In order to investigate the molecular mechanism by which ligands control the degree of activation of the metabotropic glutamate receptor (mGluR), a class C G-protein Coupled Receptor (GPCR), we monitored the kinetics of the conformational re-orientation that occurs within the extracellular ligand-binding domain, and which has been shown mandatory for G-protein activation. Using single molecule Förster Resonance Energy Transfer, we demonstrate that the receptor continuously oscillates between a resting- and an active- state on a sub-millisecond timescale, and that the role of ligands is exclusively to influence the transition rates between them, in agreement with the conformational selection theory. In addition, this study reveals that the mechanism of action of partial agonists is to finely tune the transition rates between these extreme states, rather than stabilizing a static alternative conformation. Altogether, these results represent a most-valuable contribution to the better understanding of the activation mechanism of mGluRs, and possibly GPCRs in general, and might contribute to facilitate the characterization of new potential drugs.

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Modulation of EGFR Dimer Stability by Manipulation of Phosphorilation in Situ

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Deregulation of the epidermal growth factor receptor (EGFR) signalling has been correlated with the development of a variety of human carcinomas. Receptor dimerization and phosphorylation are amongst the earliest events in signal transduction. Binding of EGF is thought to induce a conformational change which unfolds an ectodomain loop required for dimerization. It may also induce important allosteric changes in the cytoplasmic domain. Despite these findings, ensemble-averaging methods could not resolve the details of receptor activation *in situ.* Here, we used two-color single-molecule imaging to study the effect of ATP-competitive small molecule tyrosine kinase inhibitors (TKI) and phosphatase-based manipulation of EGFR phosphorylation on live cells. Ligand bound receptors were tracked on the plasma membrane with a sophisticated Bayesian segmentation tracking algorithm and the distribution of dimer lifetimes was fitted to a single-exponential to extract dimer off-rates (k_{off}). Our data show that, pre-treatment with type I TKI, gefitinib (active conformation binder) stabilizes the EGFR homodimer. Over-expression of EGFR specific DEP-1 phosphatase was also found to have a stabilizing effect on the homodimer. When a nonactivating anti-EGFR antibody, Snap-425 single-chain variable fragment that competes for EGF binding was used as a ligand, no significant difference in the k_{off} of the dimer could be detected. Conformational changes of the cytoplasmic part of the receptor upon gefitinib pre-treatment were also confirmed by changes in Fluorescence Resonance Energy Transfer (FRET) efficiency detected by ensemble FRET/FLIM (Fluorescence Lifetime Intensity Microscopy). These results provide direct evidence that receptor phosphorylation is linked to dimer stability. The phosphorylated and non-phosphorylated receptors exhibit different kinetics and cytoplasmic conformations which may account for the heterogeneity in ligand-binding affinity observed through a concave-up Scatchard plot.

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Single-Molecule Microscopy Deciphers the Relation between Trafficking and Signaling of the NK1 Receptor in Livings Cells

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Lateral diffusion enables efficient interactions between membrane proteins leading to the transmission of chemical signals within and across the plasma membrane. An open question in this context is how central cell surface receptors are distributed over space and time and thereby influence the transmembrane signaling network. Here we study the mobility of a prototypical G protein coupled receptor, the neurokinin 1 receptor (NK1R) during its different phases of cellular signaling. The investigation of thousands of single NK1R trajectories reveals a very characteristic mobility distribution pattern with two major receptor populations, one showing high mobility and low lateral restriction, the other low mobility and high restriction. The mobility distribution pattern changes in a characteristic manner upon (i) agonist activation, (ii) receptor desensitization, (iii) receptor endocytosis, and (iv) receptor recycling. The study uncovers the functional role of the heterogeneous spatiotemporal distribution of individual GPCRs during the entire cellular signalling cycle.

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Activation of the M2 Muscarinic Receptor and Computer-Aided Design of Receptor-Selective Allosteric Drugs

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G-protein coupled receptors (GPCRs) are important cell signaling membrane proteins that have been targeted by ~30-40% of marketed drugs for treating many human diseases including cancer and heart failure. The X-ray crystal structure of the M2 muscarinic receptor, a key GPCR that regulates human heart rate and contractile forces of cardiomyocytes, was determined in an inactive antagonist-bound state. Through accelerated molecular dynamics (aMD) enhanced sampling simulation, we captured activation of the M2 receptor that occurs on the millisecond timescales(1). The receptor activation is characterized by large-scale structural rearrangements of the transmembrane helices via an intermediate state. With activation-associated conformers of the M2 receptor revealed from aMD simulation, a fragment-based site mapping program FTMAP is applied to explore the receptor surface. Seven allosteric sites are identified with two distributed in the solvent-exposed extracellular and intracellular mouth regions and five in the lipid-exposed pockets formed by transmembrane helices of the receptor(2). Virtual screening is then performed to select small-molecule drugs that bind these allosteric sites with differential affinity and thus stabilize the receptor in the corresponding conformational states. In contrast to highly conserved residues in the orthosteric site where endogenous ligands bind, residues in the allosteric sites exhibit large diversity across different GPCR subtypes. Therefore, ligands that bind GPCR allosteric sites are able to provide potential receptor-selective therapeutics.

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Nature of the M_2 Muscarinic Receptor Signaling Complex Revealed by Dual-Color FCS and FRET

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The mechanism of signaling via G protein-coupled receptors (GPCRs) remains unresolved, and two questions have been the focus of much attention and debate: namely, the oligomeric status of the receptor, and the nature of its interaction with the G protein. Here, we examine those questions with the M_2 muscarinic receptor using a combination of dual-color fluorescence spectroscopy (dcFCS) and Förster resonance energy transfer (FRET). The unambiguous identification of a complex afforded by cross-correlation in dcFCS allows recruitment to be distinguished from conformational effects as the basis of changes in FRET. Both FRET and dcFCS were measured using a spectrally well-separated pair of fluorescent proteins, eGFP and mCherry (mCh), which were fused at the *N*- or the *C*-termini of the receptor and at position 91 of the α_{i1}-subunit of G_{i1}. Insertion of the fluorophore in α_{i1} did not disrupt function, as measured by ligand-induced changes in the fluorescence of Trp 211 in switch region II. Oligomers of the receptor (eGFP-M_2 and mCh-M_2) and of the G protein (eGFP-$\alpha_{i1}\beta_1\gamma_2$ and mCh-$\alpha_{i1}\beta_1\gamma_2$) were identified at the membrane of live CHO cells. In the absence of an agonist, there is neither FRET nor cross-correlation between receptor and G protein (M_2-mCh and eGFP-$\alpha_{i1}\beta_1\gamma_2$). Activation of the receptor by carbachol leads to a rapid increase in FRET and the appearance of cross-correlation, which suggests that G proteins bind only to activated receptors. Cross-correlation also revealed oligomers of the G protein (eGFP-$\alpha_{i1}\beta_1\gamma_2$ and mCh-$\alpha_{i1}\beta_1\gamma_2$) in solution which persisted after the addition of AlF_4^-. Taken together, our data suggest that an agonist promotes the *transient* interaction of an *oligomer* of receptors with an *oligomer* of heterotrimeric G proteins.

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G Protein Activation: A Dynamic Process

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The Gα subunit of heterotirmeric G Proteins is responsible for regulating intracellular signals through guanine nucleotide binding and release, the catalytic rate limiting step. Recent studies have shown interactions of Gα with Guanine