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Fluorescence/Bioluminescence Resonance Energy Transfer Techniques to Study G-Protein-Coupled Receptor Activation and Signaling[S]

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***Abstract*——Fluorescence and bioluminescence resonance energy transfer (FRET and BRET) techniques allow the sensitive monitoring of distances between two labels at the nanometer scale. Depending on the placement of the labels, this permits the analysis of conformational changes within a single protein (for example of a receptor) or the monitoring of protein-protein interactions (for example, between receptors and G-protein subunits). Over the past decade, numerous such techniques have been developed to monitor the activation and signaling of G-protein-coupled receptors (GPCRs) in both the purified, reconstituted state and in intact cells. These techniques span the entire spectrum from ligand binding to the receptors down to intracellular second messengers. They allow the determination and the visualization of signaling processes with high temporal and spatial resolution. With these techniques, it has been demonstrated that GPCR signals may show spatial and temporal patterning. In particular, evidence has been provided for spatial compartmentalization of GPCRs and their signals in intact cells and for distinct physiological consequences of such spatial patterning. We review here the FRET and BRET technologies that have been developed for G-protein-coupled receptors and their signaling proteins (G-proteins, effectors) and the concepts that result from such experiments.**

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[S] The online version of this article (available at http://pharmrev.aspetjournals.org) contains supplemental material.

I. Introduction

A. G-Protein-Coupled Receptors and Methods of Their Localization

Signaling by G-protein-coupled receptors (GPCRs[1]) is one of the key processes that regulate physiological functions. Although GPCRs seem to share an overall very similar seven-transmembrane helix structure and also similar

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principles of activation, their large number, their specificity for a huge diversity of ligands ranging from photons to large protein hormones and, hence, their immense potential to serve as drug targets make them prime topics of pharmacological research.

Research on GPCRs began with the determination of physiological responses in organ preparations or in intact organisms, combined with the use of various ligands to elicit such responses or to block them. This has led to the definition of receptor families and their subtypes based on distinct concentration-response profiles (Starke, 1981; Black, 1996). A second era of GPCR research began with the elucidation of their biochemical signaling machinery (i.e., the G-protein activation cycle) and the major downstream pathways, such as the generation of cAMP and inositol trisphosphate by various subtypes of adenylyl cyclases and phospholipase C-β isoforms (Hepler and Gilman, 1992; Pierce et al., 2002; Berridge, 2009). These studies relied mostly on biochemical studies involving cell fractionation and protein purification/reconstitution; they also included the cloning of their cDNAs and the identification of several hundred GPCR genes in the human and many other genomes (Fredriksson et al., 2003; Hill, 2006; Milligan and Kostenis, 2006; Lagerström and Schiöth, 2008).

Although these studies beautifully elucidated the interactions of the components of the GPCR signaling machinery, they essentially lacked visualization. Two recent lines of technologies and data have now contributed such visualization data to the field. The first is the elucidation of the structures of many GPCRs, encompassing both inactive and, more recently, active forms of the receptors, but also their downstream G-proteins (Rasmussen et al., 2007; Park et al., 2008; Scheerer et al., 2008; Rosenbaum et al., 2009, 2011; Choe et al., 2011b). The second is the visualization of the receptors themselves as well as their signaling cascades by optical methods; these methods are based on the genetic or chemical labeling of GPCRs and their downstream signaling proteins and allow the monitoring of both their cellular localization and their activity (Kallal and Benovic, 2000; Boute et al., 2002; Lohse et al., 2008b; Balla, 2009). Figure 1A gives an overview of the improvements over the past 25 years of our concepts of the build-up of GPCRs, ranging from the first understanding of their seven-transmembrane helix structures derived from the cloning of their cDNAs to the very recently completed structure of a receptor/G-protein complex (Rasmussen et al., 2011).

Visualization of GPCRs has been a major topic for many years. Apart from cross-functional and radioligand binding data, the earliest tool for GPCR localization was receptor autoradiography. This involved the binding of radioligands to tissue sections, followed by exposure of films or nuclear emulsions (Gehlert et al., 1984; Palacios et al., 1992); the use of radioiodinated ligands enabled the detection of receptors even at low densities (Weber et al., 1988). Immunohistochemical and immunocytochemical localization of GPCRs has, in contrast, been slow to develop, mostly because development of antibodies against these receptors has proved to be very difficult, presumably because of both the lack of immunogenicity and the low expression levels of these proteins (Daly and McGrath, 2003). Therefore, receptor localization studies benefited very significantly from the development of labeling techniques that allowed the introduction of fluorescent moieties into GPCRs. In particular, the discovery and further development of the fluorescent proteins from *Aequoria victoria* (Shaner et al., 2005; Giepmans et al., 2006) has greatly helped the investigation of GPCR localization in isolated cells, in organs, and even in intact animals (Barak et al., 1997b; Kallal and Benovic, 2000; Kieffer and Evans, 2009). Labeling of GPCRs and their downstream signaling proteins with fluorophores not only permitted observation of their localization by microscopy, with the usual resolution limit of light microscopy (i.e., several hundred nanometers), but also, by using two different labels, each attached to a specific site, allowed the study of the interactions of these two labels by resonance energy transfer techniques. These interactions occur at distances <100Å and are very sensitive to distance alterations; hence, they can be used as a "spectroscopic ruler" (Stryer, 1978), which has an optimal sensitivity on the order of the size of these proteins (≈50Å).

This review focuses on the optical techniques that have been developed over the last decade to visualize GPCRs and their signaling machinery and to study their activation and interactions by resonance energy transfer techniques in intact cells. These studies have been made possible by the use of fluorescent proteins and of small molecular dyes that can be used to label GPCRs. Among these studies, we will mostly concentrate on those experiments that are done by fluorescence resonance energy transfer (FRET) or bioluminescence resonance energy transfer (BRET). Such studies were initially described for G-proteins in yeast (Janetopoulos et al., 2001) but subsequently also for GPCRs themselves (Vilardaga et al., 2003) as well as G-proteins (Bünemann et al., 2003) and downstream signaling in mammalian cells.

FRET and/or BRET sensors have meanwhile been developed for essentially all steps in GPCR-mediated signaling. These steps begin with ligand binding to the receptors and end with classic G-protein-dependent signaling or

[1]Abbreviations: AGT, O^6-alkylguanine-DNA alkyltransferase; BRET, bioluminescence resonance energy transfer; CFP, cyan fluorescent protein; FlAsH, fluorescein arsenical hairpin; FLIM, fluorescence lifetime imaging microscopy; FRAP, fluorescence recovery after photobleaching; FRET, fluorescence resonance energy transfer; GFP, green fluorescent protein; GIRK, G protein-coupled inwardly rectifying potassium channel; GPCR, G-protein-coupled receptor; GRK, G-protein-coupled receptor kinase; HEK, human embryonic kidney; ICI 118,551, (±)-1-[2,3-(dihydro-7-methyl-1*H*-inden-4-yl)oxy]-3-[(1-methylethyl)amino]-2-butanol; NF, norphenylephrine; PTH, parathyroid hormone; RGS, regulators of G-protein signaling; Rluc, *Renilla reniformis* luciferase; TR-FRET, time-resolved FRET; TRH, thyrotropin-releasing hormone; W84, *N*,*N'*-bis[3-(1,3-dihydro-5-methyl-1,3-dioxo-2*H*-isoindol-2-yl)propyl]-*N*,*N*,*N'*,*N'*-tetramethyl-1,6-hexanediamminium; YFP, yellow fluorescent protein.

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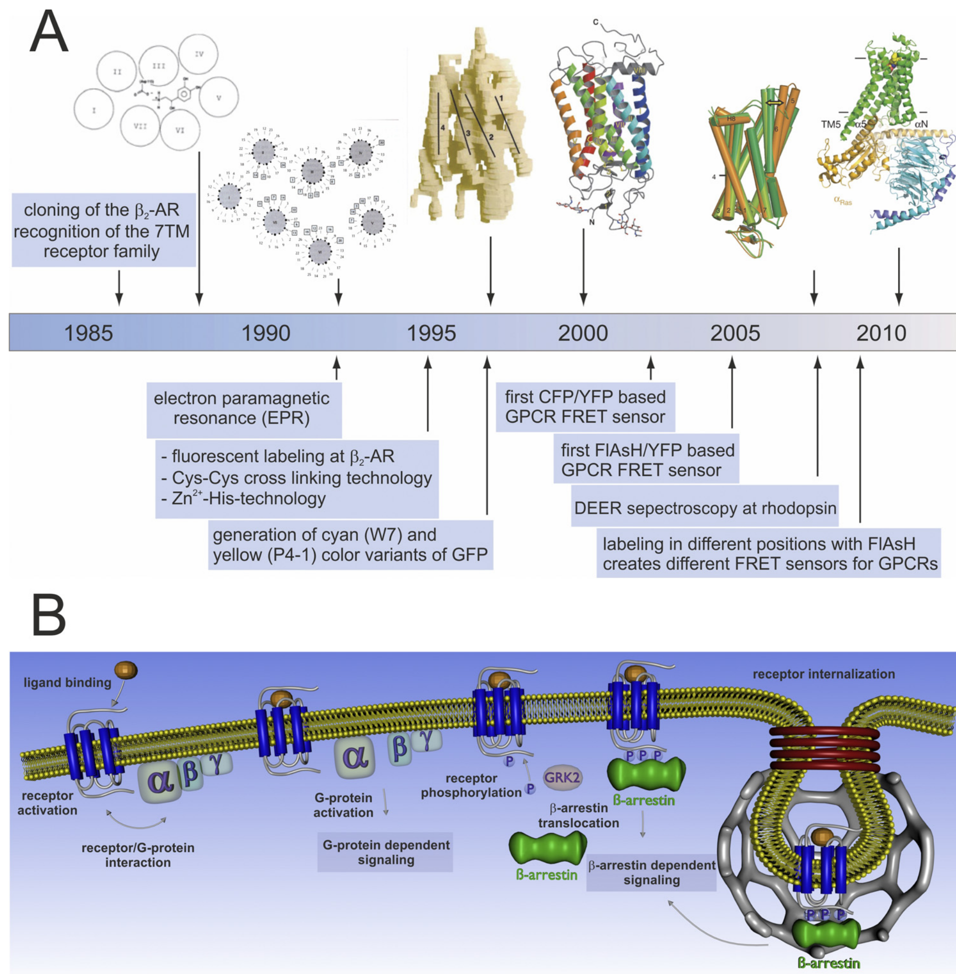

FIG. 1. A, timeline of the key discoveries in GPCR structures. Marked are key structural findings ranging from the appreciation of the seven-transmembrane (7TM) helix structure derived from cDNA cloning in the mid-1980s (Dixon et al., 1986) to the first models of the ligand binding site. [Reproduced from Strader CD, Sigal IS, Candelore MR, Rands E, Hill WS, and Dixon RA (1988) Conserved aspartic acid residues 79 and 113 of the beta-adrenergic receptor have different roles in receptor function. *J Biol Chem* **263:**10267–10271. Copyright © 1988 American Society for Biochemistry and Molecular Biology. Used with permission; and Baldwin JM (1994) Structure and function of receptors coupled to G proteins. *Curr Opin Cell Biol* **6:**180–190. Copyright © 1994 Elsevier. Used with permission.] This was followed by the first proof of the 7TM structural arrangement by the use of two-dimensional electron microscopy on frozen-hydrated two-dimensional frog rhodopsin crystals (Unger et al., 1997). A solid wood model was generated from those data. [Reproduced from Hargrave PA (2001) Rhodopsin structure, function, and topography the Friedenwald lecture. *Invest Ophthalmol Vis Sci* **42:**3–9. Copyright © 2001 Association for Research in Vision and Ophthalmology. Used with permission.] The first X-ray structure of a GPCR was published when the structure of bovine rhodopsin was solved; the figure here from that article illustrates that milestone. [Reproduced from Palczewski K, Kumasaka T, Hori T, Behnke CA, Motoshima H, Fox BA, Le Trong I, Teller DC, Okada T, Stenkamp RE, Yamamoto M, and Miyano M (2000) Crystal structure of rhodopsin: a G protein-coupled receptor. *Science* **289:**739–745. Copyright © 2000 American Association for the Advancement of Science. Used with permission.] The first crystal structure of an active GPCR was solved with the publication of the structure of opsin. [Reproduced from Park JH, Scheerer P, Hofmann KP, Choe HW, and Ernst OP (2008) Crystal structure of the ligand-free G-protein-coupled receptor opsin. *Nature* **454:**183–187. Copyright © 2008 Nature Publishing Group. Used with permission.] The current state of our structural understanding of family A GPCRs is represented by the cocrystal structure of the β_2-adrenergic receptor in complex with a G_s-protein. [Reproduced from Rasmussen SG, DeVree BT, Zou Y, Kruse AC, Chung KY, Kobilka TS, Thian FS, Chae PS, Pardon E, Calinski D, et al. (2011) Crystal structure of the β2 adrenergic receptor-Gs protein complex. *Nature* **477:**549–555. Copyright © 2011 Nature Publishing Group. Used with permission.] Below the time line, time points of crucial methodological and technical developments are marked. All technical developments are discussed in greater detail in section I.B. B, signaling and internalization of GPCRs. Shown are the steps ranging from GPCR activation by agonists to binding of β-arrestins, β-arrestin-dependent nonclassic signaling and internalization.

with nonclassic signaling, which involves receptor phosphorylation of the activated receptors by G-protein-coupled receptor kinases, GRKs, followed by binding of β-arrestins, which then recruit further proteins to trigger both receptor internalization and nonclassic signaling (Fig. 1B). These steps involve either conformational changes within proteins (such as the agonist-dependent activation of receptors) or protein-protein interactions (such as the receptor/G-protein interaction); both of these can be investigated with RET technologies, provided that the labels are inserted at suitable sites in the relevant protein(s) (Lohse et al., 2003b; Pfleger and Eidne, 2005; Marullo and Bouvier, 2007; Hoffmann et al., 2008b; Ciruela et al., 2010).

We describe here the construction and use of the various sensors that can be used to assess these individual steps, and we discuss how these new tools have expanded our knowledge of GPCR-mediated signaling. Finally, we attempt to illustrate how these studies have helped us to

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begin to decipher the spatial and temporal patterns of GPCR-mediated signaling and to realize that these processes are far more versatile than previously thought.

B. Principles and Methods of Resonance Energy Transfer Techniques

This section will briefly cover the basic principles and technologies that are required to follow the discussions in the next paragraphs with respect to conformational changes of proteins and protein-protein interactions along the signaling pathway. Among the techniques to study conformational changes or protein-protein interactions, we will concentrate in those that have been intensively applied in GPCR research. In Fig. 1A, the initial reports for the use of these technologies at GPCRs are marked with arrows along the lime line.

Resonance energy transfer techniques generally require the introduction of labels. Small labels can be attached to reactive amino acid side chains, most frequently of cysteine and lysine (Sletten and Bertozzi, 2009). Fluorescent labels are required for FRET and BRET studies, but labels also include fluorescent proteins, light-emitting enzymes (which are needed for BRET), or alternatively labels for other resonance energy transfer methods, such as electron paramagnetic resonance. Electron paramagnetic resonance techniques have greatly helped to elucidate the structures of GPCRs and will be briefly mentioned here. The first GPCR studied with electron paramagnetic resonance was rhodopsin (Farahbakhsh et al., 1993). Rhodopsin was chemically labeled with small cysteine-reactive probes that allowed selective labeling of individual residues either by depletion through mutagenesis of unnecessary cysteines or by differential reactivity of individual cysteines toward the labeling reagent and was studied in its dodecyl maltoside solubilized state; this preserved its ability to couple to transducin and to be phosphorylated by rhodopsin kinase (Resek et al., 1993; Thurmond et al., 1997). Soon after the initial report, the approach was extended to perform dual labeling experiments in different positions of rhodopsin (Yang et al., 1996). This technique allowed different types of experiments, including site-directed spin-labeling, the measurement of cross-linking kinetics of disulfides, or the measurement of sulfhydryl group reactivity (Hubbell et al., 1998). The chemical labeling procedure can generate covalently linked nitroxide groups that can be used in electron paramagnetic resonance techniques and report on the environment of the nitroxide group or the relative distance of two groups if a second paramagnetic group is present (Hubbell et al., 2003). Because of the design of signal detection, the distance of interactions that could be monitored using the site-directed spin-labeling technique was initially limited to <20 Å and thus compromised the free combinations of labeling positions within rhodopsin to some degree (Altenbach et al., 2001, 2008). This initial limitation has been dramatically improved using double electron-electron resonance technique (DEER; Altenbach et al., 2008). This technique uses a dead-time free measurement of dipole-dipole interactions (Pannier et al., 2000) and thus the recorded data do not contain possible movements of the labels themselves. This improvement extends the distance of measurements to 20 to 60 Å with a 1-Å accuracy (Altenbach et al., 2008).

The simplest case of fluorescent labeling involves a single label that can be studied either by fluorescence quenching (i.e., the loss of fluorescence in certain environments) or in conjunction with naturally occurring acceptors or donors. The first such studies involved, as in the case of rhodopsin, purified receptors and labeling with probes at defined cysteine residues with fluorescent groups that report upon mobility, intensity, or lifetime of the fluorophore attached (Kobilka, 2007). A series of such studies was done with the purified β_2-adrenergic receptor using cysteine reactive labeling groups by the team of Brian Kobilka (Gether et al., 1995; Ghanouni et al., 2001b). Their initial report of the approach (Fig. 1A) used the environmentally sensitive fluorophore *N*,*N′*-dimethyl-*N*(iodoacetoxy)-*N′*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl) ethylene-diamine, which allowed monitoring changes in the local environment of the labeled site when agonists were applied (Gether et al., 1995). Labeling of the receptor with a fluorophore in one position and with a fluorescent quencher in a second position allowed the study of fluorescence quenching by resonance energy transfer and made it possible to monitor the relative movements of receptor domains during the activation process (Ghanouni et al., 2001b). The results and implications of these studies will be discussed in more detail in section III.A.

To avoid the need to purify the protein of interest, a number of techniques have been developed to genetically encode defined labeling sites. A very attractive approach aims to genetically encode additional properties in a protein by using non-natural amino acids. The approach is based on the use of a read-through system for the amber codon (UAG) that is not normally used to encode for a specific amino acid. This codon can be used in combination with the appropriate tRNA to introduce non-natural amino acids at the position of interest and does not require the deletion of other amino acids. Until now, only one publication has used this technique for fluorescence resonance energy transfer studies at a GPCR, the NK2 receptor (Turcatti et al., 1996). The limitations of the technique were due to the difficulties in producing large amounts of chemically aminoacylated suppressor tRNA that needs to be injected into large numbers of cells to produce significant amounts of protein. This was not feasible to be performed on mammalian cells and thus limited the technique to use in oocytes (Ye et al., 2008). This has now been overcome; the technique can be used in modified HEK293 cells (Ye et al., 2008), and a versatile arsenal of modified amino acids can be used (Liu and Schultz, 2010). Hence, this technique has since been applied to rhodopsin (Ye et al., 2009, 2010) but has not yet been used for studies using resonance energy transfer in other GPCRs. However, this technique

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was recently used in combination with double electron-electron resonance spectroscopy to study resonance energy transfer between genetically encoded spin-labels in the T4-lysozyme (Fleissner et al., 2009). Thus, it is probably only a matter of time before this approach is used for other GPCRs as well.

The cloning of the green fluorescent protein (GFP) from the jellyfish *A. victoria* in 1992 (Prasher et al., 1992) opened a new era in fluorescence studies in cell biology. In 1996, different spectral variants of GFP were published (Heim and Tsien, 1996), including the basic variants cyan (CFP) and yellow fluorescent protein (YFP). Their introduction improved the accessibility of FRET approaches and the use of CFP/YFP in combination is still the way most researchers currently perform FRET experiments (Miyawaki, 2011).

FRET is a radiationless energy transfer between two fluorophores that depends on three parameters (see Fig. 2): 1) spectral overlap (the donor emission and the acceptor excitation spectra need to overlap), 2) distance between the fluorophores (FRET generally occurs at <100 Å), and 3) relative orientation of their dipole moments toward each other (highest FRET for parallel dipole orientation) (Förster, 1948; Jares-Erijman and Jovin, 2003, 2006; Miyawaki, 2011). FRET can be measured in several ways

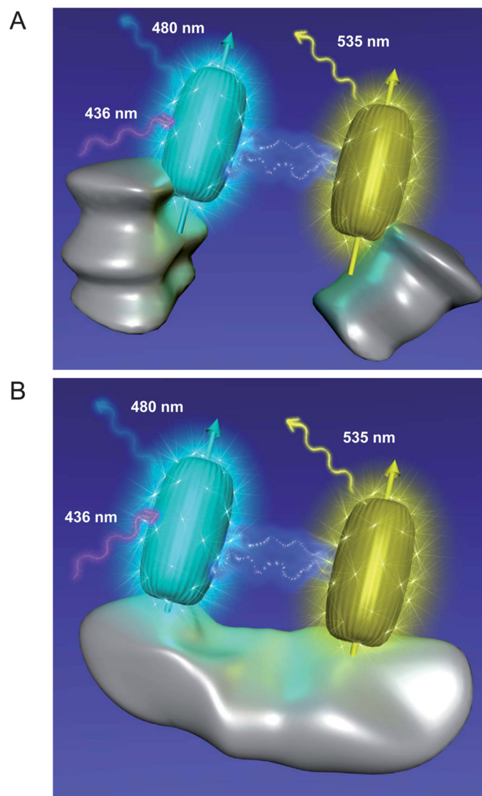

FIG. 2. Principle of inter- (A) and intramolecular (B) FRET. Shown are proteins (gray) labeled with the fluorophores CFP (cyan) and YFP (yellow). If the fluorophores are close to each other (<10 nm), the excited CFP transfers energy to YFP and yellow emission results. A small change in the distance between CFP and YFP may result from the approximation of the two proteins in A or a conformational change of the protein in B and result in marked changes in FRET.

(Jares-Erijman and Jovin, 2003; Miyawaki, 2011). The most frequently used approaches are 1) acceptor photobleaching (emission of donor increases after bleaching of the acceptor with bright light; increase corresponds to FRET efficiency), 2) sensitized emission (acceptor emits when excited by a donor via FRET), or 3) fluorescent lifetime-imaging (the lifetime of the donor fluorescence decays more rapidly after excitation with a pulsed laser; increase in decay speed depends on FRET efficiency). FRET is most commonly measured as intermolecular FRET to study the association of two proteins, each one carrying a label (Fig. 2A). Alternatively, it can be used as intramolecular FRET to study conformational changes in a protein that carries two labels that move relative to each other when the conformation of the protein changes (Fig. 2B). In the second case, equal concentrations of the two labels are automatically assured; in the first case, equal levels should be determined experimentally to avoid artifacts. Particularly when sensitized emission is measured a number of further controls are needed to assure that changes in acceptor emission are indeed due to FRET (and not, for example, fluorescence quenching of either the donor or the acceptor (Jares-Erijman and Jovin, 2003).

In practice, first a suitable pair of donor and acceptor fluorophores with good spectral overlap is chosen and then used to label suitable positions in the protein(s) of interest. Among the fluorescent proteins, CFP/YFP is the most popular pair, along with its improved variants (Miyawaki, 2011). Several variants of the original CFP and YFP have been generated within the last few years and can influence the experiments as well. A recent review compiles an overview of the currently available GFP variants, their advantages and disadvantages, and the corresponding references (Newman et al., 2011). Despite their great usefulness, it is important to keep in mind that these fluorescent proteins consist of 230 amino acids and thus are rather large proteins (27 kDa) that are attached to the protein(s) of interest. Hence, it is important to test that the integrity of the protein under investigation was not altered as a result of the incorporation of a GFP variant (Hoffmann et al., 2005). Another important issue to note is the dimerization tendency of the original fluorescent protein, which is reduced in variants containing an A206K mutation (Shaner et al., 2005).

If FRET is measured by observation of the fluorescence lifetime [fluorescence lifetime imaging microscopy (FLIM)], the decay of the activated state should be monoexponential. This is not the case for the older variants of CFP. However, there are improved variants of CFP, most notably Cerulean (Rizzo et al., 2004), and the most recent variant mTurquoise has especially been optimized for monoexponential decay of fluorescence (Goedhart et al., 2010). FRET measurements by FLIM also have the advantage of being largely independent of the fluorophore expression level (Bastiaens and Squire, 1999; Jares-Erijman and Jovin, 2003) and need only the spectral wavelength of the donor to be monitored. This effect would open other

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parts of the spectrum to monitor a second or third process within the same cell at the same time (Schultz et al., 2005). A disadvantage is the more demanding equipment to measure FLIM. If FRET is measured by sensitized emission, the appropriate controls need to be included. Apart from equal expression (see above), it is necessary to correct for bleed-through of CFP into the YFP channel and direct excitation of YFP at the CFP excitation wavelength to obtain corrected FRET ratios (Berney and Danuser, 2003). Thus, several control constructs need to be generated that contain only one of the two fluorophores.

The relative orientation of the donor absorption and acceptor transition moments are given by the orientation factor κ^2, which can theoretically vary between 0 and 4 based on relative fluorophore orientation (Jares-Erijman and Jovin, 2003). The dipole moments of the fluorophore are often unclear, and a random orientation is assumed. Based on the relative mobility of the labels, the fluorophores may rotate fast in relation to the lifetime of the fluorescent state, and thus an estimated value of 2/3 for the factor κ^2 can be used. If the relative orientation of the dipole moments of the fluorophores is thought to cause problems in the sensor design, circular permuted variants of fluorescent proteins are an alternative (Nagai et al., 2004). These variants have mostly the same spectral properties of the original fluorescent protein, but their dipole moments are different and thus may result in improved FRET sensors (Nagai et al., 2004).

Similar thoughts are true for the choice of the YFP-like protein, where also improved variants have been developed (Griesbeck et al., 2001; Nagai et al., 2002). An alternative to YFP and its variants is the small fluorescein derivative FlAsH (fluorescein arsenical hairpin binder), a compound that binds to a six-amino acid short sequence (CCPGCC). This labeling approach was originally introduced in 1998 (Griffin et al., 1998) and through a series of further optimizations, including the enhancement of the specific binding sequence (Adams et al., 2002; Martin et al., 2005), it was used to replace YFP as acceptor fluorophore in FRET experiments (Hoffmann et al., 2005). FlAsH has a dramatic advantage in size, being only 1/40 the size of GFP (Hoffmann et al., 2005), and fluoresces only upon binding to its target sequence; a disadvantage is that the labeling procedure requires a number of washing steps. This procedure has recently been described in a detailed protocol (Hoffmann et al., 2010). Several color variants of FlAsH have been synthesized (Adams and Tsien, 2008; Soh, 2008) and allow labeling of a given site with different colors (e.g., for pulse-chase experiments) (Gaietta et al., 2002). Because binding motifs with distinct affinity have also been reported, we recently developed an orthogonal labeling strategy that allowed specific labeling of two different proteins (PTH receptor and β-arrestin2) in the same cell with individual colors and to measure their interaction by FRET (Zürn et al., 2010).

Another versatile labeling approach is based on the human DNA repair protein O^6-alkylguanine-DNA alkyltransferase (AGT; Keppler et al., 2003). This enzyme transfers alkyl groups from its substrate O^6-alkylguanine-DNA to one of its own cysteine residues and thereby becomes irreversibly labeled. This enzyme is commercially available (SNAP-tag; New England BioLabs, Ipswich, MA) and has the advantage that it tolerates various modifications at the O^6 position of its substrate guanine. Thus, a wide array of substrates can be used to transfer different groups to the enzyme (Keppler et al., 2006). As in the case of FlAsH, this approach does not require cloning of a novel construct if a different color for the protein is desired. A second generation of the AGT enzyme with different substrate specificity has been generated and is available as CLIP-tag (New England BioLabs) (Gautier et al., 2008). Thus, two different labeling reactions can be performed and allows the study of protein dynamics of individually labeled proteins in living cells (Gautier et al., 2008).

Time-resolved FRET (TR-FRET) is an approach that is based on the use of lanthanides as donors in FRET applications. Strictly speaking it is not a fluorescence approach because it does not involve a singlet-to-singlet transition (Selvin, 2002). The fact that the energy transition is formally forbidden results in a delayed decay of the excited state that occurs on a millisecond timescale (Selvin, 2002). In principle, however, the rules for FRET apply with respect to distance dependence but less so for orientation dependence. Because the lanthanides exhibit a very weak absorption, which is 10^4-fold lower compared with conventional organic fluorophores, they need some kind of organic chromophore as antenna, which transfers the energy to the lanthanide (Bazin et al., 2002). Several chelates or cages are in use and by themselves can contribute to the emission behavior of the probe. The two lanthanides most frequently used are europium (Eu^{3+}) or terbium (Tb^{3+}), whereas the other two possible elements, samarium and dysprosium, emit too weakly (Selvin, 2002). Today, the most frequently used chelates for lanthanides are the cryptates. One advantage of this cage is that it shields the lanthanide from water and thus prevents quenching and broadening of the emission peaks. The second effect of the cryptate is that it leads to a kinetically stable complex that is acid resistant and thus leads to a broad increase in possible applications (Degorce et al., 2009). The interesting advantage of this approach is that the emission occurs with a significant time delay, and thus a delay window is used for recording during which the autofluorescence of the sample has decayed (Bazin et al., 2002). The lanthanides are excited at 340 to 360 nm wavelength in the UV and emit characteristic, almost atom-like spectra at very discrete wavelengths (Degorce et al., 2009). Tb^{3+} and Eu^{3+} can both be combined with red fluorophores, but Tb^{3+} has an additional emission wavelength at 490 nm, which makes it suitable for combinations with green fluorophores. Originally, they were used in combination with a 105-kDa phycobiliprotein called allophycocyanin that was cross-linked for stability and hence called XL665 (Bazin et al., 2002). Acceptor partners are the organic molecule d2

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offered commercially (Degorce et al., 2009) but also Alexa Fluor 647 (Invitrogen, Carlsbad, CA) (Ciruela et al., 2010b). Because the cryptate cage also allows some degree of chemical modifications without emission line broadening, this can be used to introduce functional coupling groups that can be used to transfer the Tb^{3+} label to different target molecules such as GPCRs. At the moment, this is done by combination of the above-mentioned SNAP-tag, in which case the O^6-alkylguanine is coupled to the Tb^{3+}-cryptate complex and can be transferred to the SNAP-tag that is fused to the N terminus of a GPCR (Maurel et al., 2008; Albizu et al., 2010). Despite all improvements of the cage size from originally 105 kDa to now approximately 1 kDa (Degorce et al., 2009), this size often limits the applications to the analysis of extracellular cell surface events (Maurel et al., 2008; Albizu et al., 2010).

As already mentioned above, this approach is less prone to orientation problems as with conventional fluorophores. This is based on two facts. First, terbium has multiple electronic transitions that are degenerate and thus, terbium is an isotropic donor even if not freely rotating (Selvin and Hearst, 1994). Second, because the lanthanides have millisecond lifetimes, both acceptor and donor will probably rotate during this time; thus, the assumption of κ^2 close to 2/3 will be true, and the signal change becomes truly dependent on the distance change between donor and acceptor (Selvin, 2002).

BRET is an alternative to FRET in which a light-emitting enzyme is used instead of the donor. The BRET technology and its application to GPCR research have been reviewed several times in recent years (Hébert et al., 2006; Pfleger and Eidne, 2006), and we thus refer the reader to those reviews for more detailed technical aspects. The basic principles of FRET or BRET are very similar, as one needs a donor and an acceptor for the resonance energy transfer in both cases. In the case of BRET, the major donor class is based on *Renilla reniformis* luciferase (Rluc) or its different variations, whereas the acceptor fluorophore is a variant of GFP (Xu et al., 1999). The light source for the donor in BRET is a chemical substance that is converted by Rluc, and the energy is transferred to GFP, applying the same rules as for FRET. The major advantage is the lack of illumination by a conventional light source, which prevents disturbance of the autofluorescence effects in the sample (Pfleger and Eidne, 2006). Several rounds of optimization of the technique have led to different generations of the BRET system. The originally described system (Xu et al., 1999) is now referred to as $BRET^1$ and uses the conventional enhanced GFP in combination with Rluc; benzyl-coelenterazine (coelenterazine *h*) is used in the oxidative reaction to generate light with an emission peak at 480 nm and its signal allows detection of BRET for up to 1 h. The next generation used a different GFP variant called GFP^2, which emits light like the normal GFP but is excited at ≈400 nm. Hence, the novel GFP^2 variant is characterized by a large Stokes shift; it requires a different compound, called DeepBlueC (commercialized as Coelenterazine 400A; Biotium, Inc., Hayward, CA), for excitation. This system is referred to as $BRET^2$. $BRET^2$ suffers from a much more rapid signal decay and exhibits a >100-fold lower quantum yield compared with coelenterazine *h* (Hamdan et al., 2005). By several rounds of optimizations, Rluc2 and Rluc8, novel improved variants of Rluc, were generated (Loening et al., 2006; De et al., 2007), which are characterized by a significantly improved brightness and quantum yield for $BRET^2$ applications (De et al., 2007). These improvements make $BRET^2$ a very good tool combining high brightness with spectral resolution and good quantum yield. A very systematic study compared the combinations of the different variants of BRET systems and their individual components for the same thyrotropin-releasing hormone (TRH) receptor/β-arrestin-2 interaction as the biological process to be monitored (Kocan et al., 2008). This study achieved an optimized signal for $BRET^2$ that was stable for extended periods (hours). The latest generation of BRET systems is called $BRET^3$ and uses a combination of mOrange and Rluc8, a combination that can use coelenterazine as substrate and that can be used for bioluminescence imaging in living tissue (De et al., 2009). The recent further improvements in BRET systems, called BRET 3.1 to BRET 6.1, combine also red-shifted variants of fluorescent proteins and thus even allow BRET experiments deep in tissues and in living mice (Dragulescu-Andrasi et al., 2011).

Table 1 summarizes the different labeling strategies that have been used in GPCR research. Table 2 gives examples of such sensors for the various steps of the signaling cascade of a GPCR (see Fig. 1B).

II. Ligand Binding to G-Protein-Coupled Receptors

Fluorescent ligands for GPCRs were introduced in the mid-1970s and offer a great potential to study receptors in their native environment (McGrath et al., 1996). They offer high spatial resolution in receptor detection and imaging. Even better resolution can be achieved by using fluorescent ligands in FRET experiments. In contrast to most radioligands, a fluorescently labeled ligand is an individual chemical entity and the affinity and selectivity of the fluorescently labeled ligand need to be determined (Daly and McGrath, 2003). Ideally, the attachment of the fluorescent group does not influence the pharmacology, but this needs to be tested using conventional assays (McGrath and Daly, 2003). Design of fluorescent ligands requires the knowledge of both the ligand and the pharmacophore (Leopoldo et al., 2009; Briddon et al., 2011). The various variables of this interaction have recently been studied in a very systematic way for ligands at the adenosine A_1 receptor (Baker et al., 2010).

Two general types of FRET experiments can be performed using fluorescent ligands for GPCRs. First, one can study the interaction between ligand and receptor as the

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TABLE 1
Labeling techniques for Förster resonance energy transfer studies at GPCRs

Technique	Variant	Advantage	Disadvantage
FRET	CFP/YFP and variants	Genetically encoded fluorescent proteins; labeling possible on intra- and extracellular side.	Relatively large size (27 kDa) and sometimes the XFP represents the same size as the target protein.
	FlAsH/CFP	Small size of FlAsH (~700 Da compared to 27 kDa for YFP); flexible positioning within target protein; less likely to perturb the protein function; similar spectral properties as YFP, thus same filters can be used for FlAsH and YFP; labeling possible on intra- and extracellular side.	Labeling procedure required; background staining, which can be reduced when using the 12-amino acid high-affinity motif for FlAsH; FlAsH is a bidentate ligand and thus may not be able to freely rotate, thus possible changes in fluorophore orientation should be kept in mind.
FRET by sensitized emission			Emission cross-talk, excitation cross-talk, bleed-through.
FRET by FLIM	mTurquoise instead of CFP	Mono-exponential fluorescence decay time.	
Time-resolved FRET (TR-FRET)	Eu^{3+}- and Tb^{3+}-cryptate in combination with fluorescent protein.	Time-resolved recording, thus less background signal from autofluorescence; almost no orientation dependence of the fluorescent signal.	Labeling procedure required; chelating cage needs to be attached to specific label (antibody, substrate for SNAP-tag etc.); labels represent charged compounds, and thus the labeling is mostly restricted to the extracellular side; rather high costs for the labeling reagent compared to genetically encoded fluorescent protein.
SNAP-tag	The SNAP-tag is a nonfluorescent adapter protein that can be used in combination with fluorophores for FRET.	Highly versatile tag that can be combined with several different color fluorophores and hence one cloned protein can be used in several ways without the need to clone different constructs.	Relatively large size (20 kDa) and sometimes the tag represents the same size as the target protein; labeling procedure required, only a very limited number of fluorophores used for labeling the SNAP-tag can cross the plasma membrane, thus mostly extracellular labeling achieved.
CLIP-tag		Modified SNAP-tag with different substrate specificity; can be used for orthogonal labeling.	
$BRET^1$	RLuc and GFP (coelenterazine *h*)	No excitation by external light source required; low background.	Relatively large size (27 kDa) and sometimes the XFP represents the same size as the target protein; chemical substrate for RLuc needs to be added to generate bioluminescent light; some substrates for RLuc are short lived and permit only short time windows to be investigated; currently still rather low light intensity.
$BRET^2$	RLuc, RLuc-2, -8, and GFP^2 (DeepBlueC)		
$BRET^3$	RLuc8 and mOrange (coelenterazine)		

initial step in the signaling cascade (Turcatti et al., 1996; Castro et al., 2005). Second, FRET between two different labeled receptor-bound fluorescent ligands has been used to detect oligomeric GPCR complexes, not only in transfected cells but also in native tissue (Albizu et al., 2010). Turcatti et al. (1996) investigated the interaction between a tetramethyl rhodamine-labeled NK2 heptapeptide ligand and the human NK2 receptor. The receptor was fluorescently labeled with 3-*N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)-2,3-diaminopropionic acid by the use of incorporation of an unnatural amino acid using the amber stop codon read-through approach mentioned in section I.B. With the use of two different labeling sites in the receptor, it was possible to measure separate distances between the ligand and the receptor, which could be used as constraints for modeling the receptor-ligand interaction (Turcatti et al., 1996).

Because the fluorescent labeling of the ligand should not alter the pharmacology of the ligand, these studies have been done more frequently with peptide ligands than for smaller ligands. In a series of studies, Miller and coworkers have used this experimental approach to study the ligand/receptor interactions of agonists and partial agonists at the cholecystokinin receptor (Harikumar et al., 2004, 2008; Harikumar and Miller, 2005). The ligands were labeled with Alexa Fluor 488, whereas the receptor

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TABLE 2
Resonance energy transfer biosensors for GPCR signal transduction. This table highlights publications that contributed novel aspects to the field

Signaling Step	Resonance Technique	Comments	Reference
Ligand binding to receptor			
TMR-labeled NK2 ligand and NK2 receptor	FRET	Use of unnatural amino acids for FRET.	Turcatti et al., 1996
BODIPY(558/568)-labeled pirenzepine and N-terminally GFP-tagged M_1-ACh receptor	FRET	The observed FRET signal was robust enough to study ligand binding in 96-well plates.	Ilien et al., 2003
Alexa Fluor 488-labeled ligands of different efficacy and Alexa Fluor 546-labeled cholecystokinin receptor	FRET	Steady-state FRET; different orientation of ligands within the receptor derived from obtained data sets.	Harikumar et al., 2004, 2008; Harikumar and Miller, 2005
TMR-labeled PTH ligand and GFP-tagged PTH1 receptor	FRET	Dynamic FRET allowed to measure ligand binding kinetics in real time.	Castro et al., 2005
Ligand binding of two differently labeled ligands	TR-FRET	Detection of dimeric receptors in native tissue.	Albizu et al., 2010
Receptor activation			
PTH1 receptor sensor with CFP/YFP	FRET	First sensor for GPCR activation based on FRET, which was designed to study conformational changes in living cells.	Vilardaga et al., 2003
A_{2A} Receptor sensor with FlAsH/CFP	FRET	First sensor using the combination FlAsH/CFP, which showed no disturbance of G-protein signaling.	Hoffmann et al., 2005
α_{2A}-Adrenergic sensor with constitutive activity	FRET	Inverse agonists exhibit signals opposite to those of agonists.	Vilardaga et al., 2005
B_2-Bradykinin receptor sensor CFP/YFP	FRET	B_2-Bradykinin receptor sensor responding to mechanosensitive stimuli.	Chachisvilis et al., 2006
α_{2A}-Adrenergic sensor FlAsH/CFP	FRET	Partial and full agonists exhibit distinct receptor activation kinetics.	Nikolaev et al., 2006
β_1-Adrenergic receptor CFP/YFP	FRET	Clinically used antagonists exhibit different FRET-signals for different polymorphic receptor variants.	Rochais et al., 2007
α_{2A}-Adrenergic sensor FlAsH/CFP	FRET	Labeling with FlAsH in different positions reveals that partial agonists only induce conformational changes in some parts of the third intracellular loop.	Zürn et al., 2009
M_2-ACh receptor FlAsH/CFP	FRET	Allosteric modulation shown by alterations in the measured FRET-signal.	Maier-Peuschel et al., 2010
Movements in receptor dimers			
mGluR1 CFP/YFP	FRET	Intersubunit FRET change between protomers, but no intrasubunit FRET change.	Tateyama et al., 2004
α_{2A}-Adrenergic sensor FlAsH/CFP	FRET	Receptor cross-talk between α_{2A}-adrenenergic receptor and μ-opioid receptor shown by influence of the FRET signal.	Vilardaga et al., 2008
mGluR1 tagged with CFP or YFP	FRET	Intersubunit FRET change between protomers within 10 ms.	Marcaggi et al., 2009
$GABA_A$ Receptor CFP/YFP	FRET	Intersubunit FRET change between protomers, but no intrasubunit FRET change.	Matsushita et al., 2010
Receptor-G-protein coupling			
Purified and fluorescently labeled G-protein and β_1-adrenergic receptor	FRET	First description of direct receptor/G-protein interaction observed by FRET.	Koestler et al., 1989; Heithier et al., 1992
Interaction between α_{2A}-adrenergic receptor and G_i-protein	FRET	Receptor/G-protein interaction can be as fast as receptor. activation; no evidence for receptor/G-protein precoupling.	Hein et al., 2005
Interaction between α_{2A}-adrenergic receptor (and other GPCRs) and G_i-protein	FRET	Evidence for receptor/G-protein precoupling.	Nobles et al., 2005
Interaction between α_{2A}-adrenergic receptor (and other GPCRs) and G_i-protein	BRET	Receptor and G-protein found to be in close proximity, evidence for receptor/G-protein precoupling.	Galés et al., 2005, 2006
Interaction between B_2-bradykinin receptor and G_q	BRET	Basal receptor/G-protein interaction observed, evidence for receptor/G-protein precoupling.	Philip et al., 2007
G-protein activation			
G-protein heterotrimer activation measured by tagging yeast $G\alpha$ and $G\beta$ or $G\gamma$	FRET	First report on G-protein FRET, decrease in FRET consistent with heterotrimer dissociation.	Janetopoulos et al., 2001
Mammalian $G\alpha_i$ and $G\beta$ or $G\gamma$	FRET	Increase in FRET inconsistent with heterotrimer dissociation upon activation; activation times approximately 450 ms.	Bünemann et al., 2003
Yeast $G\alpha$ and $G\beta$ or $G\gamma$	FRET	Decrease in FRET consistent with heterotrimer dissociation; expression of RGS proteins accelerates G-protein deactivation time.	Yi et al., 2003
Mammalian $G\alpha$ and $G\beta$ or $G\gamma$	BRET	Increase in BRET inconsistent with heterotrimer dissociation upon activation.	Galés et al., 2005, 2006
Plant $G\alpha$ and $G\gamma$	FRET observed by FLIM	No decrease in FRET suggesting no dissociation upon heterotrimer activation.	Adjobo-Hermans et al., 2006

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TABLE 2—*Continued*

Signaling Step	Resonance Technique	Comments	Reference
Mammalian Gα_s and Gβ or Gγ	FRET	Activation times for G_s very similar to G_i, approximately 450 ms.	Hein et al., 2006
Receptor-GRK interaction			
Oxytocin receptor and GRK2	BRET	Very rapid signal increase in BRET within seconds. Signal declined rapidly in first minutes. Evidence for transient interaction between receptor and GRK2.	Hasbi et al., 2004
GLP-1 receptor and GRK2	$BRET^2$	Rapid interaction between receptor and GRK2, strictly dependent on agonist concentration and following receptor-occupancy curve.	Jorgensen et al., 2007
NK1 receptor and GRK2 and GRK5	$BRET^2$	Rapid interaction between receptor and GRK2 and GRK5; higher signal amplitude for GRK2 indicating potential preassociation of receptor and GRK5.	Jorgensen et al., 2008
Receptor-β-arrestin interaction			
β_2-Adrenergic receptor and β-arrestin2	BRET	Agonist-dependent signal with high sensitivity (>10-fold over basal).	Angers et al., 2000
TRH receptor and β-arrestin1 and -2	BRET	TRH1 receptor interacts equally well with β-arrestin1 and -2.	Kroeger et al., 2001
PTH1 receptor and β-arrestin2	FRET	Dynamic receptor/β-arrestin interaction follows receptor activation with time delay.	Vilardaga et al., 2003
Various GPCRs and β-arrestin	BRET	BRET assay is very robust and suitable for high-throughput screening.	Hamdan et al., 2005
β_2-Adrenergic receptor and β-arrestin2	FRET	Phosphorylation state of the receptor influences receptor/β-arrestin interaction kinetics.	Krasel et al., 2005
$P2Y_2$-Purinergic receptor and β-arrestin1 and -2	FRET	The two endogenous ligands ATP and UTP stimulate differential interaction patterns between receptor and β-arrestin1 and -2.	Hoffmann et al., 2008
PTH Receptor and β-arrestin2	FRET	FRET observed without fluorescent proteins using two tetracysteine-reactive fluorophores for selective orthogonal labeling.	Zürn et al., 2010
Conformational change of β-arrestin			
β-Arrestin2 fused with luciferase and YFP	BRET	Receptor-mediated conformational change observed for β-arrestin2 occurred within minutes.	Charest et al., 2005
β-Arrestin2 fused with luciferase and YFP	BRET	Biased ligands exhibit different signals than nonbiased ligands.	Shukla et al., 2008

NK, neurokinin; TMR, tetramethylrhodamine; GLP, glucagon-like peptide.

was modified with thiol-reactive Alexa Fluor 546. The authors could measure steady-state FRET for various combinations of the ligand/receptor complex and were thus able to calculate several distances that could determine the orientation of the ligand within the receptor. Different distance maps were obtained for antagonist or partial agonist-receptor complexes within the extracellular loops, which would be consistent with a different orientation of the different ligands within this region (Harikumar and Miller, 2005; Harikumar et al., 2008). A similar observation was made for the secretin receptor, for which it was also possible to obtain structural constraints that allowed modeling of ligand/receptor interactions within the extracellular region of the receptor (Harikumar et al., 2007). A detailed protocol about how to perform such studies was published and should enable other groups to use this approach (Harikumar and Miller, 2009).

Although the studies mentioned so far were performed as steady-state measurements, our own group used a tetramethylrhodamine-labeled PTH and a PTH receptor, which was N-terminally tagged with GFP, to study dynamic receptor-ligand interaction with FRET in real time (Fig. 3A; Castro et al., 2005). The obtained data suggested a two-step binding process of the ligand, exhibiting a fast (≈140 ms) and a slow binding component (≈1 s). The slow component was comparable with kinetic data for receptor activation (see section III), whereas the fast component was linearly dependent on the ligand concentration and thus most probably reflects a simple bimolecular interaction between ligand and receptor (Castro et al., 2005).

Dynamic FRET measurements were also reported for the muscarinic M_1-acetylcholine receptor (Ilien et al., 2003). This report described the use of N-terminally GFP- or YFP-tagged M_1-acetylcholine receptors in combination with Bodipy(558/568)-labeled pirenzepine and used the FRET signal as readout for ligand binding studies. The obtained FRET signal allowed the development of a robust 96-well assay for ligand binding of unlabeled ligands (Ilien et al., 2003). A later study using Bodipy-labeled pirenzepine and the GFP-tagged M_1-acetylcholine receptors provided evidence for a two-step binding process for small ligands at class A GPCRs (Ilien et al., 2009).

FRET between two differentially labeled fluorescent ligands has recently been reported as a novel approach how to investigate receptor oligomerization and has carried this type of experiment to native tissues (Albizu et al., 2010). The approach used the advantage of TR-FRET, which is based on lanthanides and a second long-wavelength emitting fluorophore (see section I.B). The authors studied the oligomerization of the vasopressin V_{1a} receptor, V_2 recep-

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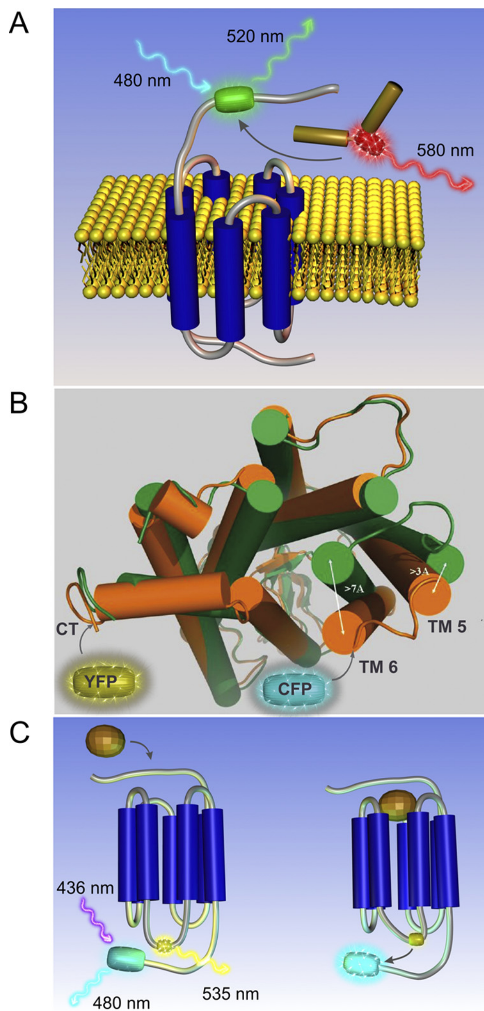

FIG. 3. Analysis of GPCR activation by FRET. A, FRET between a labeled ligand (acceptor; red) and a label in the N terminus of a GPCR (donor; green) results in FRET and can be measured as a loss in green emission [adapted from Castro M, Nikolaev VO, Palm D, Lohse MJ, and Vilardaga JP (2005) Turn-on switch in parathyroid hormone receptor by a two-step parathyroid hormone binding mechanism. *Proc Natl Acad Sci USA* **102:**16084–16089. Copyright © 2005 National Academy of Sciences, USA]. B, activation-dependent conformational change in GPCR structure. Shown are the X-ray structures of an inactive (rhodopsin, Protein Data Bank ID 1GZM) and active (opsin, Protein Data Bank ID 3CAP) state of a GPCR, viewed from the cytosolic face. The greatest difference between the two structures is visible at the cytosolic end of transmembrane domain 6 (>7Å). Insertion of two fluorescent labels (CFP and YFP) at this site plus the C terminus as a reference point results in a sensor that responds to activation with a change in FRET. [Adapted from Zürn A, Zabel U, Vilardaga JP, Schindelin H, Lohse MJ, and Hoffmann C (2009) Fluorescence resonance energy transfer analysis of α_{2A}-adrenergic receptor activation reveals distinct agonist-specific conformational changes. *Mol Pharmacol* **75:**534–541. Copyright © 2009 American Society for Pharmacology and Experimental Therapeutics. Used with permission.] C, principle of a GPCR activation sensor. A receptor carrying two labels as in B is stimulated by agonist, causing a conformational change resulting in relative movements of the two labels. If the distance between the labels increases, this causes a loss of FRET and, hence, yellow emission.

tor, and the oxytocin receptor using agonist or antagonist peptide ligands with Eu^{3+}- or Tb^{3+}-donor labels or Alexa Fluor 647 acceptor labels. FRET signals between different ligands were indicative of homo- and hetero-oligomeric receptors, not only in transfected cells but also in native tissue, and provided evidence for the existence of oxytocin receptor oligomers in mammary glands (Albizu et al., 2010). This approach has also been documented in a systematic protocol, which should help others to adopt this type of experiments to their own needs (Cottet et al., 2011).

The recent developments in the field of fluorescence techniques have allowed the development of more robust assays using fluorescent ligands as analytical tools. The combination of fluorescent ligands and N-terminally fused SNAP-tag GPCRs with lanthanide-based fluorophores as substrates for SNAP-tag labeling has greatly improved the quality of signal-to-noise ratios in TR-FRET assays and has been shown to be useful as a screening approach (Leyris et al., 2011).

The development of fluorescent ligands has been greatly advanced in recent years. It has been used not only in FRET assays, as discussed here, but also in other fields of single-cell pharmacology (May et al., 2010, 2011; Briddon et al., 2011), localization of receptor subtypes within tissues (Daly and McGrath, 2011), or localization within cellular substructures (Nikolaev et al., 2010).

III. G-Protein-Coupled Receptor Activation

Ligand binding to GPCRs causes in most instances conformational changes in the receptors. These changes can be inferred either from the downstream consequences of ligand binding (which allows us to distinguish full and partial agonists and inverse agonists), but also from an increasing number of biochemical and structural data of the receptors themselves (Choe et al., 2011a; Kobilka, 2011; Rosenbaum et al., 2011; Standfuss et al., 2011; Warne et al., 2011). The overall pattern seems to be that a small movement of the transmembrane helices around the ligand binding pocket translates into larger movements at the cytoplasmic face, such that transmembrane helix 6 moves outward and thereby opens a pocket into which the G-protein can dock (Fig. 3B). This outward movement constitutes the basis for the development of sensors that respond to activation with a change in FRET.

A movement of helix 6 relative to helix 3 as a general feature of GPCR activation had been inferred well before the availability of GPCR X-ray structures on the basis of different experimental strategies, including spin labeling studies (Altenbach et al., 1996; Dunham and Farrens, 1999), cross-linking of inserted cysteine residues (Farrens et al., 1996) or metal chelation sites (Sheikh et al., 1996), or by ligand/receptor mutagenesis pairs (Wieland et al., 1996; Zuurmond et al., 1999). These studies encompassed both rhodopsin and several class A GPCRs and led to the hypothesis that activation of GPCRs involved rigid body motions of the transmembrane helices, most notably helix 6 (called helix F in rhodopsin).

Conformational changes during GPCR activation have been studied in some detail by cross-linking of two cysteine residues that are within appropriate proximity to form a disulfide bond. This approach was initially used for rhodopsin (Yu et al., 1995) and a few years later modified for the muscarinic M_3-acetylcholine receptor (Zeng et al.,

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1999). Rhodopsin was split in two parts in various extra- or intracellular positions, the two fragments were expressed and cross-linked by Cu^{2+}-(phenanthrolene)$_3$, and the formation of "intact" rhodopsin was detected by an antibody directed against the C terminus. This approach was designed in 1995 to detect opposing amino acid residues within rhodopsin (Fig. 1A). In contrast, the muscarinic M_3-acetylcholine receptor was expressed as a whole receptor that could be split into two parts via a factor X cleavage-site introduced into the third intracellular loop of the receptor. Again, cross-linking with Cu^{2+}-(phenanthrolene)$_3$ resulted in an "intact" receptor on SDS-polyacrylamide gels; this approach has been used to study the movements in various regions of the receptor induced by ligands of different efficacy (Hoffmann et al., 2008; Wess et al., 2008b).

A somewhat similar cross-linking approach uses histidine residues that can complex Zn^{2+}-ions (Elling et al., 1995; Sheikh et al., 1996; Thirstrup et al., 1996) and thus can block helical movements. Initially, this approach helped to understand the general structural arrangement of GPCRs and their movements during activation (Elling et al., 1995, 1999). Later it was used to demonstrate differential requirements of the movements within a GPCR for G-protein signaling versus β-arrestin interactions (Vilardaga et al., 2001).

Overall, these studies led to the conclusion that during activation of GPCRs, helices 5 and 6 move as rigid bodies relative to helix 3, and that this outward movement might create a binding pocket for G-proteins at the receptors' cytoplasmic end. Such movements were studied in detail for rhodopsin in high-resolution double electron-electron resonance spectroscopy studies after the X-ray structure of rhodopsin had been solved (Altenbach et al., 2008).

A. Ligand-Induced Changes in Purified Receptors

On the basis of the concept of helix movements during GPCR activation, Gether et al. (1995) began to label purified β_2-adrenergic receptors with fluorophores to search for fluorescence signals that were influenced by receptor ligands. Their studies provided the first method to monitor directly conformational changes in a G-protein-coupled receptor and confirmed the notion of agonist-induced relative movements of helices 6 and 3. Using a series of purified β_2-adrenergic receptors with a limited number of cysteines available for chemical derivatization, they observed agonist-induced changes in fluorescence for fluorophores attached to Cys125 in transmembrane helix 3 and to Cys285 in transmembrane helix 6 (Gether et al., 1997). Use of different ligands showed, furthermore, that partial agonists caused only partial changes in the receptor fluorescence (for review, see Gether, 2000; Bissantz, 2003).

These movements could also be picked up in the G-protein-coupling region below transmembrane helix 6 (Ghanouni et al., 2001b), and they were, again, different for various β-adrenergic ligands (Ghanouni et al., 2001a). The studies mentioned above were initially based on fluorescence quenching, presumably as a result of a change in the positioning of the fluorophore within the receptor protein and/or the lipid vesicles into which the labeled receptors had been reconstituted. More specific results were obtained when fluorescence quenchers were inserted in a second position (for example at the bottom of transmembrane helix 5) so that relative movements of the two positions (and, hence, helices) relative to each other could be inferred (Ghanouni et al., 2001b).

Using a series of catechol derivatives (i.e., agonists with different affinities and efficacies) in such fluorescence quenching studies with labeled, purified, and reconstituted β_2-adrenergic receptors, Swaminath et al. (2004) proposed that agonist binding induced a sequence of conformational changes, with distinguishable kinetic properties, and that only full agonists were capable of inducing the last steps that resulted in a fully active receptor state. Subsequent studies with chemically distinct agonists—catechol and noncatechol ligands—suggested that there might be different activation modes that were characteristic for the different sets of agonists (Swaminath et al., 2005).

The agonist-induced fluorescence changes in these experiments were relatively slow, suggesting that the purified reconstituted receptors did not show the same behavior as receptors in their native environment. Subsequent studies confirmed this view by showing that careful reconstitution could increase the speed of the receptors' conformational switch and results in agonist-induced changes of fluorescence on a time-scale of approximately 30 s (Yao et al., 2006).

More recent studies from the same laboratory used purified β_2-AR constructs labeled at the cytoplasmic end of transmembrane helix 6 and two different positions on the C-tail (Granier et al., 2007; Granier et al., 2009). These experiments revealed that the receptor's C terminus was quite extended and that agonists caused small movements (1–2 Å) between these labels. Measured from the cytoplasmic end of transmembrane helix 6, full agonists decreased the distance to the proximal C terminus but increased it to the distal C terminus. In contrast, the partial agonist dopamine caused a small increase of both distances. This supports the contention that partial agonists may produce alterations in the receptors' conformation that are not only smaller but also qualitatively different.

Taken together, these fluorescence quenching studies with purified GPCRs confirmed that activation involves motions of entire helices, most notably transmembrane helix 6, and revealed the existence of multiple active structures, which may be specifically induced by distinct agonists and may be achieved in a sequential manner.

B. Receptor Conformational Changes in Intact Cells

An alternative to the investigation of GPCR activation using purified, reconstituted receptors was the development of FRET-sensors suitable for imaging in intact cells, as outlined in section I.B. To permit labeling in intact cells, the initial sensors contained genetically encoded fluores-

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cent proteins as fluorophores. Prototypes for such GPCR sensors were the somewhat analogous sensors for cAMP based on blue fluorescent protein- and GFP-labeled regulatory and catalytic subunits of PKA (Zaccolo et al., 2000) and the similarly constructed sensors for heterotrimeric G-proteins in yeast (Janetopoulos et al., 2001). This means that the sensors contained a GPCR, often somewhat modified as described below, and two fluorescent labels, usually CFP and YFP. The placement of these labels is schematically depicted in Fig. 3, B and C and is based again on the movement of helix 6 and the adjacent intracellular region during agonist-induced activation. YFP or a similar FRET acceptor is usually placed in the third intracellular loop and CFP or a similar FRET donor at the C terminus, but the reverse order is also possible.

The first example of such a sensor was a PTH receptor that carried a CFP below transmembrane helix 6 and an YFP at the end of a (shortened) C terminus (Vilardaga et al., 2003). This sensor showed FRET from CFP to YFP with an efficiency of approximately 50%, and activation with the agonist PTH resulted in a decrease in FRET by approximately 15%. An analogous sensor (but with the positions of CFP and YFP inverted) was generated for the α_{2A}-adrenergic receptor, which showed an agonist-induced reduction in FRET by up to 5%. Several lines of evidence indicated that the decrease in FRET in these receptor constructs does indeed reflect the agonist-induced conformational change that results in the active state(s) of a GPCR. These include the fact that this state was stabilized by G-proteins, that it could also be observed in urea-treated cell membranes (and was thus due to the receptor itself), and the observation that partial agonists caused only partial responses. Furthermore, the kinetics were rapid and compatible with the fast physiological responses triggered by these receptors; the activation of the class A α_2-adrenergic and the class B PTH receptor sensors occurred with time constants τ of <40 ms and 1 s, respectively.

A large number of similar GPCR activation sensors has been developed since then, including the β_1- and β_2-adrenergic, A_{2A}-adenosine, M_1-, M_2-, M_3-, and M_5-muscarinic, and B_2-bradykinin receptors (Vilardaga et al., 2003, 2005; Hoffmann et al., 2005; Chachisvilis et al., 2006; Rochais et al., 2007; Jensen et al., 2009; Maier-Peuschel et al., 2010; Reiner et al., 2010; Ziegler et al., 2011; see Table 2). The attachment of two relatively large fluorescent proteins is surprisingly well tolerated by many GPCRs, as evidenced by cell surface expression, maintained ligand binding, and often even signaling properties. However, in some instances, the fluorescent protein in the 3rd intracellular loop (i.e., below transmembrane helix 6) disturbs coupling to G-proteins (Hoffmann et al., 2005). A major improvement in such cases is the use of the FlAsH label (see section I), which, because of its much smaller size, is less likely to perturb protein function. In the case of the A_{2A}-adenosine receptor, a YFP insertion in the 3rd intracellular loop completely prevented coupling to G_s, whereas a FlAsH labeling site did not interfere with receptor signaling (Hoffmann et al., 2005). An additional advantage of the corresponding CFP/FlAsH-labeled receptor was the ≈5-fold increase in signal amplitude compared with the corresponding CFP/YFP-labeled construct. There is no systematic comparison of FlAsH- versus YFP-labeled FRET sensors to provide a clear explanation for this increase; a plausible cause is the more rigid attachment of the FlAsH label via the four cysteines to its binding sequence, which may add an orientational component to the FRET signal. After this initial description, a large number of CFP/FlAsH-based GPCR sensors have been reported (Nikolaev et al., 2006c; Zürn et al., 2009; Maier-Peuschel et al., 2010; Reiner et al., 2010; Ziegler et al., 2011).

From these reports, a number of common principles emerge. First, most but not all (Ziegler et al., 2011) such GPCR sensors respond to agonist stimulation with a decrease in FRET, compatible with an increase in the distance between the two labels. Although the size and the flexible attachment of the labels precludes any molecular interpretation, this increase would be compatible with the movements of the transmembrane helices upon GPCR activation as can be deduced from X-ray structures (see Fig. 3B).

Second, a few studies have investigated the effects of partial and inverse agonists and have reported that partial agonists produced partial effects in FRET, whereas inverse agonists produced effects in the opposite direction compared with agonists (Vilardaga et al., 2003; Nikolaev et al., 2006c; Reiner et al., 2010). These observations are in agreement with the view that the FRET signals report conformational changes in the GPCRs similar to those seen by the G-proteins. Further studies are needed to assess whether this is generally the case for this type of GPCR sensors and whether biased agonism (i.e., the differential signaling to G-proteins and other downstream pathways such as β-arrestins) can be differentially picked up by GPCR FRET sensors. An interesting result in this context comes from placing one of the two labels at different sites of the 3rd intracellular loop of the α_{2A}-adrenergic receptor (Zürn et al., 2009). The different sensors reacted differently to partial versus full agonists; the sensor with the label closest to transmembrane helix 6 was the most sensitive to partial agonists, whereas those with the label closer to transmembrane helix 5 were less sensitive. This raises the possibility that different conformations are induced by partial and full agonists at GPCRs, and that these different conformations might be picked up by receptor sensors labeled in different positions of the receptor. Similar ligand-specific conformations have recently been observed with FRET sensors for M_2- and M_3-muscarinic receptors (Bätz et al., 2011).

Several lines of evidence have recently suggested that in fact GPCRs may adopt multiple active conformations (reviewed by Hoffmann et al., 2008b). Multiple active states have been assumed on the basis of theoretical and modeling considerations (Kenakin, 1995; Leff et al., 1997;

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Buranda et al., 2007; Onaran and Costa, 2009; Kenakin and Miller, 2010) and because of ligand-specific "biased" effects of various agonist/receptor systems (Violin and Lefkowitz, 2007; Drake et al., 2008; Shukla et al., 2008; Rajagopal et al., 2011). Metal chelating strategies, originally developed to prevent the activation of GPCRs (Sheikh et al., 1996; Elling et al., 1999, 2006), revealed for the PTH receptor that different conformations were involved in coupling to G-proteins versus β-arrestins (Vilardaga et al., 2001); furthermore, the determinants of this receptor for β-arrestin binding and internalization were shown to be different (Vilardaga et al., 2002). Similar distinctions between requirements for activation and endocytosis were seen for the complement 5a receptor (Whistler et al., 2002). A second cross-linking strategy, sulfhydryl cross-linking, again revealed ligand-specific conformational changes in the M_3-muscarinic receptor (Li et al., 2007). Multiple active conformations were also inferred from the fluorescent studies on purified β_2-adrenergic receptors mentioned above (Ghanouni et al., 2001a; Swaminath et al., 2004; Yao et al., 2006), where partial and full agonists produced stepwise changes in fluorescence with distinct kinetics. More recently, the concept of agonist-specific states has been extended also to distinct antagonist-occupied states of GPCRs (for example, Brea et al., 2009; Moukhametzianov et al., 2011).

In FRET studies using GPCR sensors in intact cells, distinct agonists and partial agonists induced signals with highly variable kinetics; in studies with the α_{2A}-adrenergic receptor (Vilardaga et al., 2005; Nikolaev et al., 2006c), it was shown that full agonists produced the fastest conformational changes (time constants <50 ms) and that inverse agonists produced the slowest changes (time constants >1 s), whereas partial agonists occupied the entire range between these values, the speed being proportional to the amplitude of their effects. In a similar set of experiments on two naturally occurring β_1-adrenergic receptor variants (Gly389 and Arg389) with inverse agonists, it was shown that carvedilol induced larger conformational changes in the Arg389 variant, whereas other inverse agonists did not distinguish between the two variants (Rochais et al., 2007). All these data are consistent with the view that distinct ligands induce distinct conformations of GPCRs and that these distinct conformations are adopted with distinct kinetics and, possibly, in a sequential manner.

The third presumably general aspect of these FRET sensors for GPCRs concerns their ability to discern effects of allosteric ligands. Allosteric agonists have in recent years received increasing attention (Conn et al., 2009a,b; May et al., 2007a; Keov et al., 2011). There is the hope that allosteric ligands might provide a specific and safe way of modulating receptor function, analogous to the effects that benzodiazepines exert at the $GABA_A$ receptor (Sigel, 2002; Sigel and Lüscher, 2011). Allosteric ligands have been described for numerous GPCRs (Conn et al., 2009a; De Amici et al., 2010) and have been subdivided into various subgroups depending on their direct (direct agonists/inverse agonists; no direct effects) and on the type of allosteric modulation (positive/negative; ligand-specific). Numerous allosteric ligands for muscarinic receptors have been identified, some of which show some subtype selectivity for the M_2-muscarinic receptor [i.e., the negative allosteric modulators gallamine and *N,N′*-bis[3-(1,3-dihydro-5-methyl-1,3-dioxo-2*H*-isoindol-2-yl)propyl]-*N,N,N′,N′*-tetramethyl-1,6-hexanediamminium (dimethyl-W84)], whereas others are rather unselective (May et al., 2007b). Radioligand dissociation studies had suggested that their effects can be quite rapid without being able to resolve the kinetics of these effects (Tränkle et al., 2003, 2005).

In a recent study on M_2-muscarinic receptors, it has been shown that an analogous FRET receptor construct also reported the effects of negative allosteric modulators (Maier-Peuschel et al., 2010). These compounds produced no effects on the FRET signal by themselves but they reduced the agonist-induced signals. It is noteworthy that these effects were also quite rapid, with rates similar to those of the effects of direct agonists and antagonists (see below), and confirm the view that allosteric modulators act at the GPCRs themselves.

A very special case of allosteric regulation in GPCRs is the signaling in GPCR dimers and the communication between the two GPCRs within such a dimer. These mechanisms can also be picked up by a variety of FRET sensors (Tateyama et al., 2004; Vilardaga et al., 2008; Marcaggi et al., 2009) and are discussed in section III.C.

A particular ability of FRET sensors is their ability to report the kinetics of the underlying events in intact cells (Lohse et al., 2007a). This is also true for the various GPCR sensors that report their activation by a change in FRET (Lohse et al., 2007b, 2008a,b). For the activation step of class A GPCRs, there seems to be a relatively constant activation rate, with time constants on the order of 50 ms. Under conditions trying to optimize the speed of GPCR activation (i.e., rapid application of agonists; observation of only single cells or subcellular regions), similar activation times have been observed for all class A GPCRs (i.e., α_{2A}-, β_1-, and β_2-adrenergic, A_{2A}-adenosine, and M_1-, M_2-, M_3-, and M_5-muscarinic). In contrast, the activation rate of the class B PTH receptor, as mentioned already above, was on the order of 1 s (Vilardaga et al., 2003). It is possible that this slow activation is dependent on the complex two-step binding mode of PTH to its receptor, which is itself relatively slow (see section II; Castro et al., 2005). The slow activation rate may also be a general property of class B GPCRs. However, no similar studies have yet been performed on other class B GPCRs.

These activation times are in the range that can be expected from physiological data. For example, it is an everyday experience that the cardiac frequency can be increased within a single heartbeat (i.e., within less than a second). Experimental electrophysiological recordings of GPCR-regulated channels have also shown activation times of well below 1 s. For example, the opening of GIRK

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potassium channels by M_2 muscarinic or by α_{2A}-adrenergic receptors has shown that an entire GPCR-signaling chain can be activated within 200 to 500 ms (Pfaffinger et al., 1985; Bünemann et al., 2001). On the other hand, activation of rhodopsin, the best-studied class A GPCR, is well documented to occur much faster. In fact, activation of the light receptor rhodopsin can be observed within 1 ms of light triggering (Kahlert and Hofmann, 1991; Pugh and Lamb, 1993; Makino et al., 2003), and even the downstream closure of the cGMP-gated cation channel—which requires the intermediate steps of transducin activation and cGMP hydrolysis by the transducin-activated phosphodiesterase—is observed within 200 ms (Makino et al., 2003). Thus, it is obvious that GPCR activation can, in principle, occur much faster than observed so far with GPCR FRET sensors. There are two major possible reasons for this discrepancy. The first is that rhodopsin is a very special GPCR, tuned by evolution for the sensitive and rapid perception of signals, and that it is indeed approximately 2 orders of magnitude faster than all other GPCRs. The second is that our current knowledge of the activation times of nonrhodopsin class A activation times is limited by technical or other factors beyond the receptors' true activation time. Potential reasons for such limitations include both technical limitations—such as the application of agonists by perfusion systems—and the fact that aggregate data are obtained from an entire cell or a large region of a cell, where large numbers of receptors may switch in an only imperfectly synchronized manner. Further experimentation will be required to distinguish between these possibilities and to determine the "true" activation rates of nonrhodopsin GPCRs.

We have mentioned already above that in a study on a FRET sensor for the α_{2A}-adrenergic receptor using a series of partial agonists, the kinetics of the conformational change varied with the amplitude of the change—larger effects were induced faster than smaller ones (Nikolaev et al., 2006c). It remains to be seen how these observations are compatible with those obtained on purified β_2-adrenergic receptors, where partial and full agonists seemed to produce stepwise incremental conformational changes (Swaminath et al., 2004).

Taken together, FRET data on GPCRs in intact cells indicate that activation of these receptors can be quite rapid, taking less than 100 ms, and that different ligands act with different speeds and may induce distinct conformations. An open question is whether the distinct conformations of a GPCR are achieved in a sequential manner or via distinct routes. Other open questions concern the possibility that the extent and speed of activations are regulated locally by cell- and stimulus-specific factors (see section VII).

C. Activation in Receptor Dimers

GPCRs have traditionally been perceived as monomeric proteins, with a single receptor activating a single G-protein. Recent data have confirmed that at least class A GPCRs are fully functional and signaling competent as monomers. Single (and therefore monomeric) β_2-adrenergic receptors as well as rhodopsin and μ-opioid receptors reconstituted into small lipid vesicles couple to their respective G-proteins (Whorton et al., 2007, 2008; Kuszak et al., 2009), and monomeric rhodopsin in solution activated its G-protein transducin at the diffusion limit (Ernst et al., 2007).

However, many GPCRs seem to form dimers and presumably also higher order oligomers in intact cells (Hébert and Bouvier, 1998; Overton and Blumer, 2000; Milligan et al., 2003; Fuxe et al., 2010; Lohse, 2010). Early evidence for GPCR dimerization included various biochemical assays, functional complementation of dysfunctional receptors, coprecipitation of differently tagged GPCRs, dimer visualization in SDS-polyacrylamide gels and their disruption by receptor peptides, and large sizes of GPCRs in radiation inactivation studies (Bouvier, 2001). Later, precipitation of GPCR dimers with antibodies specifically recognizing the dimer provided evidence for native GPCR dimers (Rozenfeld and Devi, 2011). The strongest physiological evidence for functioning of GPCRs in dimers comes from the class C receptors, which are obligate dimers. For example, the $GABA_B$ receptor is a dimer of a $GABA_{B1}$ and a $GABA_{B2}$ subunit, where the $GABA_{B1}$ subunit binds the agonist (but does not couple), and the $GABA_{B2}$ subunit couples to G-proteins (but does not bind agonist) (Kaupmann et al., 1998). More recent studies have shown in fact that native $GABA_B$ receptors are heteromultimers with a family of auxiliary subunits (Schwenk et al., 2010). Larger oligomers have recently also been inferred for class A β_2-adrenergic receptors based on mobility studies with fluorescence recovery after photobleaching (FRAP) (Dorsch et al., 2009). Similar FRAP studies using immobilization of one type of receptor to see whether it affects the mobility of another receptor have suggested that in many cases (e.g., β_2-adrenergic and D_2-dopamine receptors), the dimerization may occur only transiently (Dorsch et al., 2009; Fonseca and Lambert, 2009). The ability of GPCRs to form homo- and heterooligomers seems to provide a whole spectrum of regulatory and signaling properties (Woolf and Linderman, 2004; Milligan, 2007; Gurevich and Gurevich, 2008; Pin et al., 2009; Ciruela et al., 2010a,b; Kniazeff et al., 2011; Rozenfeld and Devi, 2011).

Among the many methods to study GPCR di- and oligomerization, BRET and FRET studies have played a prominent role (Angers et al., 2000; Bader and Beck-Sickinger, 2004; Milligan and Bouvier, 2005; Pfleger and Eidne, 2005; Pin et al., 2007; Xu et al., 2009). These studies used primarily fusions of fluorescent and luminescent proteins to the receptors' intracellular C termini. An alternative approach uses antibodies or tags that can be attached to the extracellular face of the receptors and then can be fluorescently labeled to produce FRET (Maurel et al., 2008). Most of these studies reported for a variety of GPCRs that they were already synthesized as dimers (Issafras et al., 2002; Overton and Blumer, 2002; Terrillon et

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al., 2003; Herrick-Davis et al., 2006). Specificity of these interactions was suggested by the fact that in many cases, the formation of dimers did not seem to depend on the receptor expression level (Herrick-Davis et al., 2004), as would be expected for random collisions between receptors ("bystander BRET"). For some, receptors, however (e.g., CXCR1 and -2 receptors), higher expression levels seem to promote di- and oligomerization, possibly indicating a dynamic, reversible assembly of such di-/oligomers (Milligan et al., 2005). The question of whether GPCR dimers are stable has already been mentioned in a prior paragraph; this seems to depend on the receptor type. Furthermore, BRET studies suggest that GPCR dimers may also fall apart when the receptors are internalized (Lan et al., 2011).

In most instances, the presence of agonists had little or no effect on FRET between the two protomers in a dimer (Bouvier, 2001; Canals et al., 2003, 2004). It is currently not clear whether the lack of effect was due to technical limitations or dimer conformations are indeed little affected by agonists. In a few cases, ligand-induced oligomerization was observed, for example for somatostatin receptors (Patel et al., 2002). In a study on MT_2 melatonin receptors, it was shown that ligand-induced dimerization was increased by the presence of agonists as well as antagonists and inverse agonists, suggesting that the mere binding event rather than a specific conformational change in a preformed dimer was responsible for the change in BRET (Ayoub et al., 2002). A similar observation was made for CXCR4-CCR2 receptor dimers (Percherancier et al., 2005). Likewise, binding of inverse agonists to β_2-adrenergic receptors reconstituted in a model lipid bilayer led to significant increases in FRET efficiencies, suggesting that they promote tighter packing of protomers (Fung et al., 2009).

Although there has been some discussion of the interpretation and the experimental techniques of such BRET and FRET as well as functional studies to show GPCR dimerization (James et al., 2006; Lohse, 2006; Bouvier et al., 2007; Chabre et al., 2009), there seems to be overriding consensus that many GPCRs can form dimers and that they do this not only in overexpression systems but also in vivo. A corresponding database has been recently defined and implemented (Skrabanek et al., 2007; Khelashvili et al., 2010).

FRET experiments have also been done to explore the molecular nature of GPCR oligomers. For instance, FRET studies using defined extracellularly labeled $GABA_B$ receptors have indicated that the receptors seem to form tetramers, with two $GABA_{B2}$ protomers being distant from each other, whereas the $GABA_{B1}$-protomers are close to each other (i.e., presumably from the center of the tetramer) (Maurel at al., 2008).

FRET studies have recently achieved the clear demonstration of GPCR dimers in native tissues (Albizu et al., 2010; Ambrosio and Lohse, 2010; Cottet et al., 2011). These studies relied not on fluorescent receptors, as did the studies discussed so far, but instead used labeled ligands and time-resolved FRET. This allowed the demonstration of FRET between ligands bound closely to each other, such as would be found only in di- or oligomers. Such FRET was demonstrated not only for various GPCRs in transfected cells but also for oxytocin in native mammary gland tissue.

Finally, dynamic FRET experiments have been done to understand the functional interactions between the members of a GPCR di- or oligomer. These studies differ from those mentioned above in that they did not measure basal, constitutive FRET but attempted rather to identify dynamic changes that were induced by ligands. There seem to be various modes how the subunits in a GPCR dimer might interact. Although for the $GABA_B$ receptor, there must be a trans-activation, because the ligand binding B1-subunit has to activate the G-protein coupling B2 subunit, in many other receptors, there seems to be a negative cooperativity, such that only one of the subunits needs to be active to cause G-protein activation. Many lines of evidence argue for a model where an (asymmetric) GPCR dimer binds to only one G-protein, one protomer contacting the Gα-subunit and the other protomer interacting with the G$\beta\gamma$-complex (Damian et al., 2006, 2008; Arcemisbéhère et al., 2010). In these dimers (for example the BLT2 receptor binding leukotriene B_4, which has been investigated extensively in its purified, reconstituted form), there is consequently no transactivation between the protomers (Damian et al., 2008). Negative cooperativity can be inferred from the observation that the receptor monomers activate their G-protein G_{i2} more efficiently than dimers (Arcemisbéhère et al., 2010). Likewise, in metabotropic glutamate receptors, it seems that activation of a single protomer is sufficient for G-protein activation (Hlavackova et al., 2005), even though activation of both protomers may produce larger effects (Kniazeff et al., 2004).

In dynamic FRET experiments investigating a dimer of an α_{2A}-adrenergic receptor and a μ-opiate receptor, Vilardaga et al. (2008) reported basal constitutive FRET between these different types of receptors, compatible with the notion that these two receptors form dimers in transfected cells as well as in various native tissues, most notably in brain (Jordan et al., 2003). When an α_{2A}-adrenergic receptor FRET sensor was coexpressed with the μ-opiate receptor, the noradrenaline-induced signals of the FRET sensor became sensitive to opioid ligands; specifically, agonists such as morphine inhibited the noradrenaline-induced FRET signal. This suggests a type of trans-inhibition between the two protomers, such that in this dimer only one of the two protomers is active—reminiscent of the data mentioned for glutamate and leukotriene receptors, where activation of one protomer in a dimer was sufficient for G-protein activation (Hlavackova et al., 2005; Damian et al., 2006, 2008).

The kinetic analysis of this trans-inhibition gave a rate constant of $\approx$400 ms, which is slower than the GPCR activation itself ($\approx$50 ms) but slightly faster than activation of G_i by the same receptors ($\approx$500 ms; see below). This

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is compatible with a direct interaction between the two protomers as the basis for this trans-inhibition—rather than an indirect effect such as binding to and competition for a common pool of G-proteins (Quitterer and Lohse, 1999). Such a direct trans-inhibition was further suggested by the fact that it was insensitive to pertussis toxin, which inactivates G_i, or to 6 M urea, which inactivates G-proteins as well as most other non-GPCR membrane proteins.

Inhibitory effects across GPCR dimers have also been described on the basis of functional studies for other receptors (e.g., for the 5-HT_{2C} receptor) (Herrick-Davis et al., 2005; Tubio et al., 2010). Contrasting with these data is another very recent study on the 5-HT_{2C} receptor in which activation of both protomers in the dimer seemed to be more efficient in turning on its G-protein (Pellissier et al., 2011).

An interesting case are GPCRs that form obligatory dimers, as is the case for many class C GPCRs, including the $GABA_B$ and metabotropic glutamate receptors. In these receptors, FRET experiments have revealed intersubunit movements that were monitored with GFP variants inserted into various intracellular loops. It is noteworthy that these studies have revealed intersubunit changes upon agonist stimulation but have not seen intrasubunit changes, which were characteristic for the activation of class A receptors (Tateyama et al., 2004; Marcaggi et al., 2009; Matsushita et al., 2010); in one study, these intersubunit signals were of small amplitude but extremely fast (≈10 ms) (Marcaggi et al., 2009). It remains to be seen whether class C receptors indeed have a different activation mechanism involving no conformational change of the individual transmembrane domains or if the sensors used so far just happened to pick up no such changes.

Thus, it seems that many GPCRs can form dimers or larger oligomers, and that even though GPCRs seem to function well as monomers, their assembly conveys additional signaling properties. These GPCR di- and oligomers offer many ways of interaction between the protomers, including synergism as well as antagonism, absence and presence of trans-activation or -inactivation. Many questions concerning these different modes of interactions remain to be elucidated. Do they share common principles or do they represent distinct types of interactions? Is there a relationship between the structural type of assembly and the functional interactions? Do assembled receptors constitute a receptor reserve, are they silent, or do they continue to interact? Are receptor assemblies regulated by receptor function and/or by other receptor-associated proteins? And finally: Can they be exploited for therapeutic purposes?

IV. G-Protein-Coupled Receptor/G-Protein Interactions

A. Receptor / G-Protein Interaction

Once activated, GPCRs need to interact with G-proteins to transmit their signals. This interaction then triggers the activation of G-proteins by triggering dissociation of GDP from the G-protein α-subunit and binding of GTP, which induces activation of the G-protein and enables it to couple to and activate downstream effectors. This interaction between receptors and their G-proteins is presumably one of the best-studied protein-protein interactions, and many details have been resolved. (Bourne, 1997; Hamm, 1988, 1998; Birnbaumer, 2007; Johnston and Siderovski, 2007; Oldham and Hamm, 2008; Hofmann et al., 2009).

It is clear that, in principle, at least, this coupling can be extremely fast and is also quickly reversible; in the well studied case of the coupling of rhodopsin to its G-protein G_t (or transducin), one activated rhodopsin can catalytically activate several thousand molecules of G_t per second (Heck and Hofmann, 2001; Ernst et al., 2007). Even though evidence has been presented for rhodopsin forming dimers and higher order oligomers (Palczewski, 2006), maximal activation has been reported to occur in a 1:1 complex of rhodopsin/G_t (Ernst et al., 2007), and, as mentioned in section III.C, monomeric rhodopsin can fully activate G_t (Bayburt et al., 2007; Ernst et al., 2007; Banerjee et al., 2008; Whorton et al., 2008). For other GPCRs, the amplification seems to be much less; for example, photoinactivation experiments with A_{2A}-adenosine receptors have shown that in intact cells each receptor activates only a few G_s molecules (Lohse et al., 1991). Again, for nonrhodopsin GPCRs, efficient signaling has been observed in 1:1 receptor/G-protein complexes (Whorton et al., 2007; Kuszak et al., 2009) as predicted by classic rhodopsin signaling theories (Chabre and le Maire, 2005). However, more complex models have also been developed, ranging from the 2-receptor/1-G-protein models alluded to in section III.C (Damian et al., 2006, 2008; Arcemisbéhère et al., 2010; Pellissier et al., 2011) to complex assemblies involving multiple or even arrays of receptors and G-proteins (Jahangeer and Rodbell, 1993; Rodbell, 1997; Liang et al., 2003; Palczewski, 2010).

The contact sites on the receptors as well as the G-protein have been mapped with various approaches, again in most detail for the rhodopsin/G_t pair. These data make it apparent that both Gα and Gβγ contribute to the interaction. The main coupling site in the receptor is the region in its cytoplasmic face that opens up upon activation, encompassing in particular the ends of transmembrane helices 5 and 6, (see Fig. 3B), whereas in the G-proteins, the main contact site is the C terminus of Gα (Scheerer et al., 2008, 2009; Choe et al., 2011a,b). Various studies (Onrust et al., 1997; Bae et al., 1999; Cai et al., 2001; Itoh et al., 2001; Oldham and Hamm, 2008) indicate the participation of additional regions of Gα (N-terminal α-helix, α3-β5 loop and α4-β6 loop) in contacting the activated receptor. In addition to the Gα subunit, the Gβγ complex also binds receptors and stabilizes the receptor-Gα interface (Taylor et al., 1994, 1996; Azpiazu et al., 1999; Oldham and Hamm, 2008). It has been suggested, again for the rhodopsin/G_t interaction, that these various contact points might be used in a sequential manner (Herrmann et al., 2004);

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according to this model, the receptor first contacts G$\beta\gamma$ and then "rolls over" to interact with the Gα C terminus. Very recently, many more details of this important interaction have been clarified with the long-awaited crystal structure of a GPCR/G-protein complex, the complex between the agonist-occupied β_2-adrenergic receptor and trimeric G_s (Rasmussen et al., 2011). This structure shows that the agonist-induced outward movement of helix 6 is even larger in the complex than for the receptor alone, amounting to 14 Å at the cytoplasmic face. The interactions with the G-protein in this complex occur exclusively with Gα and involve the second intracellular loop and helices 5 and 6 of the receptor, and the α4-helix, the αN-β1 junction, the top of the β3-strand and the α4-helix of the ras-like domain of Gα. The most dramatic and surprising change is a complete reorientation of the helical domain of Gα in the complex; presumably, this domain is quite flexible in the complex. Such flexibility can also retrospectively be concluded from a BRET study using multipositioning of the BRET probe within Gα (Galés et al., 2006).

Even though the receptor/G-protein interaction is presumably one of the best-studied examples of protein/protein interactions, some key questions of this interaction are still unresolved. The main unanswered questions in this context concern the specificity of coupling between receptors and G-proteins (Gudermann et al., 1996). Although specificity is evident not only from the specific physiological effects of most receptors, but also in early experiments elucidating this interaction in reconstitution models, with specific knock-out of subunits and by mutagenesis of both receptors and G-protein subunits (e.g., Cerione et al., 1985; Kobilka et al., 1988; Kleuss et al., 1991, 1992, 1993), the basis for specificity is still uncertain. Whereas in several instances specific motifs required for specific receptor/G-protein coupling have been identified (see, for example, Liu et al., 1995), no defined general sequence or structure motifs seem to assure specific coupling of a GPCR to a specific G-protein, and it is unclear whether common structural motifs do in fact exist. How specificity of a given receptor to its cognate G-protein(s) is generated is unclear, and the contributions of protein-protein specificity versus spatial organization at the cell surface or via additional proteins remain to be resolved.

The second unresolved issue concerns the dynamics of this interaction. Two different models have been proposed how receptors and G-proteins might be organized. The "collision coupling" model (Orly and Schramm, 1976; Tolkovsky and Levitzki, 1978a,b) assumes that receptors and G-proteins are both mobile and that this leads to collisions between them; if during such a collision a receptor is active (i.e., agonist-occupied), it may activate the G-protein in the course of such a collision. The second model assumes that receptors and G-proteins are "precoupled" (Wreggett and De Léan, 1984; Neubig et al., 1988; Neubig, 1994; Tian et al., 1994) such that activation of a receptor leads to immediate activation of the precoupled G-protein. In the first case, receptors can catalytically activate many G-proteins; in the second case, the number of G-proteins accessible to a receptor is only one or is at least limited.

BRET and FRET have been used by several investigators to address GPCR/G-protein assembly and interaction. In fact, studies of FRET between purified, reconstituted, and fluorescently labeled G-protein subunits and β_1-adrenergic receptors were the first examples of the use of FRET in the study of GPCRs (Koestler et al., 1989; Heithier et al., 1992). In these studies, it was discovered that reconstituted β_1-adrenergic receptors showed direct interactions with the G$\beta\gamma$-complex and that these were virtually the same for agonist-stimulated and nonstimulated receptors, in line with a precoupling model of the receptor/G-protein interaction. G$\beta\gamma$-specificity was suggested in these studies by the observation that stronger interactions were seen with G$\beta\gamma$ purified from bovine brain (containing a mixture of different Gβ- and Gγ-subunits) than with retinal G$\beta\gamma$ (i.e., from G_t or transducin). The role of the G$\beta\gamma$-complex in receptor interactions coincided with the increasing perception of its active role in G-protein-mediated signaling (Birnbaumer, 1992; Iñiguez-Lluhi et al., 1993; Kisselev et al., 1995; Müller and Lohse, 1995; Neer, 1995; Dupré et al., 2009).

Only many years later were these studies transferred to intact cells using genetically encoded fluorescent proteins (for FRET) or luciferase (for BRET) fusions rather than chemical labeling. The strategy for these studies is depicted in Fig. 4B. Receptors were generally labeled at their C termini, which has been shown for many receptors and in many types of experiments to preserve most of the receptors' properties. Various sites of labeling have been used for the G-proteins: the N termini of both Gβ and Gγ (Ruiz-Velasco and Ikeda, 2001; Bünemann et al., 2003; Galés et al., 2005, 2006) are the most frequently used sites for the G$\beta\gamma$-complex. Surprisingly, the attachment of GFPs to the N termini of Gβ or Gγ does not markedly impair their ability to form a heterotrimer, to regulate effectors, or to interact with receptors (Ruiz-Velasco and Ikeda, 2001). Labeling of Gγ is also possible at the C terminus (Bünemann et al., 2003); this removes the C-terminal CAAX box that is responsible for lipid modification of Gγ (Muntz et al., 1992), results in localization of Gγ throughout the cell, and disturbs effector coupling but still allows productive interactions with Gα (Bünemann et al., 2003). Gα-subunits can also be labeled for such studies, but such fluorescently labeled Gα-subunits are most important for experiments measuring G-protein activation by FRET or BRET and are discussed in section IV.B.

The first studies involving fluorescently labeled G-proteins (discussed in section IV.B) were done to assess G-protein activation (Janetopoulos et al., 2001; Bünemann et al., 2003; Yi et al., 2003; Azpiazu and Gautam, 2004). Subsequent studies addressed directly the issue of receptor/G-protein coupling, either by FRET (Hein et al., 2005, 2006; Nobles et al., 2005) or by BRET (Galés et al., 2005, 2006; Kuravi et al., 2010). These studies agree in some key results but disagree in others.

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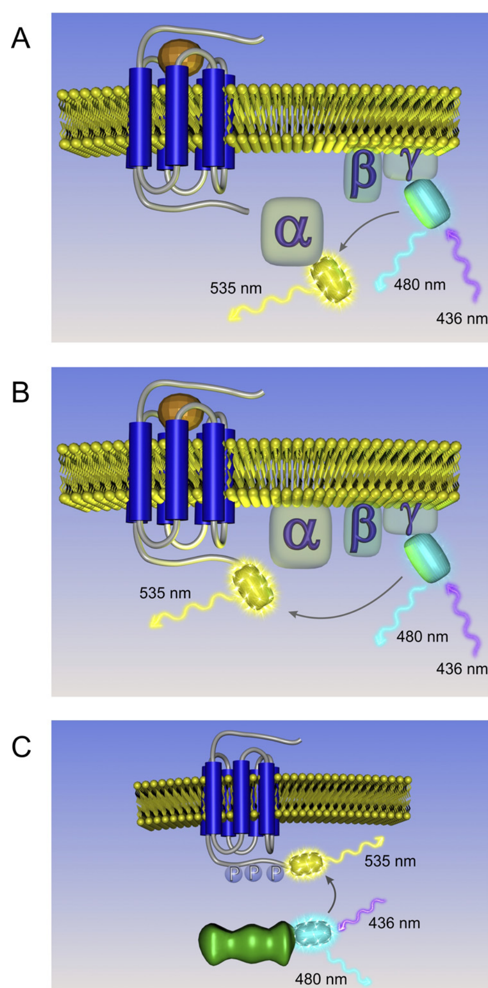

FIG. 4. FRET between receptors, G-proteins, and β-arrestins. A, FRET between a YFP-labeled G-protein α-subunit and a CFP-labeled γ-subunit. Activation of the corresponding GPCR induces rearrangement or dissociation of the G-protein subunits and a change in FRET. B, FRET between a C-terminally labeled GPCR and a G-protein. G-proteins can be labeled for such studies in any of their subunits. Stimulation of the receptor leads to an association of receptor and G-protein and thereby to an increase in FRET. C, FRET between a C-terminally labeled GPCR and β-arrestin. Agonist-stimulated receptors are phosphorylated by GRKs (see Fig. 1B) and then recruit β-arrestins from the cytosol. If receptor and β-arrestin carry a suitable fluorophore (YFP and CFP, respectively), this recruitment results in an increase in FRET.

There is overall agreement that receptor/G-protein coupling can be very fast. FRET studies designed to assess the kinetics found that the activation speed of receptors (determined by GPCR FRET sensors as described in section III.B) and that of the receptor/G-protein interaction are indistinguishable, with time constants on the order of 50 ms, provided that enough G-protein is expressed (Hein et al., 2005, 2006). Likewise, BRET studies (which have slower registration speeds because of lesser light emission compared with FRET) also observed subsecond kinetics of the receptor/G-protein interaction (Galés et al., 2005, 2006). The different studies also agree in their observation that the agonist-dependent increase in FRET between receptor and G-protein lasts as long as agonist is present (Galés et al., 2005, 2006; Hein et al., 2005, 2006). This is surprising given the generally assumed model that the receptor/G-protein complex dissociates once the Gα-subunit has bound GTP (Hepler and Gilman, 1992; Bourne, 1997). It suggests that either a small fraction of receptors and G-proteins remain tightly coupled and produce large FRET signals, or—more likely—that the receptor-G-protein complexes studied (β_1-, β_2-adrenergic and A_{2A}-adenosine receptors with G_s; α_{2A}-adrenergic receptors with G_i) do not completely disassemble after activation but rather rearrange so that the G-protein can signal to effector(s) but remains in close contact with the receptor. BRET studies with the pair β_2-adrenergic receptor/G_s found an off-reaction in the range of several seconds when the inverse agonists (±)-1-[2,3-(dihydro-7-methyl-1*H*-inden-4-yl)oxy]-3-[(1-methylethyl)amino]-2-butanol (ICI 118,551) was added, but agonist removal was not tested because of technical limitation using suspended cells (Galés et al., 2005). In similar FRET studies with α_{2A}- and β_1-adrenergic and A_{2A}-adenosine receptors with their G-proteins, the FRET signals declined, showing time constants of several seconds upon agonist washout (Hein et al., 2005, 2006).

The rapid activation kinetics of the receptor/G-protein interaction may suggest that receptors and G-proteins might be nearby or preassembled before activation and thus be taken as evidence for a precoupled model (Galés et al., 2005, 2006). This would also be in line with the kinetic studies suggesting no or little disassembly of the complex in the continued presence of agonists and possibly even beyond. Precoupling was further supported in BRET studies reporting basal BRET signals between many receptors (α_{2A}- and β_2-adrenergic, vasopressin V_2, thromboxane A_2 TPα, sensory neuron-specific SNSR-4, and calcitonin gene-related peptide receptors) and their G-proteins in the absence of agonists (Galés et al., 2005, 2006). Basal BRET was also observed between the B_2 bradykinin receptor and G_q (Philip et al., 2007), between the protease activated receptor-1 and $G\alpha_{i1}$ (Ayoub et al., 2007), and between δ-opioid receptors and $G\alpha_{i1}$ (Audet et al., 2008). BRET studies investigating a β_2-adrenergic receptor variant, which could not reach the cell surface, together with G_s have concluded that receptor/G protein complexes may already be formed before plasma membrane localization (Dupré et al., 2006). In FRET studies, similar experiments gave contradictory results—basal FRET signals for α_{2A}-adrenergic, D_2 dopamine, M_4 muscarinic, and A_1 adenosine receptors with G_o and IP prostacyclin receptors and G_s in one study (Nobles et al., 2005), but no basal FRET for α_{2A}-adrenergic receptors (with G_i) or β_2-adrenergic receptors (with G_s) in others (Hein et al., 2005). The same was true for the combination β_2-adrenergic receptors (with G_s) using BRET (Kuravi et al., 2010). There are several potential explanations for these discrepancies. First, BRET has a lower background and, compared with FRET, may thus be the better technique for the detection of low basal levels of interaction. Second, the interactions are likely to be dependent on the affinity of receptors and G-proteins toward each other and thus dependent on their expression

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level. In this case, the question of precoupling may be a rather semantic one, because some receptors will display higher affinities for their G-proteins than others. Third, precoupling may indeed be a receptor- and G-protein-specific effect. In fact, several reports suggest that G_i-coupled receptors might be particularly tightly bound to their G-protein, as can be seen in pronounced high-affinity agonist binding, cosolubilization, and copurification of receptors with G_i; high levels of constitutive activity, and a lack of modulation of second messenger production when receptor expression levels change (Lohse et al., 1984; Klotz et al., 1986; Senogles et al., 1987; Tian et al., 1994; Roka et al., 1999).

A more recent study using BRET to investigate complex formation in the β_2-adrenergic receptor system reported that β_2 receptors constitutively associate with each other and with several other class A GPCRs but that the receptors and the G-proteins are unlikely to form stable preassembled complexes (Kuravi et al., 2010). Again, this is different from the recent publication using the M_3-acetylcholine receptor and G_q, in which fluorescence recovery after photobleaching was used to demonstrate an inactive-state preassembly between receptor and G-protein (Qin et al., 2011). As mentioned above, it is necessary to keep in mind the technical limitations of such experiments that may affect the conclusions. Further data on the issue of precoupling versus collision coupling between receptors and G-proteins have been derived from the study of G-protein activation and will be discussed in the following section.

B. G-Protein Activation

The interaction between GPCRs and their G-proteins directly results in G-protein activation. Receptors trigger the GTPase cycle of G-proteins, which switch between an active, GTP-bound state and an inactive, GDP-bound state (Offermanns, 2003). G-protein activation by receptors begins with the release of GDP from the Gα-subunit and formation of a high-affinity ternary complex among agonist, receptor, and G-protein, followed by binding of GTP by the Gα-subunit; this induces a conformational change that is generally believed to result in dissociation of the complex—i.e., release of the receptor as well as dissociation of the G-protein into the Gα-subunit and the G$\beta\gamma$-complex—which can both interact with and regulate effectors until the GTPase activity of the Gα-subunit metabolizes GTP into GDP and thereby reverts the G-protein back into its trimeric inactive state (Gilman, 1987; Bourne et al., 1990). The relatively slow catalytic rates of 2 to 4 cycles/min of the GTPase can be accelerated by 3 orders of magnitude by regulators of G-protein signaling (RGS) proteins (Ross and Wilkie, 2000; Sjögren et al., 2010). On the other side, phosducins can bind G$\beta\gamma$ and thereby lead to disruption of the GTPase cycle (Bauer et al., 1992; Schröder and Lohse, 1996; Beetz et al., 2009) and affect G$\beta\gamma$ folding (Humrich et al., 2005; Willardson and Howlett, 2007). There is good evidence that both the Gα-subunit and the G$\beta\gamma$-complex participate in the activation process of G-proteins by GPCRs (Cherfils and Chabre, 2003; Dupré et al., 2009; Hofmann et al., 2009).

The changes in the structure of G-proteins during the GTPase cycle make it possible to observe it with FRET or BRET techniques by labeling the subunits with fluorophores. These studies were historically the first to analyze GPCR-mediated signaling in intact cells, and the general strategy is depicted in Fig. 4A. The key issue is that both the Gα-subunit of a given G-protein and the G$\beta\gamma$-complex need to carry a label. Sites for labeling the β- and γ-subunits have been described above. In Gα-subunits, GFPs have been placed into internal sites, because both the N and the C termini are critical for receptor coupling (see section IV.A). Possible internal insertion sites include the connecting loops that have been shown earlier to tolerate small (or even larger) insertions, such as the αAB-loop and the αBC-loop within the α-helical domain of the Gα_{i1}-subunit—sites that had been used earlier to insert various sequences into Gα-subunits and also to tolerate GFP insertions (Hughes et al., 2001; Janetopoulos et al., 2001; Yu and Rasenick, 2002). In the yeast Gα-homolog Gpa1p, insertion of CFP has also been done directly after the N-terminal myristoylation site (Yi et al., 2003). Overall, these modified G-protein subunits retained their functions, including GTPase activity, receptor coupling, and effector activation (Hughes et al., 2001; Janetopoulos et al., 2001; Yu and Rasenick, 2002; Bünemann et al., 2003; Yi et al., 2003; Hein et al., 2005, 2006), even though doubts have been raised about the functionality of some internal insertions in Gα (Gibson and Gilman, 2006).

To observe G-protein activation by FRET or by BRET, one label is placed in the Gα-subunit, and a second label is placed in either the Gβ- or the Gγ-subunit. Overall, the experiments reported so far agree about the kinetics of G-protein activation in intact cells. It is apparent that this process is, again, fairly rapid, but not as rapid as the activation of GPCRs or the interactions of activated GPCRs with G-proteins. Although these two processes occur with time constants on the order of 50 ms, FRET signals resulting from G-protein activation are approximately 10 times slower, even under conditions optimized for speed. This means they occur with activation time constants of approximately 500 ms (Bünemann et al., 2003; Galés et al., 2006; Hein et al., 2006; Adjobo-Hermans et al., 2011). As to be expected from the biochemistry of G-protein GTPase, the reversal of these signals after agonist removal (i.e., the deactivation of G-proteins) is even slower, with time constants of many seconds (Bünemann et al., 2003; Galés et al., 2006; Hein et al., 2006). In some instances, it has been shown that the G-protein deactivation depended on the receptor used to stimulate the G-protein; for example, G_s deactivation after stimulation via A_{2A}-adenosine receptors occurred with a time constant of almost 40 s, whereas it was 15 s after stimulation via β_1-adrenergic receptors (Hein et al., 2006). RGS proteins markedly increase the speed of G-protein deactivation; for

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example, in yeast, the rate constant of deactivation of the G-protein under basal conditions was 0.004 s^{-1}, whereas under overexpression of the RGS homolog Sst2p, it was 0.1 s^{-1} (i.e., 25-fold faster) (Yi et al., 2003). The physiological relevance of these activation and deactivation times has been underlined by the observation that the time courses of GIRK channel opening and of FRET signals in its regulator G_{i1} follow the exact same time course (Bünemann et al., 2003). The deactivation of receptors, receptor/G-protein interaction, and G-protein activity seems to occur in this sequence; for example, in the case of A_{2A}-adenosine receptors and G_s, the half-times of deactivation were ≈2 s for the receptor, ≈10 s for the receptor/G_s interaction, and ≈25 s for G_s (Hein et al., 2006).

G-protein sensors have also been used to address the issue of receptor/G-protein precoupling versus collision coupling (see above; Hein and Bünemann, 2009). By investigating the effects of various receptors present at different densities in CHO cells, Azpiazu and Gautam (2004) reported that different types of receptors (e.g., muscarinic and serotonin) shared common pools of G-proteins to which they seemed to have free access—a result that conflicts with a model of organized, preassembled receptor/G-protein complexes. This is in line with data mentioned above, which show that the kinetics of the FRET signal between α_{2A}-adrenergic receptors and G_i were dependent on the G-protein level and that the kinetics of GIRK channel activation were likewise dependent on the extent of receptor activation (Hein et al., 2005). These data suggest that the activation of G-proteins by receptors occurred by collision coupling. A collision coupling model is further suggested by the often large amplification between receptors and G-proteins, which FRET studies indicate. An example is shown in Fig. 5, which displays the FRET signals of α_{2A}-adrenergic receptors and G_i in transfected HEK293 cells (from Nikolaev et al., 2006c). It can be seen that, compared with the full agonist norepinephrine (100%), the partial agonist norphenylephrine (NF) produced signals of only ≈15% at the receptor but ≈50% at the G-protein; similar data were observed with other partial agonists, suggesting that it is a general phenomenon. Such amplification (also called "receptor reserve") of a signal from the receptor to the G-protein level is most likely due to the fact that one receptor activates these G-proteins, and that receptors have (more or less) free access to multiple G-proteins—as suggested by early experiments on the β-adrenergic receptor system in turkey erythrocytes that led to the formulation of the collision coupling model (Tolkovsky and Levitzki, 1978a,b).

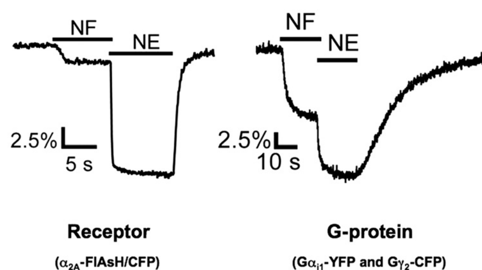

FIG. 5. Evidence for a receptor reserve between a GPCR and its G-protein. Shown are the traces of activation of an α_{2A}-adrenergic receptor (left; labeled with FlAsH and CFP), and of the corresponding G-protein, G_{i1} (right; labeled with YFP in the $G\alpha_{i1}$-subunit and with CFP in the $G\gamma_2$-subunit). The receptors were stimulated for the indicated periods with the partial agonist norphenylephrine (NF) or the full agonist norepinephrine (NE). The experiments were done in transfected HEK293 cells. Note the relatively much larger effect of NF at the G-protein level compared with the receptor level. [Adapted from Nikolaev VO, Hoffmann C, Bünemann M, Lohse MJ, and Vilardaga JP (2006c) Molecular basis of partial agonism at the neurotransmitter α_{2A}-adrenergic receptor and G_i-protein heterotrimer. *J Biol Chem* **281:**24506–24511. Copyright © 2006 American Society for Biochemistry and Molecular Biology. Used with permission.].

Potential solutions to discrepant results on receptor/G-protein precoupling versus collision coupling have been proposed as alternative or unifying models. The restricted collision coupling model assumes that receptors have free access to G-proteins only in a small region; this has been observed for A_{2A}-adenosine receptors coupled to G_s (Braun and Levitzki, 1979; Gross and Lohse, 1991). It has been proposed that such limiting regions might be due to receptors and G-proteins residing in membrane compartments that differ in lipid composition (Ostrom et al., 2000; Patel et al., 2008). A study on A_{2A}-adenosine receptors suggested, on the basis of FRAP experiments, that A_{2A} receptors couple to G_s in cholesterol-rich domains, whereas coupling to mitogen-activated protein kinases occurs elsewhere (Charalambous et al., 2008). Likewise, Pontier et al. (2008) proposed that β_2-adrenergic receptors did not partition within the liquid-ordered lipid phase, whereas G-proteins and adenylyl cyclases were sequestered in these domains, and that their mobilization led to increased receptor-mediated signaling. Milan-Lobo et al. (2009) suggested, on the basis of FRET and FRAP studies, that the restricted mobility of corticotropin-releasing factor receptors (CRFR2) may be increased by agonist activation of the receptors, suggesting a transfer into a different membrane compartment or loss of contact with the cytoskeleton.

Such potential organization of signaling proteins in or by lipid rafts has been a major hypothesis for many years (Patel et al., 2008; Lingwood and Simons, 2010), even though high-resolution microscopy experiments have revealed that sphingolipids and glycosylphosphatidylinositol-anchored proteins interact with cholesterol-mediated complexes only for very short time periods (10–20 ms) and in small (<20 nm) complexes (Eggeling et al., 2009)—sizes and times that are not compatible with a function as organizers of transmembrane signaling. Caveolae are another structural element that has been proposed to organize GPCR signaling at the cell surface (Patel et al., 2008). No FRET or BRET studies have been done so far to image directly such lateral organization of GPCRs and their downstream signaling proteins in the cell membrane.

Another approach to reconcile the apparently discrepant data on receptor/G-protein coupling comes from mathematical modeling of signal transduction in dependence of the expression levels and mobility of receptors, G-proteins, and downstream signaling proteins (Ugur and Onaran,

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1997). Thus, Brinkerhoff et al. (2008) pointed out that variations in the expression levels of the individual components (receptors, G-protein subunits) may affect the collision coupling model to behave as if access of receptors to G-proteins were limited. These authors therefore concluded that the collision coupling model, without compartments of receptors or G-proteins, is sufficient to explain a great variety of experimental observations that might, at least, be assumed to be in discordance with collision coupling model predictions.

However, free access to each other caused by free diffusion of receptors and G-proteins at the cell surface may not be typical for all receptor and cell types. In fact, recent data using a FRET sensor for cAMP (see section VI) suggests that GPCRs may indeed have specific subcellular localizations (Nikolaev et al., 2010). In this study, it was observed that in isolated cardiac myocytes, cAMP signals could be elicited by β_2-adrenergic receptor stimulation only from T-tubules, whereas β_1-adrenergic receptor stimulation caused cAMP signals from all over the cell surface. Such organization of receptors and G-proteins at the surface of cells might be a property of differentiated cells such as cardiomyocytes or possibly neurons and reflect their highly organized overall structure.

A similar point of discussion that emanates from FRET studies is the question of whether G-proteins dissociate during activation. The classic view, based on a plethora of biochemical data, is that Gα and G$\beta\gamma$ dissociate upon binding of GTP by Gα (Gilman, 1987; Bourne et al., 1990) and that in some instances, Gα even moves from the cell membrane to the cytosol (Ransnäs et al., 1989; Rudolph et al., 1989). If this were also the case in intact cells, then FRET between Gα and G$\beta\gamma$ should decrease upon G-protein activation and should be close to zero at full activation. Most studies have indeed observed such a decrease in FRET (Janetopoulos et al., 2001; Bünemann et al., 2003; Yi et al., 2003; Azpiazu and Gautam, 2004; Hein et al., 2006) or BRET (Galés et al., 2005, 2006; Kuravi et al., 2010). However, a full loss of FRET has rarely been observed (Azpiazu and Gautam, 2004), and in most instances, a high proportion of FRET or BRET remained even after full agonist activation (Bünemann et al., 2003; Hein et al., 2006; Lambert, 2008). Such residual FRET might be a consequence of incomplete activation of the respective G-proteins, because in most cells, the levels of a given receptor are well below the levels of the corresponding G-protein(s), so that even when a single receptor activates many G-proteins, it does not activate all of them. A second explanation for an incomplete loss of FRET is that during the GTPase cycle, only a fraction of activated G-proteins is in the dissociated state. A third possibility, however, is that Gα and G$\beta\gamma$ do not dissociate completely, but rather rearrange such that some degree of FRET remains even between GTP-bound Gα and G$\beta\gamma$. This view is supported by studies showing that with suitably placed fluorophores, FRET between Gα and G$\beta\gamma$ may even increase upon activation (Bünemann et al., 2003; Galés et al., 2006). Such an increase might be caused by a relative movement of Gα and G$\beta\gamma$ so that some regions of the proteins come closer to each other, whereas the interface between Gα and G$\beta\gamma$, which is required for coupling to effectors (Ford et al., 1998; Davis et al., 2005; Lin and Smrcka, 2011), becomes available. Although the full functionality of one of the internally labeled Gα constructs that have been used in these studies has been doubted (Gibson and Gilman, 2006), the concept of G-protein rearrangement rather than dissociation is in agreement with older studies showing that nondissociable G-protein constructs are still capable of transmitting signals (Rebois et al., 1997; Klein et al., 2000; Levitzki and Klein, 2002). Furthermore, the ability of G-proteins to show such increases in FRET upon activation seems to depend on the subtype: it has been observed for G_{i1}, G_{i2}, G_{i3}, and G_z but not for G_o, and it has been attributed to a G_i-specific region in the $\alpha_{B/C}$-region of Gα (Frank et al., 2005). It has also been reported for G_q, depending on the type of G$\beta\gamma$ (Chisari et al., 2009). In the end, it may well be that—as in the case of receptor/G-protein coupling—the issue is more semantic than fundamental: if G-protein subunits have finite affinities toward each other in their different activation states, then they may partly dissociate when the affinity is reduced (i.e., upon binding of GTP), but a smaller or larger fraction may remain associated if the concentrations of Gα and G$\beta\gamma$ are high [i.e., in the range of their dissociation constant(s)].

V. Interactions with G-Protein-Coupled Receptor Kinases and β-Arrestins

After the transmission of a signal from GPCRs to G-proteins, many GPCRs become phosphorylated by G-protein-coupled receptor kinases (Benovic et al., 1986, 1989; Krupnick and Benovic, 1998) and some GPCRs also by second messenger-activated kinases such as protein kinases A (PKA) and C (Clark, 1986; Hausdorff et al., 1990; Lohse et al., 1990). Although—on the basis of detailed studies of the β_2-adrenergic receptor—the latter kinases are thought to directly phosphorylate regions of the receptors that are involved in G-protein coupling and thereby inhibit receptor/G-protein interactions, phosphorylation by the GRKs leads to binding of β-arrestins (Lohse, 1993). The GRK/ β-arrestin mechanism was initially perceived as a pure desensitization mechanism preventing further G-protein activation by the agonist-activated receptors (Lohse et al., 1990b), but it was subsequently realized that it also triggers endosomal receptor internalization, which is followed by dephosphorylation, receptor recycling, and resensitization or by receptor degradation (Pippig et al., 1995; Ferguson et al., 1996; Goodman et al., 1996; Krupnick and Benovic, 1998; see Fig. 1B). Finally, it has become apparent that receptor-bound β-arrestins elicit "nonclassic" signals such as activation of the extracellular signal-regulated kinase pathway by interacting with multiple signaling proteins, including Src and extracellular signal-regulated kinase 1/2 (Luttrell et al., 1999, 2001;

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Shukla et al., 2011). The multiplicity of functions requires that the sequence of G-protein activation/GRK-mediated phosphorylation/β-arrestin binding/internalization/β-arrestin-mediated signaling/degradation or recycling must be well orchestrated to shape the final output of receptor stimulation and its temporal and presumably also spatial patterns.

A. G-Protein-Coupled Receptor/G-Protein-Coupled Receptor Kinase Interactions

Phosphorylation of agonist-activated GPCRs by GRKs is the first step in this cascade of events. GRKs are a family of seven serine/threonine kinases that fall into three subfamilies: 1) GRK1 and -7 are retinal kinases that phosphorylate rod and cone photoreceptors; 2) GRK2 and -3 are ubiquitously expressed cytosolic kinases that are targeted to the cell membrane via a pleckstrin homology domain that binds to $G\beta\gamma$ as well as membrane lipids; and 3) GRK4, -5, and -6 are ubiquitous (GRK5 and -6) or testes-specific (GRK4) and are constitutively membrane-bound as a result of lipid modifications (Benovic et al., 1986, 1989; Lohse et al., 1996; Pitcher et al., 1998). GRKs are activated by their substrates, the active form of GPCRs, plus via several regulatory mechanisms, including activation by protein kinase C and by Ca^{2+}/calmodulin (Chuang et al., 1995; Winstel et al., 1996; Pronin et al., 1997; Krasel et al., 2001) and degradation via several β-arrestin-dependent pathways (Nogués et al., 2011). There is no known linear consensus sequence for GRK-mediated phosphorylation, and the phosphorylation sites within receptors are mostly ill-defined; a so-called "bar code" hypothesis assumes that the exact phosphorylation sites in a given receptor may depend on both the type of GRK and the specific agonist and that these specific phosphorylation patterns may determine the type of regulation that ensues (Ren et al., 2005; Violin et al., 2006; Busillo et al., 2010; Butcher et al., 2011). These data suggest that multiple domains of the GRKs interact with multiple domains of the activated receptors to generate a productive interaction and to elicit full catalytic activity (Boguth et al., 2010), and the latter seems to require a conformational change in the GRKs involving interactions between their N-terminal and the central kinase domains (Pao et al., 2009; Huang et al., 2011).

Only a few studies have investigated the interaction between receptors and GRKs by resonance energy transfer techniques. Hasbi et al. (2004) fused luciferase to the oxytocin receptor C terminus and YFP to the GRK2 C terminus and observed a BRET signal that started immediately after agonist addition and achieved almost 80% of the maximum at the first time point of their measurements at 4 s. The signal showed a peak of this interaction for approximately 1 min and then declined, reaching approximately a third of the maximum at 8 min. These data indicate that the receptor/GRK interaction is transient in nature and that GRKs dissociate from the phosphorylated receptor. A similar time course of oxytocin receptor/GRK2 interaction was found in coimmunoprecipitation and in membrane translocation assays.

In BRET[2] studies of the luciferase-tagged glucagon-like peptide (GLP-1) receptor and GFP[2]-tagged GRK2, Jorgensen et al. (2007) likewise observed a rapid interaction that peaked at 1 to 2 min. In concentration-response curves with the full agonist GLP-1 and the partial agonist oxyntomodulin, they found approximately the same concentration dependence for the interactions of the receptors with either GRK2 or β-arrestins (see section V.B), suggesting that both of these interactions are strictly agonist-dependent and therefore follow the receptor occupancy curve (Lohse et al., 1990a). In contrast, cAMP signals elicited by the same compounds were left-shifted by 2 orders of magnitude, more for the full agonist GLP-1 than for the partial agonist oxyntomodulin, as expected for an amplification process or a receptor reserve (see section IV.B).

In similar BRET[2] studies on the neurokinin NK1 receptor, Jorgensen et al. (2008) investigated the interaction of the receptor with GRK2 and GRK5. Both showed a rapid signal that peaked after 10 to 20 s, but the GRK2 signal started from much lower basal values and was therefore of a larger amplitude; the authors interpreted this as a preassociation of receptors and (membrane-localized) GRK5, whereas GRK2 needs to be recruited from the cytosol. Binding of GRK2 to the receptors (as assessed from BRET[2] saturation experiments) had a high-affinity component that was kinase-independent and was lacking in GRK5; this component may represent an additional binding event that may be needed for the recruitment of cytosolic GRK2. Perhaps in line with this high-affinity component, a further difference was that the NK1 receptor/GRK2 complex seemed fairly stable, whereas the complex with GRK5 was not.

In summary, the interaction of GRKs with GPCRs can be monitored by BRET and has been shown to occur rapidly after agonist stimulation (within seconds). A comparison with the recruitment (≈50 ms) and activation (≈500 ms) times of G-proteins (Galés et al., 2005, 2006; Hein et al., 2005, 2006) is difficult because of the different experimental setups used, but these times are in line with the obvious notion that G-protein-mediated signals need to be initiated before they can be switched off. An interesting approach to solve this problem was introduced by the group of Michel Bouvier, who combined independent BRET and FRET readouts for receptor/G-protein interaction, receptor/GRK interaction, and G-protein activation. Although theoretically all steps could be measured in the same cell using multiple controls and corrections, for simplicity they kinetically analyzed only one step per well. Because all cells were derived from the same transfection, kinetic data from these experiments could be compared and showed that receptor/G-protein interaction is fast and is followed by G-protein activation and receptor/GRK interaction (Breton et al., 2010).

Huang et al. (2011) have recently described a conformational change in GRKs that is thought to occur upon bind-

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ing to receptors. This relatively large change, in principle, should also be amenable to investigation by intramolecular FRET, but to our knowledge, this has not yet been attempted.

In most instances, GRKs are thought to act by phosphorylating sites in GPCRs that provide binding sites for β-arrestins (see section V.B), which then switch off G-protein interactions and trigger receptor internalization and nonclassic signaling. However, in a few cases, it seems that the interactions of GRKs with the receptors but also with G-proteins by themselves suffice to inhibit G-protein-dependent signaling (Dicker et al., 1999; Pao and Benovic, 2002). Whether such direct inhibitory actions are due to a different mode of interaction with the receptors has not yet been investigated by resonance transfer or other techniques.

B. Binding of β-Arrestins

The paradigm of receptor phosphorylation followed by binding of an inhibitory arrestin molecule has initially been developed from experiments in the visual rhodopsin system. Hermann Kühn and his group discovered phosphorylation of rhodopsin (Kühn and Dreyer, 1972; Kühn, 1974) as well as the subsequent binding of visual arrestin [then called 48-kDa protein or S-antigen, because it can elicit autoimmune uveoretinitis in the eye (Dorey and Faure, 1977; Shinohara et al., 1991)]. They also discovered that this binding resulted in inhibition of signaling from rhodopsin to G_t (Kühn et al., 1984; Wilden et al., 1986). The observation that retinal arrestin can inhibit signaling by GRK2-phosphorylated β_2-adrenergic receptors (Benovic et al., 1987) led to the hypothesis that an analogous system might exist for this receptor and to the discovery of β-arrestin (Lohse et al., 1990b). Despite a high degree of homology to visual arrestin (Shinohara et al., 1987), β-arrestin and visual arrestin show specificity for binding to β-adrenergic receptors versus rhodopsin (Lohse et al., 1992). Two other members of this family, β-arrestin2 (Attramadal et al., 1992) and cone arrestin (Craft et al., 1994), were subsequently identified; together with GRK7, cone arrestins seem to be specific for the color opsins. The two β-arrestins (also termed arrestin-2 and arrestin-3) seem to differ from the visual arrestins (also termed arrestin-1 and arrestin-4) by their ability to couple to multiple other proteins and, thereby, to initiate receptor internalization as well as many nonclassic signaling pathways (Shenoy and Lefkowitz, 2011; Shukla et al., 2011). As in the case of signaling to G-proteins, it seems that monomeric GPCRs are sufficient for an interaction with (β-)arrestins; this can be concluded from experiments with reconstituted monomeric rhodopsin, which is well capable of binding visual arrestin (Bayburt et al., 2011).

β-Arrestins are subject to various types of modifications that alter their subcellular distribution as well as their function as an adaptor and signaling protein (Shenoy and Lefkowitz, 2011; Shukla et al., 2011): homo- and heterodimerization (Storez et al., 2005), ubiquitinylation (Shenoy et al., 2001; Shenoy and Lefkowitz, 2005), sumoylation (Wyatt et al., 2011), phosphorylation (Lin et al., 1997; Barthet et al., 2009), and nitrosylation (Lohse and Klenk, 2008; Ozawa et al., 2008).

The translocation from the cytosol to the cell membrane that β-arrestins undergo to bind to agonist-stimulated GPCRs (Ungerer et al., 1996) makes this process particularly suitable for optical assays, including BRET and FRET. One of the first such assays was developed with a β-arrestin2-GFP conjugate that showed a strong translocation to the cell surface in response to stimulation of a large variety of GPCRs (Barak et al., 1997a). This assay even proved suitable for the identification of ligands for orphan receptors (i.e., receptors lacking a known ligand) and was subsequently shown to work for many more GPCRs.

A BRET assay for the interaction of β-arrestins with GPCRs was first described by Angers et al. (2000) for the β_2-adrenergic receptor, labeled at its C terminus with luciferase, and β-arrestin, labeled at its C terminus with YFP; they observed a large (>10-fold above background) signal, which was agonist-dependent with high sensitivity and affinity (EC_{50} of isoproterenol 0.4 nM). Similar studies followed for the thyrotropin-releasing hormone receptor (Kroeger et al., 2001), where receptor-specific differences were subsequently shown for β-arrestin1 versus β-arrestin2. The TRH1 receptor interacted equally well with β-arrestin1 and -2, whereas the TRH2 subtype preferred β-arrestin2 (Hanyaloglu et al., 2002). A better interaction with β-arrestin2 over β-arrestin1 has been observed for many GPCRs, and these are called class A GPCRs, whereas class B receptors do bind equally well to both β-arrestins (and also to visual arrestins; Oakley et al., 2000). BRET-based assays for the GPCR/β-arrestin interaction are so robust that they are suitable for high-throughput screening (Bertrand et al., 2002; Hamdan et al., 2005), and they have been developed for many receptors (e.g., chemokine, opiate, dopamine, and prostanoid receptors) (Hamdan et al., 2005; Qiu et al., 2007; Coulon et al., 2008; Klewe et al., 2008; Masri et al., 2008; Leduc et al., 2009). The assays also revealed that receptors that undergo β-arrestin-independent internalization, such as the gonadotropin releasing hormone receptor, also did not show receptor/β-arrestin BRET signals (Kroeger et al., 2001).

A number of studies have investigated the GPCR/β-arrestin interaction using FRET, with β-arrestins and various receptors (α_2, β_1-, and β_2-adrenergic, PTH, μ-opiate, and various P2Y purine receptors (Vilardaga et al., 2003; Krasel et al., 2005, 2008; Hoffmann et al., 2008a; Reiner et al., 2009, 2010; Frölich et al., 2011). The strategy is very similar to that of receptor/G-protein coupling and is depicted in Fig. 4C. The receptors were usually C-terminally labeled with either CFP or YFP or with the small FlAsH label, and the β-arrestins were C-terminally labeled with CFP or YFP. An alternative has been developed that uses specific, orthogonal labeling of receptor and of β-arrestin with two small fluorophores, FlAsH and red arsenical hair-

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pin binder; although this technology has the distinct advantage of using two small labels, the fluorescence of red arsenical hairpin binder is weak and thus only weak FRET signals were observed (Zürn et al., 2010).

FRET studies have proven to be particularly well suited for the kinetic analysis and, thus, the sequence of these events, because they allow fast sampling rates and, consequently, accurate recordings. Vilardaga et al. (2003) compared the kinetics of receptor activation and β-arrestin2 recruitment for the PTH receptor and described a lag time between rapid receptor activation and slower β-arrestin2 recruitment; this short time represents the period when the receptors are available to signal to G-proteins. It is due mainly to the fact that the β-arrestin2 recruitment proceeds more slowly than the receptor activation, which, as detailed in section III.B, can occur in the 50-ms time range. Furthermore, a lag time was observed between agonist stimulation and the beginning of β-arrestin2 recruitment, whereas there was none for the receptor activation (Vilardaga et al., 2003). A similar lag time before the onset of β-arrestin2 recruitment was observed in BRET analyses of this process. Specifically, in their study on oxytocin receptors, Hasbi et al. (2004) showed that β-arrestin recruitment caused a BRET signal that started only 10 s and achieved its maximum only 35 s after agonist addition. There is good evidence to believe that the delay and the slow time course of β-arrestin2 recruitment are limited by GRK-mediated receptor phosphorylation (Hasbi et al., 2004; Krasel et al., 2005). For example, the β-arrestin recruitment was much faster when phosphorylation-insensitive mutants were used, when GRKs were overexpressed, or when the receptors had been prephosphorylated by a prior stimulus (Krasel et al., 2005). For the β_2-adrenergic receptors in HEK293 cells, the half-times of β-arrestin2 binding were ≈20 s for nonphosphorylated receptors, but only ≈2 s under conditions in which phosphorylation was not required (Krasel et al., 2005). A mathematical model has been developed that describes this "memory" effect of prior and relatively long-lasting receptor phosphorylation as a means of providing rapid desensitization of receptors (Vayttaden et al., 2010).

FRET studies in intact cells showed a clear requirement for both agonist occupancy and GRK-mediated phosphorylation for β-arrestin binding to receptors (Krasel et al., 2005), a finding that had not been fully visible from experiments with isolated purified systems (Söhlemann et al., 1995; Gurevich and Gurevich, 2006). After removal of the agonist, β-arrestins dissociate from the receptors within seconds, with a speed that is closely linked to the dissociation of the agonist from the receptors (Krasel et al., 2005).

A lot of data have accumulated to suggest that agonists at GPCRs may differ in their ability to trigger G-protein-mediated versus β-arrestin-mediated signaling (see section III.B). This concept of "biased signaling" or "functional selectivity" assumes that different agonists may induce different active conformations of a receptor and that these different conformations may have different abilities to couple to and trigger G-proteins and/or β-arrestins (Urban et al., 2007; Violin and Lefkowitz, 2007). Quantification of these interactions with BRET and FRET assays has greatly helped to support and further develop this concept. It is noteworthy that FRET assays have also been able to show that not only synthetic but also endogenous agonists may differ in their effects. For example, at the $P2Y_2$ receptor, UTP induced equal recruitment of β-arrestin1 and -2, whereas ATP resulted in much better recruitment of β-arrestin2 than β-arrestin1 (Hoffmann et al., 2008a). This would classify the UTP-stimulated $P2Y_2$ receptor as a class B receptor, whereas the same receptor stimulated with ATP would be classified as a class A receptor according to the classification of Oakley et al. (2000). Differences between endogenous ligands were also reported for the β_2-adrenergic receptor, where norepinephrine and epinephrine were equally effective in eliciting G_s activation at the β_2-adrenergic receptor, but norepinephrine caused less β-arrestin2 recruitment (Reiner et al., 2010).

It seems that the recruitment of β-arrestins from the cytosol and their binding to GPCRs may be a multistep process, where different intracellular regions of the receptors may be sequentially involved (Krasel et al., 2008). The binding step seems to be further modulated by other proteins that bind to the cytosolic face of GPCRs, such as the sodium proton exchanger regulatory factor NHERF1, which has been shown to bind to the C terminus of the PTH receptor and to facilitate its interaction with β-arrestin2 (Klenk et al., 2010).

In line with a multistep binding model of the GPCR/β-arrestin interaction, there is good evidence of a conformational change in β-arrestins upon receptor binding. On the basis of crystallographic as well as mutagenesis data, it seems that during binding of β-arrestins to GPCRs, the N- and the C-terminal domains loosen their interactions, which liberates the C terminus for interactions with clathrin and adaptin (AP2; Gurevich and Gurevich, 2006). Charest et al. (2005) used BRET between luciferase and YFP flanking the β-arrestin2 sequence to monitor this conformational change in intact cells. They reported an increase in BRET upon receptor activation, suggesting that the C terminus of β-arrestin2 moved closer to its N terminus. This change occurred over several minutes, suggesting that it followed the initial receptor/β-arrestin2 interaction, which was also measured by BRET between labeled V_2-vasopressin or β_2-adrenergic receptors and β-arrestin2. In line with the concept of biased agonism, Shukla et al. (2008) investigated conformational changes of this BRET sensor for β-arrestin2 in response to biased agonists at several receptors or at biased receptor mutants. They reported a common pattern of BRET changes: an increase in BRET for unbiased ligands and a decrease for β-arrestin-biased ligands or β-arrestin-biased receptor mutants. The authors suggest that an increase in BRET reflects a conformation of β-arrestin that mediates both desensitization and signaling via β-arrestin, whereas a decrease in BRET represents a conformation that only

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signals without mediating desensitization (Shukla et al., 2008).

β-Arrestin1 and -2 have been suggested to have different cellular localizations. β-Arrestin1 traffics to the nucleus whereas β-arrestin2 does not, presumably owing to a nuclear export signal in the β-arrestin2 C terminus (Scott et al., 2002); in the nucleus, β-arrestin1 has been reported to enhance gene transcription via recruitment of the histone acetyl transferase p300 (Kang et al., 2005). Subcellular trafficking of β-arrestins and its modulation by the formation of homo- and heterodimers has been investigated with the help of BRET and FRET (Storez et al., 2005). These studies led to the conclusion that coexpression of β-arrestin2 and β-arrestin1 prevented β-arrestin1 accumulation in the nucleus; this suggests that such heterodimerization may serve to regulate cytosolic versus nuclear effector mechanisms of β-arrestins.

And finally, β-arrestins have been shown to become ubiquitinylated via an Mdm2-dependent process, which is thought to be important for the formation of high-affinity complexes with receptors and their subsequent internalization (Shenoy and Lefkowitz, 2011). This ubiquitinylation has likewise been monitored in intact cells by $BRET^2$, using luciferase-labeled β-arrestin1 and GFP^2-labeled ubiquitin (Perroy et al., 2004). It is noteworthy that distinct ubiquitinylation kinetics were observed in response to activation of different receptors—transient ubiquitinylation for β_2-adrenergic receptor stimulation and sustained after stimulation of V_2 vasopressin receptors.

VI. Downstream Signaling

GPCRs interact directly with ligands, G-proteins, GRKs, and β-arrestins, and the study of these interactions with resonance energy transfer techniques has been described in the preceding sections. It is obvious that protein-protein interactions also occur beyond these immediate steps in GPCR activation and signaling and that these interactions can also be investigated by FRET and BRET techniques. This is true for the interactions between G-proteins and effectors, which have been analyzed in a few studies by resonance energy transfer studies, but also for the downstream intracellular signaling pathways that can be triggered by GPCRs. There is a plethora of studies that cover the immediate determination of second messengers as well as the subsequently activated protein kinases and other second messenger-regulated proteins (see Várnai and Balla, 2007; Prinz et al., 2008; Newman et al., 2011; Mehta and Zhang, 2011; Kiyokawa et al., 2011 for recent reviews).

In addition, there are interactions of GPCRs with additional proteins that are just beginning to be explored by resonance energy transfer and other imaging techniques. For example, some GPCRs have to associate with nonreceptor proteins to form functional receptors (Ferré et al., 2009). An example of such proteins are the so-called receptor activity-modifying proteins, which combine with several class B GPCRs such as the secretin, glucagon, vasoactive intestinal peptide, calcitonin, and calcitonin receptor-like receptors and thereby form compound heterodimers with new types of pharmacology (Parameswaran and Spielman, 2006; Wootten et al., 2010; Archbold et al., 2011). The assembly of such heterodimers has been observed in intact cells using BRET by Héroux et al. (2007a,b). Combining BRET with bimolecular fluorescence complementation, the authors reported that functional calcitonin gene-related peptide receptors are asymmetric units consisting of a homo-oligomer of the calcitonin receptor-like receptor and a single receptor activity-modifying protein-1 (Héroux et al., 2007b). Such asymmetric assemblies have been discussed in section III.C for receptor dimer/G-protein assemblies but have also been reported for receptor heterodimers together with a β-arrestin (for example, for a complex consisting of an A_{2A}-adenosine receptor, a D_2-dopamine receptor, and β-arrestin2) (Borroto-Escuela et al., 2011).

Asymmetric complexes have also been described for melatonin MT_1 receptor, G_i, and RGS20 (Maurice et al., 2010) or activator of G-protein signaling-4 together with $G\alpha_{i1}$ and α_2-adrenergic receptors (Oner et al., 2010). Such multimeric complexes allow an almost unlimited array of complex pharmacologies that will certainly be a major future topic, because of both the fundamental issues of kinetics and specificity of such complex formation and the potential for new types of drug actions. BRET and FRET assays using multiple labeled fluorophores will help to study these complex assemblies.

G-protein/effector interactions have only begun to be studied with resonance transfer techniques. The available data seem to suggest a relatively tight association. This has been observed by both coimmunoprecipitation and BRET experiments to show that G-protein $G\alpha_s$- and $G\alpha_i$-subunits as well as $G\beta\gamma$-complexes form stable complexes with adenylyl cyclase and Kir3.1 channels in intact cells (Rebois et al., 2006). Preliminary FRET data also suggest tight interactions between G-protein subunits and adenylyl cyclase type V (Milde and Bünemann, 2011). Functional and kinetic studies comparing the activation of G_i-proteins and GIRK channels by FRET and by patch clamping also support the notion of a tight kinetic and presumably also spatial coupling of G-proteins and their effector channels (Bünemann et al., 2003; Lohse et al., 2008b). This is further supported by the notion that GIRK channel regulation involves both Gα subunits and, primarily, the $G\beta\gamma$ complex (Hommers et al., 2003; Berlin et al., 2010). This was additionally shown by a combined approach using FRET and total internal reflection microscopy that demonstrated a preformed complex of G-protein and GIRK channel; this preformed complex would allow a precise temporal control of activation and add to the selective activation of the channel (Riven et al., 2006).

From these data, a concept emerges that involves catalytic, collision-coupling interactions between GPCRs and

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G-proteins, so that one agonist-occupied receptor can activate many G-proteins, followed by noncatalytic, tightly coupled interactions between G-proteins and effectors (Vorobiov et al., 2000; Dascal, 2001; Lohse et al., 2008b; Hein and Bünemann, 2009).

A large number of techniques have been developed to image second messengers in intact cells with specific FRET sensors. A discussion of these is beyond the scope of this review, but we briefly mention key issues that are important for the understanding of GPCR-mediated signaling and for future studies.

FRET sensors have been generated for the key second messengers: inositol trisphosphate (Morii et al., 2002; Tanimura et al., 2004, 2009; Shirakawa et al., 2006), calcium (Miyawaki et al., 1999; Palmer and Tsien, 2006), and cAMP (Zaccolo et al., 2000; DiPilato et al., 2004; Nikolaev et al., 2004, 2006b; Ponsioen et al., 2004; Klarenbeek et al., 2011) as well as cGMP (Honda et al., 2001; Nausch et al., 2008; Nikolaev and Lohse, 2009). These sensors have been used in a multitude of experiments, mostly in cell lines or in primary isolated cells, but attempts are also being made to image second messengers in vivo (Shafer et al., 2008; Gervasi et al., 2010).

Second-messenger-regulated proteins have also been used for imaging of the step directly downstream from second messengers. The best examples here are sensors for protein kinases, which is achieved by either monitoring labeled constructs of the respective kinase itself or by measuring responses to phosphorylation of engineered substrates. This has been studied in great detail for the cAMP-dependent protein kinase A (Nagai et al., 2000; Zhang et al., 2001; Zaccolo and Pozzan, 2002; Berrera et al., 2008), but also for the Ca^{2+}-dependent kinase protein kinase C (Violin et al., 2003; Schleifenbaum et al., 2004; Brumbaugh et al., 2006), as well as for downstream kinases such as protein kinases B (Calleja et al., 2003) and D (Kunkel et al., 2007).

These studies have, in particular, concentrated on several complex questions of receptor signaling discussed in section VII. These concern issues of compartmentalization of signals, of their temporal and spatial organization and interaction, methods to image multiple signals simultaneously, and, finally, ways to image in vivo.

VII. Outlook

FRET and BRET imaging have greatly contributed to our understanding of GPCR signaling and regulation. They have, in particular, helped to elucidate the kinetics of the individual steps in isolated cells and led to models of the sequence of events (Lohse et al., 2008a,b; Jensen et al., 2009). Table 3 summarizes our current knowledge of the kinetics of the individual steps of the GPCR signaling chain from ligand binding to effector activation and second messenger production, as compiled from resonance energy transfer-based studies.

An open question is whether these kinetics reflect those of an individual signaling chain or represent aggregate responses that are presumably much slower than those of individual receptors. These difficulties will probably be overcome either by achieving single molecule sensitivity in the detection of GPCR activation and signaling or by synchronous triggering of activation (as in the case of rhodopsin), for example via caged agonists or other ultrarapid techniques of agonist delivery to receptors.

A second major issue is that of spatial control of signals and their compartmentalization. Compartmentalization of GPCRs and their signals and, in particular, of cAMP, is a topic that has been discussed for several decades (Hayes and Brunton, 1982; Zaccolo et al., 2002; Warrier et al., 2007; Berrera et al., 2008) and is discussed also in other fields of cellular signaling (Dehmelt and Bastiaens, 2010; Vartak and Bastiaens, 2010). It has been studied in much detail for cardiac myocytes, where signals triggered by different receptors may remain either localized or become ubiquitous, and where different receptors seem to trigger different types of cAMP signals (Fischmeister et al., 2006; Berrera et al., 2008; Xiang, 2011). An example of such different signals are the two β-adrenergic receptors, β_1 and β_2; both are coupled to G_s and increase production of cAMP, but the β_1-subtype has long-term detrimental effects (apoptosis, fibrosis, loss of function), whereas the β_2-subtype does not (or at least fewer effects) (Singh et al., 2000; Lohse et al., 2003a; Steinberg, 2004; Xiao et al., 2006). On the basis of functional (Rich et al., 2001) as well as FRET imaging studies (Zaccolo and Pozzan, 2002), these differences have been attributed to a number of factors, including different subcellular localization of PKA isoforms as a result of anchoring proteins (Di Benedetto et al., 2008), anchoring of the PKA substrates (Zhang et al., 2001), modulation by phosphodiesterases, which may also be locally anchored (Mongillo et al., 2004; Leroy et al., 2008), modulation by cGMP (Stangherlin et al., 2011), and modulation by Ca^{2+} (Warrier et al., 2007; Iancu et al., 2008). Specific localization of receptors also seems to contribute to localized signaling. Thus, experiments with local delivery of agonists through the pipette of a scanning ion conductance microscope has shown, that responses to β_2 receptor stimuli could only be elicited when agonists were applied to T-tubules, whereas responses β_1 receptor stimuli were elicited all over the cell; the cAMP signals generated by T-tubular receptors remained local, whereas those generated at the outer cell surface were generalized (Nikolaev et al., 2010)—confirming earlier data indicating localized cAMP signals in response to β_2 receptor stimuli and generalized cAMP responses to β_1 receptor stimuli (Nikolaev et al., 2006a). These studies are at the limits of resolution of FRET images by wide field or confocal microscopy. In the future, improved images using super-resolution microscopy (Hell, 2007; Patterson et al., 2010) might help to resolve objects that cannot be distinguished today. Adaptation of these techniques to FRET imaging will require

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TABLE 3

Kinetics of the individual steps of GPCR activation and signaling as observed in resonance energy transfer studies.

Values in brackets denote approximate values estimated from the respective publications. In the case of multiprotein complexes, the labeled proteins (or subunits) are bold.

Step, Method, and Time Constant (τ)	Example(s)	References
Ligand binding		
FRET		
140 ms /1 s	PTH1R	Castro et al., 2005
Receptor activation		
FRET		
40 ms	$\alpha_{2A}AR$	Vilardaga et al., 2003
1 s	PTH1R	
66–88 ms	$A_{2A}R$	Hoffmann et al., 2005
<40 ms	$A_{2A}R$	Hein et al., 2006
60 ms	β_1AR	Rochais et al., 2007
<1.6 s	PTH1R	Ferrandon et al., 2009
<100 ms	M_1R	Jensen et al., 2009
50–130 ms	β_2AR	Reiner et al., 2010
87–101 ms*	M_1R, M_3R, M_5R	Ziegler et al., 2011
Receptor/G-protein interaction		
FRET		
<100 ms	$\alpha_{\mathbf{2A}}\mathbf{AR}/G\alpha_{i1}G\beta_1G\gamma_{\mathbf{2}}$	Hein et al., 2005
<433 ms*	$\beta_{\mathbf{2}}\mathbf{AR}/G\alpha_{s}G\beta_{\mathbf{1}}G\gamma_2$	Galés et al., 2005
	$\beta_{\mathbf{2}}\mathbf{AR}/G\alpha_{s}G\beta_1G\gamma_{\mathbf{2}}$	
<50 ms	$\mathbf{A_{2A}AR}/G\alpha_{s}G\beta_1G\gamma_{\mathbf{2}}$	Hein et al., 2006
	$\beta_{\mathbf{1}}\mathbf{AR}/G\alpha_{s}G\beta_1G\gamma_{\mathbf{2}}$	
200 ms	$\mathbf{M_1R}/G\alpha_{q}G\beta_{\mathbf{1}}G\gamma_2$	Jensen et al., 2009
<1.6 s	$\mathbf{PTH1R}/G\alpha_{s}G\beta_1G\gamma_{\mathbf{2}}$	Ferrandon et al., 2009
		Ayoub et al., 2007, 2010
BRET		
6 s*	$\mathbf{PAR_1}/G\alpha_{\mathbf{i1}}$	
G-protein activation		
FRET		
[<5 s]	$G\alpha_{\mathbf{2}}G\beta G\gamma$	Janetopoulos et al., 2001
1.4–2.9 s*	$\alpha_2AR/G\alpha_{\mathbf{i1}}G\beta_{\mathbf{1}}G\gamma_2$	Bünemann et al., 2003
	$\alpha_2AR/G\alpha_{\mathbf{i1}}G\beta_1G\gamma_{\mathbf{2}}$	
<500 ms	$A_2R/G\alpha_{\mathbf{s}}G\beta_1G\gamma_{\mathbf{2}}$	Hein et al., 2006
<450 ms	$\beta_1AR/G\alpha_{\mathbf{s}}G\beta_1G\gamma_{\mathbf{2}}$	
<2 s	$PTH1R/G\alpha_{\mathbf{s}}G\beta_1G\gamma_{\mathbf{2}}$	Ferrandon et al., 2009
<3 s	$M_1R/G\alpha_{\mathbf{q}}G\beta_{\mathbf{1}}G\gamma_2$	Jensen et al., 2009
505 ms*	$H_1R/G\alpha_{\mathbf{q}}G\beta G\gamma_{\mathbf{2}}$	Adjobo-Hermans et al., 2011
500 ms	$M_3R/G\alpha_{\mathbf{q}}G\beta_1G\gamma_{\mathbf{2}}$	Qin et al., 2011
Effector activation		
Patch clamp		
300–500 ms (GIRK)	$\alpha_{2A}AR$, $\alpha_{2C}AR$: $GIRK_{1,4}$	Bünemann et al., 2003
FRET		
1.3 s	$G\alpha_q/PLC\beta_1$ interaction	Jensen et al., 2009
cAMP accumulation		
FRET		
[25 s]	PKA-based cAMP sensor	Zaccolo et al., 2000
<23 s*	cAMP sensor Epac2-cAMPS/β_2AR	Nikolaev et al., 2004
[20 s]	cAMP sensor ICUE3/β_2AR	Violin et al., 2008
21 s*	cAMP sensor ICUE3/β_2AR	Di Pilato and Zhang, 2009
[60 s]	cAMP sensor ICUE3/PTH1R	Wan et al., 2011
[30 s]	cAMP sensor Epac1-cAMPS/β_2AR	Gesellchen et al., 2011
β-Arrestin binding		
FRET		
[3 min]	V_2R/β-arrestin2	Azzi et al., 2003
28.3 s*	β_2AR/β-arrestin2	Krasel et al., 2005
0.7/3.6 min*	β_2AR/β-arrestin2	Violin et al., 2006
<10 s	β_2AR/β-arrestin2	Reiner et al., 2010

cAMPS, adenosine-3′,5′-cyclic monophosphorothioate; ICUE, indicator of cAMP using Epac.
* Values calculated from data in the respective publication.

further work, both on the respective sensors and on the microscopic techniques.

A third emerging topic is the temporal control of GPCR signaling and, in particular, of GPCR-triggered second messenger signals. Oscillations in the concentrations of intracellular second messengers have been shown to play a functional role in cellular responses decades ago (Carafoli et al., 1966; Roos et al., 1977). However, only the advent of imaging technologies first by fluorescence (Tsien and Tsien, 1990) and later by FRET (Miyawaki, 2003) allowed the discovery of the full spectrum of spatiotemporal patterns in second-messenger concentrations. These studies revealed not only that concentrations in calcium as well as cAMP can show complex patterns of oscillations but that these may be interlinked by various intracellular mechanisms such as Ca^{2+}-regulated phosphodiesterases and adenylyl cyclases (Zaccolo and Pozzan, 2003; Landa et al., 2005; Harbeck et al., 2006; Willoughby and Cooper, 2006; Kim et al., 2008; von Hayn et al., 2010; Ni et al., 2011; Werthmann et al., 2011). Oscillations have also been de-

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scribed for the activity of protein kinase C (Violin et al., 2003). Deciphering the code of such oscillatory signals and their interdependence will no doubt be a major research topic for future years.

To be able to analyze such temporal patterns for multiple parameters, it will be important to improve the simultaneous detection and imaging of different signaling steps. A sensor has been described for the simultaneous analysis of protein kinases A and C (Brumbaugh et al., 2006). Simultaneous imaging of cAMP and Ca^{2+} has been achieved in several studies using, for example, Fura-2 to measure Ca^{2+} and different FRET sensors for cAMP (Landa et al., 2005; Harbeck et al., 2006; Adachi et al., 2009; Niino et al., 2009). More recently, the simultaneous determination of both cAMP and cGMP plus Ca^{2+} has been described (Niino et al., 2010). Bimolecular complementation (i.e., the formation of functional donor or acceptor molecules for FRET or BRET studies from two parts of the respective fluorophore attached to different proteins) is often advocated as an additional tool for further interactions (Hébert et al., 2006; Molinari et al., 2008; Shyu et al., 2008). However, it should be kept in mind that the affinity of the two parts toward each other will contribute to the overall affinity of the two fusion proteins for each other, and careful controls are needed to assure specificity of the interaction.

Such simultaneous studies for many more steps in GPCR signaling will be important to understand not only the kinetics of individual steps but also their interplay in eliciting cellular and physiological responses. This is again a topic that is not specific for GPCR-mediated signaling but is similarly studied in other signaling pathways (Ananthanarayanan et al., 2008; Ouyang et al., 2008).

Finally, to understand the physiological significance of these signaling mechanisms, it will be important to transfer FRET imaging techniques from isolated cells to intact organs and ultimately in vivo. Although it has been possible to generate images of primary cells or of functional units such as thyroid follicles or pancreatic islets isolated from mice transgenically expressing second-messenger sensors (Kim et al., 2008; Calebiro et al., 2009; Mironov et al., 2009; von Hayn et al., 2010; Werthmann et al., 2009, 2011), the high degree of background fluorescence calls for several improvements for in vivo microscopy, including red-shifted sensors and FRET analysis by multiphoton and second harmonic generation microscopy (Provenzano et al., 2009). In addition, BRET studies have been performed in cells isolated from transgenic mice expressing luciferase-labeled β_2-adrenergic receptors and GFP2-labeled β-arrestin2 (Audet et al., 2010). The studies of species more suitable for microscopy will certainly facilitate such measurements. It is noteworthy that FRET imaging has been achieved in zebrafish (Fan et al., 2007; Tao et al., 2011) as well as *Drosophila melanogaster* (Lissandron et al., 2007; Shafer et al., 2008; Gervasi et al., 2010; Cooper et al., 2011), and the latter studies have helped to elucidate the role of adenylyl cyclases and cAMP in learning processes in the *D. melanogaster* brain.

These examples show that BRET and FRET studies do not only help to assess molecular mechanisms of GPCR activation and signaling, but that they can also reach into the physiological dimension and help us understand in unprecedented detail, how GPCRs exert their many physiological functions and how different cellular signals are integrated to produce an overall response.

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Wrote or contributed to the writing of the manuscript: Lohse, Nuber, and Hoffmann.

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