminus and the gamma-subunit C-terminus. Direct information about diffusion and interaction of GPCRs and their G-proteins is mandatory for an understanding of the signal transduction mechanism. By using fluorescence microscopy and single particle tracking we showed that the encounters of the alpha-subunit C-terminus with the GPCR rhodopsin change after receptor activation revealing inhomogeneous and restricted diffusion of the receptor (1). To obtain further information about the underlying membrane structure in the signaling state of rhodopsin we now constructed high-resolution transducin visits maps on rhodopsin disk membranes using the inherent information from the single molecule traces.

(1) Kim, T.Y., Uji-i, H., Moeller, M., Muls, B., Hofkens, J. and Alexiev, U. *Biochemistry* 48, 3801-3803(2009)

1512-Pos

Molecular Dynamics Simulations of Active Receptor-G Protein Complex in a Lipid Bilayer

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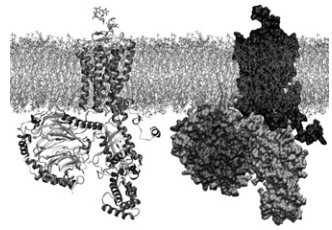

The crystal structure of opsin in its putative active state, the G-protein interacting conformation (Ops*-$G_t\alpha CT_{K341L}$), is arguably the most important breakthrough since the reports of structures for ground-state rhodopsin and the β_2-adrenergic receptor. We use this structure as a template and propose a structural model of the complex with full-length transducin (Ops*-$G_t\alpha\beta\gamma$) based on additional experimental structures including dark-state rhodopsin and holotransducin (Gt, $G_t\alpha\beta\gamma\bullet$GDP). We dock Gt with a reconstructed model of C-terminal α5 helix of $G_t\alpha$ to the open binding site in Ops*. Our model differs from others that propose a requirement for a 40°-tilt of $G_t\alpha\beta\gamma$ relative to the α5 helix in order to avoid steric clashes between $G_t\beta\gamma$ and the membrane. We further report a new method based on grid potentials to embed the complex into a POPC bilayer membrane. Compared with our previous molecular dynamics (MD) studies of the inactive states of rhodopsin[1] and β_2-adrenergic receptor,[2] these new simulations shed light on the role of the protonation state of the opsin residues K296(7.43) and E134(3.49) in stabilizing the receptor-G-protein complex. 1) T.Huber, et al. (2004) Biophys.J. 86:2078-2100. 2) T.Huber, et al. (2008) Biochemistry 47:11013-11023.

1513-Pos

Studying the Diffusion Characteristics of Different Activity States of the Human Adenosine-A3 Receptor Using Fluorescence Correlation Spectroscopy

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The adenosine-A_3 receptor is one of four known G-protein coupled receptors activated by the nucleoside adenosine. Here we use fluorescence correlation spectroscopy (FCS) in conjunction with pharmacological and molecular biology approaches to investigate the diffusion characteristics of different activity states of the human A_3-receptor. Initial FCS experiments using Chinese hamster ovary (CHO) cells expressing the wild type human A_3-receptor and the fluorescent adenosine receptor antagonist XAC-X-BY630 revealed both fast and slow moving complexes at the cell membrane, with average diffusion co-efficients of 1.58 ± 0.16 μm^2/s (τ_{D2}) and 0.081 ± 0.007 μm^2/s (τ_{D3}), respectively. At concentrations of XAC-BY630 ranging from 1-10 nM the amount of τ_{D3}, but not τ_{D2}, increased in a concentration-dependent manner. Pre-incubation of cells with the A_3-receptor specific antagonist MRS1220 at concentrations ranging from 0.3-300 nM significantly reduced the amount of slow moving (τ_{D3}) complexes in a concentration-dependent manner, indicating that they represent receptor bound ligand. Parallel experiments in which CHO cells were transfected with GFP tagged wild-type A_3-receptor (wt), a G-protein uncoupled mutant (W243A, W243F), or a constitutively active