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Recording Kinetics of Adrenergic Receptor Activation in Live Cells

Jean-Pierre Vilardaga

Laboratory for GPCR Biology, Department of Pharmacology and Chemical Biology, University of Pittsburgh, School of Medicine, Pittsburgh, PA, USA

I. OVERVIEW

G protein-coupled receptors (GPCR) biosensors have been recently developed that measure intramolecular conformational changes by recording fluorescence resonance energy transfer (FRET) between fluorescent protein tags introduced at two intracellular sites in the receptor. This technique allows the spatial and temporal recording of agonist (full, partial, and inverse)-induced receptor conformational changes in live cells in real time. This review discusses the kinetics of receptor activation, the direct measurement of ligand efficacy at the level of the receptor, and how ligand efficacy can be modulated by receptor heteromers.

II. INTRODUCTION

Specialized transmembrane proteins known as G protein-coupled receptors (GPCRs) serve as cell surface switches to transmit extracellular signals into cells (Miyawaki & Tsien, 2000; Pierce, Premont, & Lefkowitz, 2002). This large receptor family includes receptors for small neurotransmitters (e.g., adrenaline, noradrenaline, dopamine), and larger peptide hormones (e.g., parathyroid hormone, vasopressin) involved in the function of human body systems as diverse as the endocrine, the skeletal, and the cardiovascular systems among others. As key initiators of a large palette of physiological and psychological functions, these receptors are also involved in many diseases, and are the targets of many approved drugs (e.g., β -blockers). Signal transduction through GPCRs proceed through a succession of biochemical events that take place initially at the cell membrane and involve ligand binding ($L + R \rightarrow LR$, where L and R represents a ligand and a GPCR, respectively), which switch the receptor into an active-state conformation ($LR \rightarrow LR^*$) (Wess, Han, Kim, Jacobson, & Li, 2008). The activated receptor can then interact with heterotrimeric G proteins ($G\alpha\beta\gamma$) to form a transient LR^*G complex, which exhibits higher affinity for the “agonist” ligand than does the initial L-R state. This interaction further promotes a conformational change-induced exchange of GDP for GTP on the $G\alpha$ subunit with concomitant release of the activated GTP-bound $G\alpha$ ($G\alpha$ -GTP) along with the $G\beta\gamma$ subunits from the LR complex. The subsequent activation by $G\alpha$ -GTP of cell membrane-bound effectors such as adenylyl cyclases catalyses the synthesis of second messengers such as cAMP. Signaling responses are rapidly attenuated by receptor desensitization, typically involving receptor phosphorylation of intracellular parts of the receptor (loops and C-tail) by GRKs, which facilitates interaction of β -arrestins to the receptor (Lefkowitz, Hausdorff, & Caron, 1990; Lefkowitz & Shenoy, 2005; Lohse, Benovic, Codina, Caron, & Lefkowitz, 1990; Reiter & Lefkowitz, 2006). This interaction results in the physical uncoupling of G proteins from the

receptor and thus terminates agonist-mediated signaling. Arrestin translocation to the receptor can also recruit cAMP-specific phosphodiesterase 4 (PDE4) at the plasma membrane to rapidly degrade cAMP (Hanyaloglu & von Zastrow, 2008; Perry et al., 2002), and can mediate receptor endocytosis (Hanyaloglu & von Zastrow, 2008). Receptor endocytosis has at least two outcomes. The first is directing the receptor to intracellular compartments, thus contributing to signal desensitization by reducing the number of cell surface receptors. The second is the movement of the receptor to lysosomes for degradation. These processes are thought to stop production of second messengers once receptors are internalized.

Receptors are kept in an inactive state (R) until the binding of a ligand switches them into an active state (R*). The molecular nature of conformational changes associated with β_2 -adrenergic receptor (β_2 -AR) activation was initially observed in purified and reconstituted receptors labeled with fluorescent molecules sensitive to environmental and/or conformational changes (Gether, Lin, & Kobilka, 1995). These changes involve a structural rearrangement of several transmembrane helices, in particular helices 3 and 6 (Sheikh et al., 1999; Sheikh, Zvyaga, Lichtarge, Sakmar, & Bourne, 1996). The importance of the relative motion between helices 3 and 6 associated with receptor activation has also been confirmed for other different GPCRs such as the PTHR (Sheikh et al., 1999; Vilardaga, Frank, Krasel, Dees, Nissenson, & Lohse, 2001). The transition between the inactive and active states is associated with a relative movement and rotation of the cytoplasmic part of helix 3 away from the cytoplasmic part of helix 6 (Farrens, Altenbach, Yang, Hubbell, & Khorana, 1996). This helical movement is triggered by the release of at least two molecular constraints. The first is the disruption of an ionic interaction between the cytoplasmic face of helix 3 and helix 6 (known as ionic lock); the second involves a rotamer toggle switch in helix 6 (modulation of the helix conformation around a conserved proline-kink) (Yao et al., 2006). These transmembrane movements presumably expose receptor epitopes at the cytosolic side, which are then recognized by heterotrimeric G proteins. Direct observations of conformational changes in GPCRs can also be observed in living cells by using an optical technique involving fluorescence resonance energy transfer (FRET) that permits spatial and temporal recordings of the activation/deactivation steps of diverse adrenergic receptors (Lohse, Nikolaev, Hein, Hoffmann, Vilardaga, & Bunemann, 2008; Vilardaga et al., 2009). This review discusses the principle of these FRET experiments and kinetics of receptor activation in response to ligand of diverse efficacies.

III. PRINCIPLE OF THE EXPERIMENT

The experimental system developed for recording GPCR activation mediated by ligand binding relies on an intramolecular FRET-based approach that involves the incorporation of two variants of the green fluorescent protein (GFP) to the intracellular part of the same receptor molecule (Fig. 1) (Hoffmann et al., 2005; Vilardaga, Bunemann, Krasel, Castro, & Lohse, 2003). The cyan (CFP) and the yellow (YFP) variants share a spectral overlap between the CFP's emission and YFP's absorption spectrums, and is a well-documented FRET pair used in live cell-based assays. FRET occurs when the excited CFP molecule transfers nonradiative energy to a YFP molecule in close proximity resulting in decreased CFP emission and increased YFP emission. The efficiency of FRET is very sensitive to the

distance and orientation between CFP and YFP molecules, and falls off with the sixth power of the distance between the two fluorophores. FRET is therefore well suited to measure protein–protein interactions when the two fluorophores are in two separate proteins (intermolecular FRET), or conformational changes when the two fluorophores are inserted in two separate structural domains of the same protein (intramolecular FRET). The YFP/CFP FRET pair can be easily incorporated into proteins by using well-established DNA recombinant techniques and the engineered proteins can be expressed in live cells to monitor high temporal resolution protein–protein interaction, or to quantify kinetics in protein conformational changes (Miyawaki & Tsien, 2000).

FRET-based GPCR biosensors have been engineered by introducing CFP and YFP into domains of the receptor known to be conformationally sensitive. Constructs can be made with either CFP in the third intracellular loop and YFP in the C-terminus or *vice et versa* (Fig. 1). Each receptor construct (referred to as GPCR^{CFP/YFP}) is usually well expressed and functional, albeit G protein coupling may be reduced in some cases.

Experiments are performed with live cells placed under a fluorescence microscope, and selectively excited (light at 440 nm) to induce the CFP, and hence YFP emission fluorescence of the GPCR^{CFP/YFP}. Upon fast addition of an agonist, the YFP signal decreased and simultaneously the CFP signal increased, indicating a decrease in FRET, monitored as the ratio of emission intensities of YFP and CFP fluorescence. This FRET reduction reflects a conformational switch of the receptor that is compatible with receptor activation leading to G protein signaling. This strategy has been successfully applied to the α_{2A} –, β_1 –, and β_2 -adrenergic receptors among other GPCRs (Vilardaga et al., 2009). By exploiting this novel FRET-based approach, several studies addressed critically important questions on the mechanism of adrenergic receptor signaling. What is the kinetics of ligand-mediated receptor activation in live cells? Can receptors adopt multiple active states to induce distinct signaling pathways? How do inverse agonists act on receptors?

IV. RECEPTOR ACTIVATION IN LIVE CELLS

A. Kinetics of Receptor Activation

Kinetics of FRET changes of GPCR^{CFP/YFP} in response to increasing concentrations of agonist revealed that the rate constant (k_{obs}) of receptor activation follows a hyperbolic dependence on ligand concentrations and reaches a maximal value at saturating concentrations of agonist. This is indicative of a two-step process where a fast binding step is followed by a slower conformational change. At low agonist concentrations, the rate constants increase in proportion to agonist concentrations, indicating that agonist binding to the receptor is the rate-limiting step. At saturating concentrations of the agonist, the rate constant reached a limit, indicating that a step other than the binding between agonist–receptor is rate limiting. This limit is compatible with the ability of the agonist to mediate a conformational change of the receptor. The time constant (τ) of α_{2A} –, β_1 –, and β_2 -adrenergic receptor activation is ≈ 50 ms at a saturating concentration of a full agonist (Fig. 2) (Hoffmann et al., 2005; Reiner, Ambrosio, Hoffmann, & Lohse, 2010; Rochais, Vilardaga, Nikolaev, Bunemann, Lohse, & Engelhardt, 2007; Vilardaga et al., 2003; Vilardaga, Steinmeyer, Harms, & Lohse, 2005). This speed is considerably faster than that

recorded for peptide hormone receptors such as the parathyroid hormone receptor ($\tau \approx 1$ s). This difference in the activation time course might depend on intrinsic properties of receptors themselves (family A versus family B GPCRs), and also on the type of ligand and its mode of binding to the receptor (small molecules versus peptides) (Ferrandon et al., 2009).

B. Recording Intrinsic Efficacy of Ligands

Ligands can either stimulate fully (full agonists) or partially (partial agonists), or reduce (inverse agonists) the basal receptor activity. The term intrinsic efficacy was introduced as a fundamental parameter to differentiate and to classify the varying action of ligands when they occupy the same fraction of a single receptor to produce a functional response (Kenakin, 2002a, 2002b). The determination of intrinsic efficacies currently relies on indirect measurements of downstream physiological or biochemical responses (e.g., measurements of second messengers production such as cAMP among others, protein phosphorylation, level of gene expression, or smooth muscle cell relaxation) resulting from receptor activation that are dependent on receptor, G protein, and transducer expression levels. Using FRET-based GPCR biosensors can circumvent these difficulties by directly measuring the ligand-induced change in the receptor conformation itself, and which is independent from variation in either receptor number or cell conditions.

Studies with the α_{2A} -adrenergic receptor biosensor (α_{2A} -AR^{CFP/FlAsH}) expressed in human embryonic cells (HEK293) or neuron-like PC12 cells revealed that full, partial, and inverse agonists of different chemical structures and efficacies produced full, partial, and reverse FRET signals that correspond exactly to their predicted properties (Nikolaev, Hoffmann, Bunemann, Lohse, & Vilardaga, 2006; Vilardaga, Nikolaev, Lorenz, Ferrandon, Zhuang, & Lohse, 2008; Vilardaga et al., 2005). These studies not only revealed that ligands of different efficacies induce receptor's conformational changes of a different nature, magnitude, and kinetics, but also showed a direct correlation between the intrinsic efficacy of ligands and the kinetics of the conformational change in receptors (Figs. 3 and 4): fast conformational changes for full agonists (time constant $t \approx 50$ ms), progressively slower and smaller changes for partial agonists ($t_{1/2} < 1$ s), and even slower changes ($t \approx 1$ s) in the opposite direction with inverse agonists. The direct correlation between the relative agonist efficacy (measured by the extent of G protein activation with a FRET-based Gi biosensor; Bunemann, Frank, & Lohse, 2003) and the corresponding half-time of receptor activation and Gi activation is interpreted as evidence for the stabilization of distinct active states of the receptors, which in turn set the kinetics of Gi activation, as a mechanism to modulate the threshold of the cellular response. Given that an intramolecular FRET assay with GPCR biosensors is not dependent on transducer (G-proteins), effector proteins or receptor expression levels, the amplitude and the kinetics of changes in FRET can directly monitor intrinsic efficacy of compounds acting at a given GPCR.

C. Modulation of Ligand Efficacy by Receptor Polymorphism and Receptor Heteromers

The direct recording of ligand efficacy at the level of the receptor has permitted to associate intrinsic efficacies of β -blockers with the effects of a frequent β_1 -adrenergic receptor (β_1 -AR) polymorphism found in heart failure patients (Rochais et al., 2007). Patients with the

Arg389 in the carboxy terminus tail of the β_1 AR showed a better survival rate upon treatment with the β -blocker bucindolol than those with the Gly389 variant (Bristow, 2000). Other β -blockers currently in clinical use such as metoprolol, bisoprolol, and carvedilol act as inverse agonists of the human β_1 AR, and have demonstrated significant improvement in survival in patients with severe chronic heart failure. However, the association between the genetic heterogeneity of the β_1 AR and the effect of these β -blockers on receptor signaling has been unclear until a FRET study done with the β_1 AR^{CFP/YFP} biosensor revealed that carvedilol differentiated itself from both metoprolol and bisoprolol by a selective and strong inverse agonist effect at the more frequent Arg389-variant (Rochais et al., 2007). This FRET study thus suggests that carvedilol could be a better treatment for patients carrying the Arg389-variant β_1 -AR. The FRET-based GPCR biosensors might thus be a valuable tool to fine-tune a therapy targeted at a receptor genotype.

Ligand efficacy can be modulated not only by point mutations encountered in receptor polymorphism but also by cross-conformational change that propagates from one receptor to another. This fascinating process has been experimentally demonstrated for the α_{2A} -adrenergic receptor (α_{2A} AR), which can form heterodimer (also named heteromer) complexes with the μ -opioid receptor (α_{2A} AR/ μ OR) in neurons and cultured cells (Vilardaga et al., 2008). These receptors act either singly or as heteromer complexes that stimulate common signaling pathways through the inhibitory G_i protein. When α_{2A} AR/ μ OR heteromers are formed, morphine binding to the μ -opioid receptor decreases NE-mediated activation of both G_i -protein signaling and MAP kinases (ERK1/2) phosphorylation. The kinetic of this change is rapid ($t_{1/2} < 400$ ms) and faster than that of G protein activation ($t_{1/2} = 500$ ms). Thus, the conformational cross-talk between α_{2A} AR and μ OR serves as a likely means to prevent over-stimulation of signaling pathways by rapidly adjusting both the intrinsic efficacy (the efficacy of NE is decreased in the morphine bound α_{2A} AR/ μ OR heteromer) and the extent of G protein activation to the two different ligands acting on the receptor heteromer.

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FIGURE 1.

Recording and watching α_2A -adrenergic receptor activation. The *upper panel* represents the principle of FRET experiment with the chemical structure of Flash. The *central panels* show changes in the fluorescence of CFP and Flash (*left*) or CFP and YFP (*right*), and corresponding FRET ratio F_{YFP}/F_{CFP} in response to saturating concentration of norepinephrine (NE, 100 μ M) recorded from a single HEK-293 cell expressing $\alpha_2AAR^{Flash/CFP}$ or $\alpha_2AAR^{YFP/CFP}$. Initial values of relative fluorescence (dark grey traces for CFP, and light grey traces for YFP or Flash) and the FRET ratio were set to one. Horizontal bars represent the duration of ligand application. The *lower panels* show FRET imaging of receptor activation in HEK293 cells transiently transfected with $\alpha_2AAR^{Flash/CFP}$. The *left* panel shows the epifluorescence image and the next two panels present the pseudocolored FRET ratio before and after stimulation by NE.

FIGURE 2.

Kinetics of receptor activation/deactivation measured by FRET. Time constants of PTHR, α_{2A} AR, and β_1 AR activation (measured in HEK293 cells expressing their corresponding FRET-based biosensors as shown in Fig. 1) in response to a saturating concentration of PTH (for PTHR), or norepinephrine (for adrenergic receptors). Deactivation time constants were measured after ligand washout.

FIGURE 3.

Action of ligand of different intrinsic efficacy on a FRET-based α_2AAR biosensor. FRET signals mediated by a saturating concentration of the agonist norepinephrine (NE), the partial agonist clonidine (Clo), and the inverse agonist rauwolscine (Rau). (Adapted from Vilardaga et al., 2005).

FIGURE 4.

Multiple receptor conformations determine distinct kinetics of G-protein activation. *Top*: chemical structures of α_2AAR agonists: norepinephrine (NE), dopamine (DA), octopamine (Oct), norphenylephrine (NF), tyramine (Tyr), m-tyramine (m-Tyr), UK-14,304 (UK), moxonidine (Mox), clonidine (Clo), oxymetazoline (Oxy), and α_2AAR -antagonist phentolamine (PA). *Bottom left*: correlation between the rate constant of receptor activation (mean $\pm$ SE) and respective extent of FRET amplitude seen with different partial agonists (mean $\pm$ S.E.). *Bottom right*: correlation between half-time of G_i protein activation and agonist efficacy (where efficacy is defined as the ability of an agonist to produce a FRET response compared with the maximal response achieved by NE) analyzed by linear regression (red line, $r^2 = 0.95$, $p < 0.0001$). (Adapted from Nikolaev et al., 2006). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this book.)