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REVIEW

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Conformational changes in G-protein-coupled receptors—the quest for functionally selective conformations is open

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The G-protein-coupled receptors (GPCRs) represent one the largest families of drug targets. Upon agonist binding a receptor undergoes conformational rearrangements that lead to a novel protein conformation which in turn can interact with effector proteins. During the last decade significant progress has been made to prove that different conformational changes occur. Today it is mostly accepted that individual ligands can induce different receptor conformations. However, the nature or molecular identity of the different conformations is still ill-known. Knowledge of the potential functionally selective conformations will help to develop drugs that select specific conformations of a given GPCR which couple to specific signalling pathways and may, ultimately, lead to reduced side effects. In this review we will summarize recent progress in biophysical approaches that have led to the current understanding of conformational changes that occur during GPCR activation.
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Abbreviations: FlAsH, fluorescein arsenical hairpin binder; FRET, fluorescence resonance energy transfer; GFP, green fluorescent protein; GPCR, G-protein-coupled receptor; TM, transmembrane domain

Introduction

The G-protein-coupled receptor (GPCR) family comprises the largest family of cell-surface receptors, which can sense information encoded by diverse stimuli and translate the encoded information into readable signals for the cell (Marinissen and Gutkind, 2001). A large fraction of drugs exert their action via interaction with these receptors (Flower, 1999). Drugs that influence GPCRs can help treat human diseases as different as pain, asthma, hypertension or chronic heart failure. For a long time, receptors were depicted as simple switches for 'on' and 'off' states, and ligands were thought to simply stimulate (agonists) or inhibit (antagonists) the receptor by promoting either the 'on' or the 'off' state. Accordingly, less efficacious agonists (partial agonists) were only thought to vary in signal strength, but all agonists were assumed to produce qualitatively the same effect as the endogenous agonist(s) (reviewed by Perez and Karnik, 2005). However, a growing body of experimental evidence forced the receptor theories to be constantly updated and led to the inclusion of the ensemble theory in GPCR dynamics (Kenakin, 2002) and the development of the concepts of collateral efficacy and permissive antagonism (Kenakin, 2005) to accommodate different conformations into the receptor theory. Various GPCRs have been studied with respect to different receptor conformations and contributed to the body of evidence supporting this concept. Among them are 5-HT$_2$-serotonin receptors (Berg *et al*., 1998), α_{2A}-adrenoceptors (Vilardaga *et al*., 2005; Nikolaev *et al*., 2006), AT$_1$ receptor (Wei *et al*., 2003), β_2-adrenoceptors (Ghanouni *et al*., 2001a; Swaminath *et al*., 2004; Trester-Zedlitz *et al*., 2005), gonadotropin-releasing hormone receptors (Lu *et al*., 2007), μ-opioid receptors (Keith *et al*., 1998; Whistler *et al*., 1999), parathyroid hormone (PTH) receptors (Bisello *et al*., 2002) and many others, thus proving that this phenomenon is widespread among GPCRs. Today, it is mostly accepted that different ligands can induce different receptor conformations (Perez and Karnik, 2005; Vauquelin and Van Liefde, 2005; Urban *et al*., 2007), and this phenomenon has been described by several synonyms such as 'functional selectivity', 'agonist-directed trafficking' or 'biased agonism'. Recently, it has been suggested that the number of different terms be limited by using the term 'functional selectivity' or 'ligand-induced differential signalling' to describe this phenomenon (Urban *et al*., 2007). Besides the mostly phenomenological description of the occurrence of different functionally selective conformations, little is known about the actual distinction at the molecular

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level. Structural data with high resolution are currently available only for the inactive state of rhodopsin (Palczewski *et al*., 2000; Schertler, 2005; Ridge and Palczewski, 2007). However, a combined effort from structural biology, molecular pharmacology and computational chemistry may help fill the gap until crystal structural data of an active receptor conformation are made available. To gain information about the switch from an inactive conformation to active receptor conformation(s), several different techniques have been developed and used over the past decade.

In the following sections, the structural information obtained from the rhodopsin system pioneered by the work from Khorana's and Hubbell's groups (Hubbell and Altenbach, 1994), the β_2-adrenoceptor system with techniques developed by Kobilka's group (Gether *et al*., 1995) and the M3 subtype of the muscarinic ACh receptor introduced by the group of Wess (Zeng *et al*., 1999) will be described in brief. All these techniques use purified and reconstituted GPCRs to investigate the mechanisms of receptor activation. Then, the metal-ion chelator approach that has been widely used by the group of Schwartz (Thirstrup *et al*., 1996) will be summarized. In comparison to those systems, the recently developed approaches that have made it possible to investigate conformational changes of GPCRs in living cells by means of fluorescence resonance energy transfer (FRET) (Vilardaga *et al*., 2003; Hoffmann *et al*., 2005) will be discussed.

The rhodopsin system

The solution structure of rhodopsin has been analysed in great detail, and the work was recently reviewed in the light of the rhodopsin crystal structure (Hubbell *et al*., 2003). The term 'solution structure' refers to purified rhodopsin from bovine retina, which has been reconstituted into dodecyl maltoside micelles. This environment is assumed to be a reasonable approximation of the normal lipid bilayer, as it has been shown to conserve functional properties of rhodopsin such as transducin activation (Resek *et al*., 1993) or receptor phosphorylation by rhodopsin kinase (Thurmond *et al*., 1997). Three major classes of experiments have been carried out, namely site-directed spin labelling, disulphide cross-linking kinetics and sulphydryl reactivity studies.

Site-directed spin labelling uses cysteine residues that have been substituted for the normal amino acid and can be reacted with nitroxide reagents. This procedure generates nitric oxide side chains that can be utilized in electron paramagnetic resonance and, dependent on the type of experiment, can report on the mobility of the side chain, the accessibility of the side chain in solution and the distance between two side chains if a second paramagnetic group is introduced into the protein of interest (Hubbell *et al*., 1998). Data from distance measurements by site-directed spin labelling can be complemented by the second technique mentioned, by disulphide cross-linking kinetics. This technique uses the fact that the rate of oxidative formation of disulphide links depends on the distance, relative orientation and flexibility of the side chain to be cross-linked (Falke and Koshland, 1987). Sulphydryl reactivity studies can report about solvent accessibility of the substituted cysteine. In this case, the reaction rate can be followed by measuring the absorption at 324 nm of a stoichiometric by-product of the reaction between the cysteine under investigation and a sulphydryl reagent 4,4′-dithiopyridine. The reaction rate is a measure of the relative accessibility of the respective side chain (Hubbell *et al*., 2003). By generating more than 100 mutations and various combinations thereof within all of the cytoplasmic domains of rhodopsin, it was possible to achieve a detailed picture of the movements of the rhodopsin structure during the activation process. As the issue of receptor activation itself has been reviewed in great detail by others (Gether, 2000; Bissantz, 2003; Schwartz *et al*., 2006), only the changes that occur will be briefly described, with emphasis on what is known or can be concluded for the cytoplasmic domains. This issue is focused on, as cytoplasmic domains are the assumed contact points for downstream signalling partners and, hence, are likely to be interesting domains for ligand-selective conformations. Upon receptor activation, transmembrane helix (TM) VI undergoes the largest movement and thus the third intracellular loop close to TMVI should follow the movement and move outward from the structure if viewed from the cytoplasmic side (Hubbell *et al*., 2003) (Figure 1). Smaller changes were observed for TMIII, and again an outward movement of the second intracellular loop would be the logical consequence (Hubbell *et al*., 2003). The first intracellular loop appears to be more rigid and does not seem to undergo large-scale movements. However, a slight outward movement can be concluded from distance measurements with different reference points within the rhodopsin molecule (Hubbell *et al*., 2003). The C-terminal domain is very flexible and totally disordered in solution after position 340 (Cai *et al*., 1997). Therefore, no information is available for its global movement, but the part close to TMVII should follow the slight outward movement of helix VII, and therefore it can be assumed to move slightly outwards. All in all, the overall movement would open a cleft in the molecule, and the process has been described as a 'blossoming' of the molecule (Meng and Bourne, 2001), thus allowing interactions with downstream signalling molecules such as transducin or visual arrestin.

Besides the vast amount of information that was achieved by the use of the rhodopsin system about the activation mechanism of GPCRs in general, the system is complicated by the fact that 11-*cis*-retinal needs to be covalently linked to opsin at position 296 to achieve its full potential as agonist when converted into 11-*trans*-retinal. Thus, the system is not easy to use for ligand screening to detect functionally selective conformations, although work with alternative ligands has been performed to investigate the role of the retinal ring in receptor activation (Bartl *et al*., 2005). A lack of the ring structure was found to be without influence on the kinetics of formation of the active conformation metarhodopsin II. However, the lack of the retinal ring was found to not only result in low amounts of metarhodopsin II but also lead to a fast decay of active conformation (Bartl *et al*., 2005). It was concluded that it has a role in stabilizing the active receptor conformation.

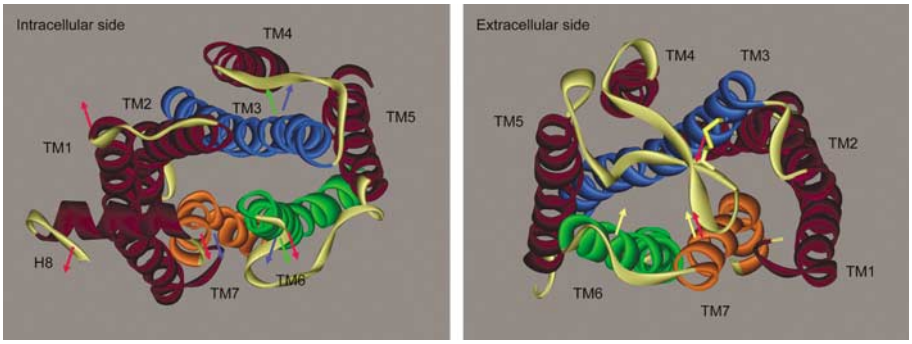

Figure 1 Schematic representation of the helix movements as delineated from experimental data described by the different approaches. The crystal structure data from bovine rhodopsin (PDB access code: 1U19, Okada *et al*., 2004) were used to generate the figure. Colour code: TMIII, blue; TMVI, green; TMVII, dark orange. Left: view from the cytoplasmic side; right: view from the extracellular side. Helix movements are indicated by arrows and are meant to indicate the general movements as mentioned in the text. Blue arrows: data delineated from the rhodopsin system; green arrows: data delineated from the β_2-adrenoceptor system; red arrows: data delineated from the M3 muscarinic ACh receptor system; yellow arrows: data delineated from the metal-ion chelator approach. PDB, Protein Data Bank; TM, transmembrane domain.

The β_2-adrenoceptor system

As two recent reviews have been published on the β_2-adrenoceptor as a model system for studying ligand-induced conformational changes (Kobilka, 2007; Kobilka and Deupi, 2007), only the key points that are relevant to the concept of ligand-selective conformational changes will be summarized. In general, the system uses purified and reconstituted β_2-adrenoceptors that have been depleted of all but the essential cysteines (Gether *et al*., 1997). The modified receptor is then reacted with small cysteine-reactive fluorescent probes that can be monitored for several different properties such as mobility, intensity or lifetime. It has been possible to label the receptor construct with probes that provide information about the polar environment in which the actual fluorophore is located (Gether *et al*., 1997), as well as the dual labelling of the receptor with a fluorophore, such as fluorescein, and a fluorescence quencher in a different position of the receptor, therefore reporting the relative movements of the two groups to each other (Ghanouni *et al*., 2001b). As already outlined in the section describing the rhodopsin system, this approach has greatly advanced our current knowledge on the molecular details about how GPCRs change their conformation upon activation. These studies have demonstrated movements of TMIII and TMVI during agonist-induced conformational changes (Gether *et al*., 1995, 1997), which were in good agreement with reports on rhodopsin (Farrens *et al*., 1996) (Figure 1). In recent years, the system was further developed to allow screening of several different ligands with respect to conformational changes in different positions of the receptor, and it also allowed the detection of intermediate states during the activation process (Swaminath *et al*., 2004, 2005; Yao *et al*., 2006). Using a receptor construct that was labelled underneath TMVI with fluorescein and employing fluorescent lifetime measurements as read-out, it was possible to detect different lifetimes for the fluorophore, which were dependent on the ligand that the receptor was exposed to. Full or partial agonists were thus shown to induce different conformational changes (Ghanouni *et al*., 2001a, b). Kinetic studies followed those initial reports and further refined our understanding of receptor activation. It was shown that the partial agonist dopamine induced a rapid change of the receptor conformation as did the full agonist noradrenaline (Swaminath *et al*., 2004). However, noradrenaline exhibited a second slower phase that is lacking for dopamine but was observed for the full agonists isoprenaline or adrenaline (Swaminath *et al*., 2004). As the only structural difference between dopamine and noradrenaline is the lack of the β-hydroxyl group, it was concluded that the presence of the β-hydroxyl group and its interaction with TMVI (Wieland *et al*., 1996) would be responsible for the induction of the slow phase, whereas the presence of the catechol structure would allow the induction of the rapid phase (Swaminath *et al*., 2004). This hypothesis was supported by the notion that the partial agonist salbutamol carrying a β-hydroxyl group, but no catechol structure, only induced the slow phase of conformational change and could be complemented by the addition of catechol (Swaminath *et al*., 2005). The kinetic resolution for receptor activation that has been achieved with this system is in the range of several seconds for the fast phase and almost a minute for the slow phase of receptor activation; therefore, it would not be compatible with the receptor function in biological systems. However, the kinetics of receptor conformational changes improved by careful reconstitution of the receptor (Yao *et al*., 2006), showing that a more natural environment may lead to kinetic measurements that are more consistent with biological functions. The latest study in this series also addressed the issue of functionally selective receptor conformations at the molecular level (Yao *et al*., 2006). Two β_2-adrenoceptor constructs were used for this study, one that reports upon movements of the TMVI and the second that reports upon changes between TMIII and TMVI. Using a selection of compounds with various degrees of efficacy, it was possible to show that full agonists such as isoprenaline, noradrenaline or adrenaline could induce conformational changes of the receptor in both cases, whereas the partial agonist salbutamol only induced conformational changes in TMVI (Yao *et al*., 2006). The structural changes of the C terminus have recently been subjected to investigation with the same set of receptor ligands. Again, a complex pattern of movements

was observed, which allowed the conclusion that each ligand has the potential to induce individual conformational changes or ligand-selective conformations (Granier *et al*., 2007). This system has so far been the most informative with respect to molecular changes being induced by ligands of different efficacy.

The M3 muscarinic ACh receptor system

Using the rat M3 subtype of the muscarinic ACh receptor, an *in situ* disulphide cross-linking strategy was developed by the group of Wess. To develop this system, an M3 muscarinic ACh receptor construct was generated that lacked most of the native cysteine residues and had the third intracellular loop largely truncated (Zeng *et al*., 1999). Into the third intracellular loop, two factor Xa cleavage sites were introduced to allow selective digestion of the proteins into two fragments. The first fragment consists of the N-terminal part of the receptor till TMV and the N-terminal part of the intracellular loop 3, whereas the second fragment consists of the C-terminal part of intracellular loop 3 plus TMVI, TMVII and the normal C terminus of the M3 receptor. By selective introduction of two cysteine residues, one in each fragment mentioned above, it is possible to cross-link the two fragments by oxidation with Cu(II)-phenanthroline if the two cysteine residues are in close enough proximity and appropriate orientation. Cross-linked fragments can then be detected by western blotting under non-reducing conditions as judged by the appearance of a full-length receptor band at the appropriate molecular weight, using an antibody against the very C terminus of the M3 receptor. The initial studies used solubilized receptors from membranes of COS-7 cells expressing the receptor construct (Zeng *et al*., 1999). However, it was recently reported that solubilized receptors in some rare cases might allow promiscuous cross-linking, and the approach was further developed to the use of native membranes (Ward *et al*., 2006) to prevent this problem. A series of publications have demonstrated the usefulness and strength of this approach. Consistent with previous publications on rhodopsin (Farrens *et al*., 1996) or the β_2-adrenoceptor (Gether *et al*., 1997), it was demonstrated that the cytoplasmic end of TMVI undergoes a rotational movement upon receptor activation (Ward *et al*., 2002, 2006) (Figure 1). Furthermore, it was found that agonist activation leads to an increase in distance between the cytoplasmic ends of TMI and TMVII (Han *et al*., 2005a), whereas the extracellular ends of TMIII and TMVII move closer to each other upon agonist stimulation (Han *et al*., 2005b), proving that this technique is not limited to studies on the intracellular site of the receptor. In the latest publication from this series, the issue of ligand-selective conformations was addressed. Agonists with different efficacy, such as full, partial or inverse agonists, were studied with respect to conformational changes induced by the respective ligand (Li *et al*., 2007). It was found that agonists such as carbachol would increase the distance between the C-terminal part of helix VIII and the cytoplasmic end of TMI, whereas inverse agonists such as atropine would decrease the distance in these parts of the receptor (Li *et al*., 2007). The opposing effects on conformational changes for agonists or inverse agonists have also been demonstrated for the α_{2A}-adrenoceptor by means of FRET (Vilardaga *et al*., 2005).

A great advantage of the *in situ* disulphide cross-linking strategy technique compared to the studies for rhodopsin mentioned above is the possibility to include several different ligands in the assay, thus having the potential to pin down different conformational changes for different classes of ligands at the molecular level. One minor drawback of this technique is the fact that only those conformational changes can be observed that will lead to cross-linking of the two fragments; therefore, the design of the fragments itself may limit the approach, as no changes within the fragment TMI–TMV, or the respective intracellular loops 1 and 2, can be investigated. As all measurements are carried out under equilibrium conditions, this approach also does not lead to any information with respect to kinetic resolution of the conformational changes.

Metal-ion chelator sites and receptor activation

An alternative approach using the introduction of histidine side chains to complex metal-ions has contributed greatly to the understanding of conformational changes that occur during receptor activation; these techniques have been reviewed recently (Schwartz *et al*., 2006). The basic principle of this technique is to introduce histidine residues that can complex Zn(II) or Cu(II), thus forming a bridge between different residues and thereby allowing the study of helix–helix interactions. A variation of this technique uses metal-ions that have been chelated with bipyridine or phenanthroline as aromatic chelators. This allows investigation of the surroundings of the attachment site within the helical bundle in more detail, and the organic metal-ion complex has been shown to mimic nicely small molecule agonists at chemokine receptors (Schwartz *et al*., 2006).

The metal-ion chelator approach has initially been applied in an inhibitory mode by cross-linking two helices and thus inhibiting receptor activation. Those studies were performed using a variety of receptors, including the κ-opioid receptor (Thirstrup *et al*., 1996), rhodopsin (Sheikh *et al*., 1996), the β_2-adrenoceptor (Sheikh *et al*., 1999) and the M_1-ACh receptor (Lu and Hulme, 2000). The approach was also successfully used to activate the receptors of interest, and this was first achieved for the β_2-adrenoceptor (Elling *et al*., 1999) and the NK_1 receptor (Holst *et al*., 2000). Using the metal-ion chelator strategy for the PTH receptor, it could be demonstrated that different receptor conformations would be required for G-protein activation and β-arrestin interaction (Vilardaga *et al*., 2001). A great and detailed work using this strategy has been performed by the group of Schwartz and has led to the development of the 'global toggle switch' model for receptor activation (Elling *et al*., 2006; Schwartz *et al*., 2006). This model suggests an outward movement of the intracellular segments ('blossoming' of the molecule in the section The rhodopsin system) and an inward movement of the extracellular segments of the transmembrane bundle (Figure 1). According to this model, the extracellular segments of TMVI and TMVII are bent inward towards

TMIII, and this conformation would be stabilized by small ligands within the helical bundle. This type of movement has recently been confirmed for the CXCR3 receptor (Rosenkilde *et al*., 2007). Large agonists such as peptides or proteins could stabilize a similar active conformation by contacting the extracellular ends of the helices or by interacting with the extracellular loops. This type of movement is consistent with a large body of data that was acquired by the different approaches outlined in this review and could be an explanation for a common activation mechanism for GPCRs.

Studies in living cell by fluorescence resonance energy transfer

With the introduction of the green fluorescent protein (GFP) and its colour variants in the late 1990s (Tsien, 1998), it became possible to create fusion proteins of almost any kind with genetically encoded fluorescent markers. The introduction of GFP into cell biological work significantly expanded the accessibility of proteins to studies in living cells. It had already been possible for a long time to use FRET to study protein function (Stryer, 1978), but usually isolated proteins had to be chemically labelled and needed to be injected back into the cell to study protein function in living cells (Adams *et al*., 1991). Such experiments became significantly easier by the availability of GFP. Many biological processes suddenly became 'visible' (Miyawaki and Tsien, 2000) and could be monitored in real time (Miyawaki, 2003). Activation of a GPCR was monitored by the use of GFP already in 1997 (Barak *et al*., 1997). However, the approach was indirect, as a β-arrestin–GFP fusion protein was used as a downstream readout for receptor activation, and it did not monitor the changes at the level of the receptor itself. The first approach to directly measure receptor activation by conformational changes in living cells was published in 2003 (Vilardaga *et al*., 2003). Two receptors from different subclasses of GPCRs were used for this study: the α_{2A}-adrenoceptor and the PTH receptor. The receptor constructs had been modified in a way that a cyan variant of GFP (CFP) was inserted into the third intracellular loop, whereas a yellow variant (YFP) had been fused to the C terminus, or vice versa (Figure 2a). The relative distance of both fluorophores was optimized by using several different truncations at the C terminus or the third intracellular loop, thus allowing optimization of the signal amplitude. The positions for insertion into intracellular loop 3 were concluded from the studies of rhodopsin (Farrens *et al*., 1996) and the β_2-adrenoceptor (Gether *et al*., 1997), which had shown significant movements of TMVI

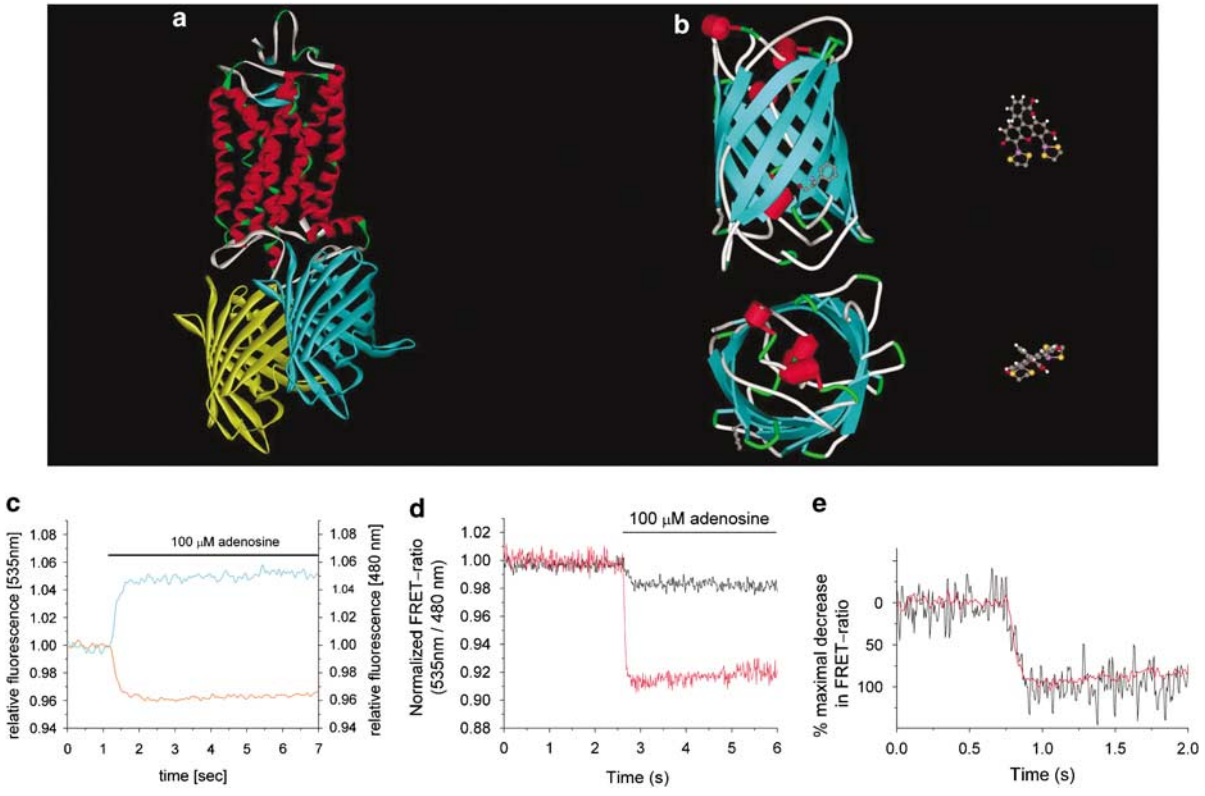

Figure 2 (**a**) Schematic representation of a GPCR modified with the cyan and yellow fluorescent protein. Crystal structure data from bovine rhodopsin and GFP were used to generate the figure. (**b**) Size comparison of GFP (left) and FlAsH (right). A phenylalanine side chain of GFP is shown to indicate that FlAsH was matched to size. Side and top view of both fluorophores are shown. (**c**) Changes in the relative fluorescence of CFP or FlAsH in response to 100 μM adenosine from a single HEK-293 cell expressing the A_{2A}-FlAsH3-CFP construct. (**d**) Comparison of FRET signals in FlAsH/CFP- and CFP/YFP-labelled receptors. Normalized FRET ratios in response to 1 mM adenosine from single HEK-293 cells expressing A_{2A}-FlAsH3-CFP (red) or A_{2A}-CFP-YFP (black) are shown. (**e**) Same data, as in panel d, with the amplitude (response to 1 mM adenosine) set to 100% for both traces. Figure is reproduced with permission from Hoffmann *et al*. (2005). FlAsH, fluorescein arsenical hairpin binder; FRET, fluorescence resonance energy transfer; GFP, green fluorescent protein; GPCR, G-protein-coupled receptor.

during receptor activation. Thus, it was assumed that the third intracellular loop would undergo a conformational change upon receptor activation and that the relative distance of the two fluorophores would change and give rise to a change in the FRET signal. These theoretical considerations were confirmed by actual experiments. When the α_{2A}-adrenoceptor was stimulated by an agonist, the change in the FRET signal occurred at a millisecond time scale, whereas the PTH receptor responded much slower with a τ of about 1 s (Vilardaga *et al*., 2003). The timescale for receptor activation was much faster than that which had been reported for the β_2-adrenoceptor but still slower than those reported for rhodopsin (see also Lohse *et al*., 2007 for a more in-depth discussion of the kinetic aspects). Several control experiments were performed to ensure that the observed signal did arise from a single GPCR subunit and not from potential rearrangements within receptor dimers, or that it was caused by interactions with G-proteins (Vilardaga *et al*., 2003). Thus, the first system was available to monitor conformational changes induced by direct agonist binding to the receptor in living cells. For the α_{2A}-adrenoceptor, agonist stimulation caused a decrease in the FRET signal, whereas inverse agonists had the opposite effect, exhibiting an increase in the FRET signal of the receptor construct (Vilardaga *et al*., 2005). Taking into account that the FRET signal is distance-dependent, one could conclude that agonist stimulation leads to an increase in distance, whereas inverse agonists would cause a decrease in distance between the C terminus and intracellular loop 3. In line with this finding is the recent report from the rat M3 receptor. Here, it was shown that agonists increase the distance between helix VIII and TMI, whereas inverse agonists lead to a decrease in distance (Li *et al*., 2007). Those movements would be consistent with the 'blossoming' receptor as mentioned above. An extension of this approach using CFP and YFP as fluorophores was reported in 2005 (Hoffmann *et al*., 2005). For the adenosine A_{2A} and the α_{2A}-adrenoceptor, an alternative approach was realized using CFP in combination with the small fluorescein-derived analogue FlAsH (*fl*uorescein *ars*enical *h*airpin binder) (Adams *et al*., 2002). This fluorescent tag is only 700 Da in size and thus significantly smaller than GFP (27 kDa) (Figure 2b), and it needs only six amino acids (CCPCCC) to be able to bind to the molecule of interest (Adams *et al*., 2002). A side-by-side comparison of both FRET approaches was made and showed that the kinetics of receptor activation were independent of the fluorophores used for detection of the signal (Hoffmann *et al*., 2005). The adenosine A_{2A} receptor was activated with a rate constant about 50 ms similar to the α_{2A}-adrenoceptor, showing that small ligand receptors might respond faster than peptide hormone receptors such as the PTH receptor. The FlAsH-based FRET approach showed a larger signal amplitude (Figures 2c–e) and better functionality of the receptor construct (Hoffmann *et al*., 2005). For the α_{2A}-adrenoceptor, the signal amplitude was improved fourfold and this allowed a much more detailed study of receptor response to ligands with varying efficacy (Nikolaev *et al*., 2006). Several structurally distinct ligands, such as noradrenaline, dopamine, clonidine and others, were shown to induce kinetically distinct conformational changes of the receptor—a notion that is similar to the finding for the β_2-adrenoceptor (Ghanouni *et al*., 2001a), proving that different ligand-induced conformations do occur in living cells and are not produced by reconstituted systems. Another point proved by this study is the readiness of this system to screen for a larger number of compounds. The approach to monitor conformational changes of a GPCR with CFP and YFP fusion constructs was rapidly adapted by other groups and applied to monitor conformational changes of the bradykinin B_2 receptor in endothelial cells, showing that the conformational dynamics of the receptor were altered under fluid shear stress in real time (Chachisvilis *et al*., 2006). The first report on receptors of a class C GPCR used the mGluR1α receptor as a model system for receptor activation (Tateyama *et al*., 2004). However, the general concept of this study was different, as the authors investigated movements within receptor dimers rather than movements within individual subunits of a receptor dimer. In this study, no agonist-dependent conformational change within a receptor dimer subunit was observed when the fluorophores were inserted into the intracellular loops 1 or 2 in combination with the C terminus. However, if receptor constructs were used that were solely tagged within the second or first intracellular loop, the outcome was different. Under those conditions, an increase of FRET was observed between the second intracellular loops, whereas a decrease of FRET was observed between the first intracellular loops. The data suggest that rearrangements within receptor dimers may occur during receptor activation. However, these data are not fully conclusive with respect to movements of the intracellular loop 1 or 2 and the C terminus within a receptor dimer subunit, as the receptor constructs were non-functional with respect to calcium signalling and may therefore have been non-responsive to the agonist even though binding was demonstrated.

A recent study using the β_1-adrenoceptor linked conformational changes induced by inverse agonists with polymorphisms occurring naturally at the receptor (Rochais *et al*., 2007). Bisoprolol, metoprolol and carvedilol function as inverse agonists at the human β_1-adrenoceptor (Hoffmann *et al*., 2004) and are used to treat chronic heart failure. Using two β_1-adrenoceptor variants (Gly389 and Arg389) as FRET sensors, it was demonstrated that only carvedilol differed in its ability to induce conformational changes and that the change was larger for the Arg389 variant. This was consistent with a larger reduction of intracellular cAMP levels for carvedilol treatment of the Arg389 variant (Rochais *et al*., 2007). No such difference was observed for bisoprolol or metoprolol. As the Arg389 polymorphism is associated with a poor prognosis for heart failure (Mialet Perez *et al*., 2003) or at least with a different responsiveness to β-blockers (Lohse, 2004), these findings could have possible consequences for the clinical use of the β-blockers.

Outlook

The biophysical and biochemical approaches outlined in this review have led to a great increase in our understanding of what happens structurally when a GPCR is activated. A very different question arises from inverse agonism, and the

clinical expectations for inverse agonists have recently been highlighted (Gilchrist and Blackmer, 2007). Two current papers have shown that the strength of inverse agonism or the change from agonism to inverse agonism is determined by rather subtle changes in the binding mode of the ligand (Holst *et al*., 2007; Miura *et al*., 2007). However, it is ill known how an active receptor conformation differs structurally with respect to different ligands, and this is also especially true for inverse agonists. Therefore, the quest for molecular explanations of functionally selective conformations is open.

The described FRET approach in living cells using FlAsH in combination with CFP is very promising, compared to the bulky YFP, as it allows a more detailed investigation of conformational changes that could occur in the third intracellular loop during agonist-induced receptor activation, a question that has not been addressed with any of the other approach yet. To our understanding, the different approaches described here are rather complementary than competitive, as all approaches have their strengths or weaknesses. For example, the FRET approaches have good kinetic resolution but currently provide little structural information, whereas the latter is true for the structural approaches. The common weakness of all approaches is currently the lack of a correlate of the observed effects with biological functions. More studies addressing the effects of different compounds on different biological functions will be needed to address this issue. The recently published work on the β_2-adrenoceptor (Galandrin and Bouvier, 2006) or the D2 receptor (Lane *et al*., 2007) should encourage colleagues to follow that path. A significant input could also come from the industrial partners by data mining their archives for valuable information from different screening assays and promising compounds as research tools.

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Conflict of interest

The authors state no conflict of interest.

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