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REVIEW

Advances in receptor conformation research: the quest for functionally selective conformations focusing on the β_2-adrenoceptor

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Nomenclature of receptors adhere to the Concise Guide to Pharmacology 2013/2014 (Alexander *et al.*, 2013).

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Seven-transmembrane receptors, also called GPCRs, represent the largest class of drug targets. Upon ligand binding, a GPCR undergoes conformational rearrangement and thereby changes its interaction with effector proteins including the cognate G-proteins and the multifunctional adaptor proteins, β-arrestins. These proteins, by initiating distinct signal transduction mechanisms, mediate one or several functional responses. Recently, the concept of ligand-directed GPCR signalling, also called functional selectivity or biased agonism, has been proposed to explain the phenomenon that chemically diverse ligands exhibit different efficacies towards the different signalling pathways of a single GPCR, and thereby act as functionally selective or 'biased' ligands. Current concepts support the notion that ligand-specific GPCR conformations are the basis of ligand-directed signalling. Multiple studies using fluorescence spectroscopy, X-ray crystallography, mass spectroscopy, nuclear magnetic resonance spectroscopy, single-molecule force spectroscopy and other techniques have provided the evidence to support this notion. It is anticipated that these techniques will ultimately help elucidate the structural basis of ligand-directed GPCR signalling at a precision meaningful for structure-based drug design and how a specific ligand molecular structure induces a unique receptor conformation leading to biased signalling. In this review, we will summarize recent advances in experimental techniques applied in the study of functionally selective GPCR conformations and breakthrough data obtained in these studies particularly those of the β_2-adrenoceptor.

LINKED ARTICLES

This article is part of a themed section on Chinese Innovation in Cardiovascular Drug Discovery. To view the other articles in this section visit http://dx.doi.org/10.1111/bph.2015.172.issue-23

Abbreviations

G_i, inhibitory G-protein; GRK, GPCR kinase; G_s, stimulatory G-protein; HDX-MS, hydrogen-deuterium exchange coupled to MS; pdb, Protein Data Bank; SMFS, single-molecule force spectroscopy; TM, transmembrane

Tables of Links

TARGETS	
GPCRs[a]	**Enzymes**[b]
5-HT_{1B} receptor	GRK2
5-HT_{2B} receptor	GRK6
β_1-adrenoceptor	
β_2-adrenoceptor	
D_{2L} receptor	
M_2 receptor	
V_2 receptor	

LIGANDS	
Adrenaline	Ergotamine
Alprenolol	Fenoterol
cAMP	Formoterol
Carmoterol	Isoetharine
Carazolol	Isoprenaline
Carvedilol	Propranolol
Cyanopindolol	Salbutamol
Dobutamine	Salmeterol

These Tables list key protein targets and ligands in this article which are hyperlinked to corresponding entries in http://www.guidetopharmacology.org, the common portal for data from the IUPHAR/BPS Guide to PHARMACOLOGY (Pawson *et al.*, 2014) and are permanently archived in the Concise Guide to PHARMACOLOGY 2013/14 ([a,b]Alexander *et al.*, 2013a,b).

Introduction

The GPCR superfamily with more than 800 members is a class of membrane proteins responsible for transmembrane (TM) signal transduction (Bjarnadottir *et al.*, 2006). Members in this family share a common seven TM α-helix structure and have very diversified secondary and tertiary structures in their extracellular and ligand-binding regions. While the ligand-binding pockets of some GPCRs bury deep inside the proteins, all of them face the extracellular surface. Thus, GPCRs can specifically recognize extracellular signals in forms as diverse as nutrients, metabolites, neurotransmitters, hormones, peptides, proteins, lipids, odours, light, and other chemical or physical stimuli. The binding of a ligand to the receptor triggers a conformational change, which propagates to the intracellular part of the protein 30 nm underneath the lipid bilayer and modulates the interaction of the receptor with its intracellular partners mainly G-proteins. This first layer of signal transduction on the receptor marks the beginning of a signal transduction process involving a multitude of downstream signalling molecules through which the signals are processed and propagated to the destination effector proteins for the execution of a biological response (Kenakin, 2006).

Classical receptor theory defines ligand efficacy for a given GPCR as a linear system in terms of a single pharmacological readout (Kenakin, 2004; 2006). Therefore, according to their efficacies, ligands can be classified into full agonists, partial agonist, neutral antagonist or inverse agonists (Costa and Herz, 1989; Bond *et al.*, 1995; Kenakin, 2004; 2006; Kobilka and Deupi, 2007). This is based on the two-state model of GPCR activation where a receptor exists in either the inactive state R or the active state R* (Gether *et al.*, 1995). In most cases, a GPCR exists in equilibrium between the two states in the absence of a ligand and therefore exhibits a basal activity (Bond *et al.*, 1995; Kenakin, 2004; Kobilka and Deupi, 2007). Depending on their intrinsic activities, agonists promote a different degree of equilibrium shift towards the R* state (Gether *et al.*, 1997; Ghanouni *et al.*, 2001a,b; Seifert *et al.*, 2001). An inverse agonist does the opposite to shift the equilibrium towards the R state (Costa and Herz, 1989; Bond *et al.*, 1995). A neutral antagonist does not change this equilibrium, but it competes with other ligands for binding to the orthosteric ligand-binding site of the receptor (Kenakin, 2004; 2006). Traditionally, the theory of GPCR activation has been best investigated on β_2-adrenoceptor (Figure 1).

Increasing experimental evidence over the last two decades has demonstrated that a GPCR does not just possess a single efficacy. Rather, a GPCR possesses pluridimensional efficacies as defined by different assays for the multiple downstream signal transduction pathways involved (Galandrin and Bouvier, 2006; Urban *et al.*, 2007). For instance, many GPCRs transduce their signals via different G-protein subfamilies, and some even via G-protein-independent mechanisms (Xiao *et al.*, 1995; 1999; Xiang and Kobilka, 2003; Lefkowitz and Shenoy, 2005). Among the non-G-protein signalling effectors of GPCRs, GPCR kinases (GRKs) and β-arrestins are the most important because they are ubiquitously involved in GPCR function. Apart from their well-defined roles in controlling the desensitization, internalization and recycling of GPCRs (Lefkowitz, 1998), experimental evidence accrued over the last decade suggests that GRKs and β-arrestins can act as signal transducers in their own right (Azzi *et al.*, 2003; Lefkowitz and Shenoy, 2005; Ren *et al.*, 2005; Rajagopal *et al.*, 2010). In particular, β-arrestins are highly dynamic inside the cell and act as multifunctional scaffolds that facilitate interaction of GPCRs with their protein partners (Luttrell *et al.*, 1999; DeFea *et al.*, 2000; Baillie *et al.*, 2003). The consequence of these interactions may be the formation of signal complexes, the activation of protein kinases or the targeting of these effectors to different subcellular compartments, and these effectors, in turn, activate other downstream intracellular targets (Luttrell *et al.*, 1999; 2001; DeFea *et al.*, 2000; Rajagopal *et al.*, 2010). Recent reports have also revealed that certain ligands, termed biased ligands, act on GPCRs to induce cellular responses through G-protein-independent and β-arrestin-dependent signalling (Lefkowitz and Shenoy, 2005; Shenoy *et al.*, 2006; Violin and Lefkowitz, 2007; Rajagopal *et al.*, 2010). In addition, a ligand can simultaneously be an agonist for one pathway while at the same time an antagonist for another pathway. For example, carvedilol has been shown to activate β-arrestin-

dependent ERK1/2 signalling via β_1- and β_2-adrenoceptors while antagonizing G_s-dependent cAMP formation on the same receptors (Wisler *et al.*, 2007; Kim *et al.*, 2008) (Figure 1). This paradigm shift in the understanding of efficacy, commonly known as functional selectivity, biased agonism, pluridimensional efficacy or ligand bias, has major implications on drug discovery (Kenakin, 2004; 2007; Galandrin and Bouvier, 2006; Kobilka and Deupi, 2007; Urban *et al.*, 2007; Violin and Lefkowitz, 2007; Evans *et al.*, 2010; Rajagopal *et al.*, 2010; DeWire and Violin, 2011).

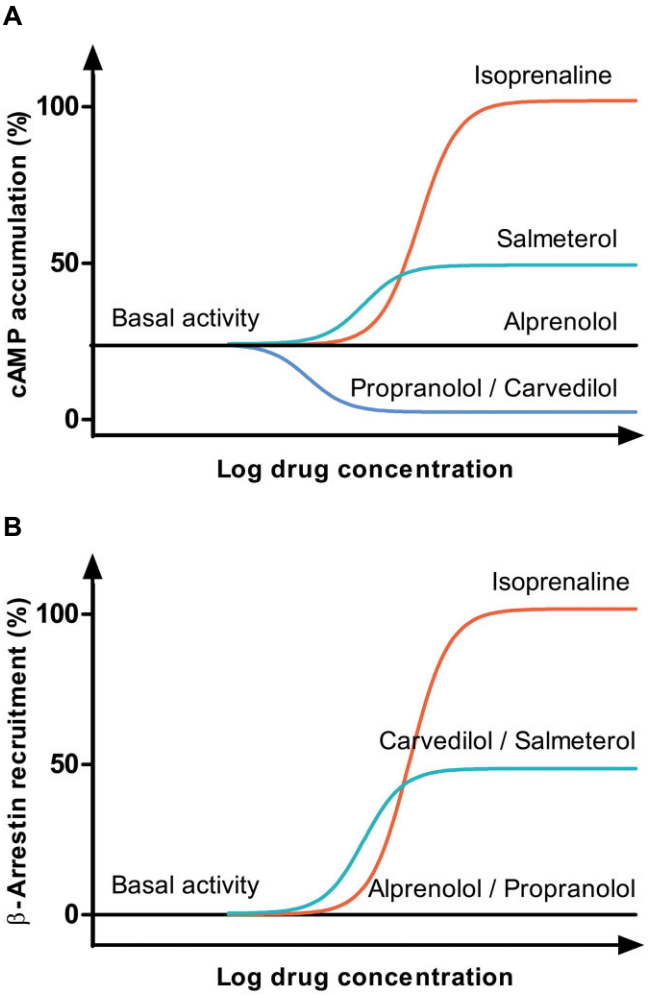

Figure 1

Concentration–response profiles of β_2-adrenoceptor ligands possessing different efficacies for cAMP and β-arrestin responses. (A) The β_2-adrenoceptor exhibits basal agonist-independent activation of the cAMP response. The inverse agonists (propranolol, carvedilol) suppress basal activity. The neutral antagonist (alprenolol) blocks the binding of other ligands, but does not produce any biological response. The full agonist (isoprenaline) produces a full response. The partial agonist (salmeterol) only produces a partial response even at saturating concentrations. (B) Under basal condition, the β_2-adrenoceptor does not recruit β-arrestins to the plasma membrane. Alprenolol and propranolol act as antagonists for β-arrestin recruitment. The full agonist (isoprenaline) produces a full response. The partial agonist (salmeterol) produces a partial response. Carvedilol behaves as a partial agonist in this case, suggesting that it is a β-arrestin-biased agonist. (Curves are schematic representations inferred from the data reported in Wisler *et al.*, 2007 and Drake *et al.*, 2008.)

Mechanisms of functional selectivity

Different mechanisms of functional selectivity have been proposed. It has been hypothesized that chemically diverse ligands stabilize a given receptor at distinct active conformations (R^{*1}, R^{*2}, . . . R^{*n}), and each of these conformations activates a unique signalling pattern. In other words, ligand-specific receptor conformations lead to ligand-specific signalling (Kenakin, 2007; Kobilka and Deupi, 2007; Woo and Xiao, 2012). Biased signalling predominantly via this mechanism has been referred as 'ligand bias' (DeWire and Violin, 2011).

In addition, the mechanisms of functional selectivity may involve downstream signalling events. For instance, phosphorylation of β_2-adrenoceptors by different GRK isoforms at the carboxy-terminus dictates the function of β-arrestins, the scaffold proteins that terminate G-protein signalling. Using MS, Nobles *et al.* (2011) have shown that the full-agonist isoproterenol and the β-arrestin-biased agonist carvedilol induce different phosphorylation patterns at the GRK2-sites and the GRK6-sites of the β_2-adrenoceptor. These phosphorylation patterns result in distinct outcomes in the recruitment of β-arrestins to the receptor and different degrees of ERK1/2 activation (Ren *et al.*, 2005). Other proposed mechanisms of functional selectivity include allosteric modulation, receptor dimerization, receptor desensitization, non-receptor modifiers of GPCR signalling, cellular microdomains and cellular contexts (Maudsley *et al.*, 2005; Kenakin and Miller, 2010).

Among these proposed mechanisms, the ligand-specific receptor conformation theory is best supported by experimental evidence. This mechanism is mainly related to the intrinsic property of a ligand–receptor complex and is system-independent. Specifically, the functional outcome of a given ligand–receptor interaction should be highly preserved in different experimental settings, including different cell types, animal species, *in vitro* or *in vivo* systems. In the following, we will use β_2-adrenoceptors as a prototypical example to illustrate recent progress in ligand-specific GPCR conformation research.

Evidence for ligand-specific receptor conformations

Recently, biophysical methods such as fluorescence spectroscopy, X-ray crystallography, MS, NMR spectroscopy and single-molecule force spectroscopy (SMFS) have been used to study ligand-specific receptor conformations and their functional consequences. In the following sections, we will review how studies using some of these techniques have advanced our understanding of ligand-specific or functionally selective GPCR conformations.

Fluorescence spectroscopy

Fluorescence spectroscopy has been one of the most widely used methods in the study of receptor conformational

transition. The first experimental evidence to support the existence of two receptor states (R and R*) was given by Gether and co-authors in 1995 in a fluorescence spectroscopic study of purified β_2-adrenoceptor covalently labelled with a fluorescence probe. Over the years, different variants of the method have emerged (for review, see Hoffmann *et al.*, 2008; Lohse *et al.*, 2008). Fluorescent spectroscopy has outperformed guanine nucleotide analogues (Seifert *et al.*, 1999) and zinc-bridge techniques (Vilardaga *et al.*, 2001) developed at the same time.

Studies using fluorescent probes have provided a large body of evidence for ligand-specific receptor conformations. Most notably, studies by Kobilka's group in the turn of the century (Gether *et al.*, 1997; Ghanouni *et al.*, 2001a,b; Seifert *et al.*, 2001) have not only experimentally determined the inactive and the active states of β_2-adrenoceptors bound with different ligands, but also identified intermediate states for partial agonist-bound receptor. These findings support the multi-state model of GPCR activation. This model hypothesized that a GPCR exists in a hierarchy of intermediate states between the inactive and the fully active states. Further studies on β_2-adrenoceptor activation in this direction have identified multiple ligand-specific receptor conformations and have led to the sequential receptor activation model (Swaminath *et al.*, 2004; 2005; Yao *et al.*, 2006). Fluorescent spectroscopy has been applied in the study of receptor conformational transition of other GPCRs such as 5-HT_4 receptor (Baneres *et al.*, 2005), α_{2A}-adrenoceptor (Gales *et al.*, 2006; Zurn *et al.*, 2009), β_1-adrenoceptor (Rochais *et al.*, 2007), leukotriene B_4 receptor (Damian *et al.*, 2006), metabotropic glutamate receptor (Tateyama *et al.*, 2004; Tateyama and Kubo, 2006), δ-opioid receptor (Audet *et al.*, 2008) and parathyroid hormone receptor (Castro *et al.*, 2005).

The introduction of the FRET technique has enabled receptor conformational studies in living cells. Development of the low molecular weight fluorescent probes such as fluorescein arsenical hairpin binder has allowed receptor conformational studies in high resolution. Using FRET or bioluminescence resonance energy transfer, ligand-specific conformations have been identified for the β_2-adrenoceptor (Granier *et al.*, 2007; Reiner *et al.*, 2010; Malik *et al.*, 2013), ghrelin receptor (Mary *et al.*, 2012), vasopressin V_2 receptor (Rahmeh *et al.*, 2012) and formyl-peptide receptor type 2 (Cooray *et al.*, 2013).

X-ray crystallography

GPCRs are challenging targets for structural determination by X-ray crystallography. The first high-resolution crystal structure of GPCR was solved in 2000 (Palczewski *et al.*, 2000). Technological advances over the past few years have led to an explosion of GPCR crystal structures. To date, the crystal structures of 25 different GPCRs have been reported. Among these GPCRs, some have been resolved in both the agonist-bound and the antagonist/inverse agonist-bound conformations. These include rhodopsin (retinal-bound in both the ground state and the photoactivated state), β_1-adrenoceptor, β_2-adrenoceptor, adenosine receptor, ACh M_2 receptor, smoothened receptor and $P2Y_{12}$ receptor. However, not all crystal structures of the agonist-bound GPCRs are in their active states. Crystallographic studies of β_1-adrenoceptor, β_2-adrenoceptor and 5-HT receptors will be reviewed below for their importance to the understanding of the structural basis of GPCR functional selectivity.

About a dozen crystal structures of the β_2-adrenoceptor have been reported. These structures include the unliganded receptor (or apo-β_2-adrenoceptor) and the receptor bound with neutral antagonists, inverse agonists, and agonists with different binding affinities or covalently linked agonists (Cherezov *et al.*, 2007; Rasmussen *et al.*, 2007; 2011a,b; Rosenbaum *et al.*, 2007; 2011; Wacker *et al.*, 2010; Ring *et al.*, 2013). Without exception, co-crystallization with the heterotrimeric G-protein (G_s) or its mimetic antibodies is required to obtain structures of agonist-bound β_2-adrenoceptors in their active state, suggesting that the agonist-bound β_2-adrenoceptor is highly dynamic. Overall, these studies have revealed subtle, ligand-specific differences in receptor conformations. According to their similarities, these conformations can be categorized into two groups, the R state represented by the carazolol-bound β_2-adrenoceptor [Protein Data Bank (pdb):2RH1] (Cherezov *et al.*, 2007) and the R* state represented by the BI167107-bound β_2-adrenoceptor-G_s complex (pdb:3SN6) (Rasmussen *et al.*, 2011a). The crystallographic studies have offered important insights into the mechanisms underlying β_2-adrenoceptor activation and functional selectivity.

Crystallographic studies have reported the structures of the turkey β_1-adrenoceptor bound with diverse ligands, including the full agonists isoprenaline and carmoterol, the partial agonists salbutamol and dobutamine, the β-arrestin-biased agonists carvedilol and bucindolol, the inverse agonist carazolol and the antagonist cyanopindolol (Warne *et al.*, 2008; 2011; 2012; Moukhametzianov *et al.*, 2011). Among them, the structure of the cyanopindolol-bound β_1-adrenoceptor is regarded as the reference conformation. This structure is similar to the crystal structures of β_1-adrenoceptors bound with agonists. It is postulated that the agonist-bound structures are inactive, non-signalling state of the receptor formed on initial ligand binding. As these data suggest, the binding of each of the full or partial agonists causes a contraction of the ligand-binding pocket by a similar extent. Carvedilol and bucindolol have been shown to activate β-arrestin signalling, while acting as inverse or partial agonists for the G-protein pathway (Galandrin *et al.*, 2008; Kim *et al.*, 2008). Interestingly, they do not cause contraction of the ligand-binding pockets, and, therefore, their stabilized structures represent that of the antagonist-bound receptor. The authors have proposed that the bulky aromatic substituents at the amine ends of bucindolol and carvedilol, which are not found in other β-blockers, may interact with additional residues in extracellular loop 2 and TM7 and these additional hydrophobic interactions may promote β-arrestin signalling.

More recently, Stevens' research group have studied the structural basis of functional selectivity of 5-HT receptors by comparing the crystal structures of ergotamine-bound human 5-HT_{1B} (Wang *et al.*, 2013) and 5-HT_{2B} receptors (Wacker *et al.*, 2013) with those of the β_2-adrenoceptor. Firstly, ergotamine predominantly signals through the β-arrestin pathway at the 5-HT_{2B} receptor, whereas it is relatively unbiased at the 5-HT_{1B} receptor. Secondly, the confor-

mation of the D(E)/RY motif in the 5-HT_{1B} receptor is similar to that in β_2-adrenoceptor-R* (pdb:3SN6), whereas the conformation of the 5-HT_{2B} receptor compares to that of β_2-adrenoceptor-R (pdb:2RH1). In addition, the NPxxY motif in the 5-HT_{1B} receptor is in an intermediate active state when compared with β_2-adrenoceptor, whereas the conformation of the 5-HT_{2B} receptor is similar to β_2-adrenoceptor-R*. These results are in agreement with the signalling patterns of ergotamine in the two receptors and lead to the deduction that ergotamine stabilizes a conformation in 5-HT_{2B} receptor with reduced G-protein signalling.

MS

While X-ray crystallography has provided considerable insights into the structural mechanism of GPCR activation, the technique can only provide static pictures of ligand–receptor complexes. In contrast, MS does not characterize discrete conformational states, but it is capable of detecting changes in the equilibrium between these states. West *et al*. (2011) have used hydrogen-deuterium exchange coupled to MS (HDX-MS) to study the ligand-induced changes in conformational states of apo-β_2- and β_2-adrenoceptors bound with different ligands including a full agonist, a partial agonist and three antagonists or inverse agonists. Results of the study suggest a good correlation of the conformational change in particular regions of the receptor to ligand efficacy, and thus support the sequential receptor activation model proposed by crystallographic (Wacker *et al*., 2010; Ring *et al*., 2013) and fluorescence spectroscopic (Swaminath *et al*., 2004; 2005) studies. Kahsai and others (2011) have employed a similar quantitative mass spectroscopic approach to study ligand-receptor interactions for nine functionally distinct β_2-adrenoceptor ligands. They have shown that ligands can produce both agonist-specific and functionally specific patterns of receptor conformational rearrangement of β_2-adrenoceptors. In particular, the β-arrestin-biased agonist carvedilol triggers a unique reactivity pattern on $Lys263^{6.25}$ and $Cys265^{6.27}$. These studies have provided direct evidence for the presence of multiple ligand-specific conformations.

NMR spectroscopy

Protein structures from X-ray crystallography are biased towards the most thermodynamically stable conformations. Therefore, many functionally important conformations may be missed in crystallographic studies simply because they are less stable. To understand better the dynamic properties of GPCRs, NMR spectroscopy has been employed.

Bokoch *et al*. (2010) have analysed the NMR spectra of seven $^{13}CH_3$-labelled lysine residues on β_2-adrenoceptor bound with the inverse agonist carazolol, the neutral antagonist alprenolol and the full-agonist (*R,R'*)-formoterol. This report has provided a unique perspective into how conformational changes at the orthosteric ligand-binding pocket and the extracellular surface of the β_2-adrenoceptor may be coupled by ligands targeting these surfaces. By monitoring nine $^{13}CH_3$-labelled methionine residues on the β_2-adrenoceptor using NMR, Kofuku *et al*. (2012) have found that the binding of ligands, including carazolol, alprenolol, tulobuterol, clenbuterol and formoterol, induce efficacy-dependent changes in the chemical shifts of three methionine residues on TM2, TM5 and TM6 respectively. Consistent with the inference from other crystallographic (Rasmussen *et al*., 2011a) and NMR (Nygaard *et al*., 2013) studies, the authors explained their data saying, 'the resonances from $Met215^{5.54}$ and $Met279^{6.41}$, which are located on the cytoplasmic side of the TM region, were not observed in the formoterol-bound state, suggesting that these signals were broadened because of the conformational exchange between the multiple conformations in the formoterol-bound state. The multiple conformations on the cytoplasmic side of the TM region in the full agonist-bound state may be effective for interacting with various effectors.'

Liu *et al*. (2012) have studied biased signalling of β_2-adrenoceptors in response to functionally diverse ligands using ^{19}F-NMR by labelling two functionally important cysteine residues ($Cys265^{6.27}$ and $Cys327^{7.54}$) at the cytoplasmic surface of β_2-adrenoceptors. Interestingly, the β-arrestin-biased ligands isoetharine and carvedilol cause large shifts of the equilibria in $Cys327^{7.54}$ towards the active state. The authors concluded that conformational change in TM7 primarily affects β-arrestin signalling. Another line of investigation by ^{19}F-NMR has identified two inactive conformations S_1 and S_2 and a conformation of the activation intermediates S_3 of β_2-adrenoceptors (Kim *et al*., 2013). The study has also suggested the existence of multiple active state conformations of the β_2-adrenoceptor although the data are less convincing as compared with those for the inactive state of the receptor.

SMFS

SMFS is an atomic force microscopy-based technique widely applied in the characterization of biomolecular systems. Recently, SMFS has revealed molecular interactions in a multitude of membrane proteins including the GPCRs (Zocher *et al*., 2012; Kawamura *et al*., 2013). Zocher *et al*. (2012) have used dynamic SMFS to investigate how ligand binding modulates the energy landscape of the human β_2-adrenoceptor. Five different ligands representing agonists (BI167107, THRX-144877 and adrenaline), inverse agonists (carazolol) or neutral antagonists (alprenolol) establish a complex network of interactions in the receptor. Compared with the unliganded or neutral antagonist-bound states, the binding of agonists or carazolol increases the width of the stabilized free energy valley of the structural core segment [TM3-cytoplasmic loop 2-TM4-extracellular loop 2-TM5.1]. Interestingly, the magnitude and the location of these changes depend on the efficacy of the bound-ligand. This implies that the conformational variability of the core segment increases in response to ligand binding. This observation is consistent with the crystallographic data (Rasmussen *et al*., 2011b) that the core segment is important for ligand binding (TM3 and TM5) and β_2-adrenoceptor activation (cytoplasmic loop 2). The SMSF data also correlate well with other structural and functional data (Rasmussen *et al*., 2011a; Kofuku *et al*., 2012; Nygaard *et al*., 2013) showing that ligand-binding enables the β_2-adrenoceptor to adopt more conformations from which certain conformations represent an active state.

Ligand-specific receptor conformations underlying-biased GPCR signalling

Existing biophysical studies have provided ample evidence for the existence of intermediate states apart from the R state and the R* state during GPCR activation. This conclusion forms the basis of the multi-state GPCR activation model, which suggests that different ligands induce ligand-specific receptor conformations and accordingly produce biological responses at different levels of efficacy. It is likely that a similar concept applies to the structural mechanism of pluridimensional efficacy, given the many crystallographic data that actually demonstrate some, although minor, differences in the conformations of a given GPCR bound with pharmacologically diverse ligands (Warne *et al.*, 2011; Ring *et al.*, 2013). In addition, recent fluorescence spectroscopic studies (Reiner *et al.*, 2010; Mary *et al.*, 2012; Rahmeh *et al.*, 2012; Cooray *et al.*, 2013; Malik *et al.*, 2013) and a few advanced biophysical studies (Kahsai *et al.*, 2011; Liu *et al.*, 2012; Wacker *et al.*, 2013) have indicated a correlation between ligand-specific receptor conformation and biased GPCR signalling. Nevertheless, it has not yet been shown how a particular ligand-receptor interaction induce a unique receptor conformation and by what mechanism this conformation causes biased signalling. Thus, ligand-specific receptor conformation remains a missing link in our present understanding of the mechanism underlying functional selectivity. More research in this area is needed to elucidate the structural basis of functional selectivity.

Mutagenic studies

Site-directed mutagenesis is widely used to characterize ligand recognition sites. Mutagenic studies on biased GPCR signalling can be dated back to the 1990s (Negishi *et al.*, 1995; Wiens *et al.*, 1998). However, a pitfall of this approach is the possibility for the mutation to induce ligand-independent alteration of the receptor conformation in such a way to disrupt receptor's natural interaction with its intracellular partners, in other words, the stability of the functional receptor–partner complex called 'signalsome'. This problem can be circumvented if a structure–functional selectivity relationship analysis based on a sound structural knowledge of the ligand recognition sites of the GPCR is incorporated into the study scheme. Therefore, mutagenic studies in the framework of contemporary structural biology can unravel the mystery how a particular ligand–receptor interaction alters the GPCR conformation and consequently leads to biased signalling. In recent years, benefitting from the availability of the crystal structures of a number of GPCRs and the computational tools for the study of ligand–receptor interactions and protein conformations or dynamics, mutagenic studies have begun to reveal the structural basis of functional selectivity (Fenalti *et al.*, 2014; Woo *et al.*, 2014).

Recent mutagenic and structure–functional selectivity relationship studies have identified amino acid residues on GPCRs important for biased signalling and have led to the discovery of new biased ligands. Tschammer *et al.* (2011) have found that mutation of His393$^{6.55}$ of the dopamine D_{2L} receptor substantially masks ligand-biased signalling. Subsequent targeted chemical modification has resulted in a novel biased ligand FAUC350, which behaves as an antagonist for cAMP accumulation and a partial agonist for ERK1/2 phosphorylation. Using a mutagenic approach, Fowler *et al.* (2012) have identified the three conserved serine residues on TM5, especially Ser194$^{5.43}$, to be the other major determinants for biased signalling at the D_{2L} receptor. Further computation analysis has revealed multiple ligand-specific receptor conformations. Based on these findings, a mechanism of functional selectivity at D_2 receptors has been proposed in which ligand-induced conformational change at TM6 determines signalling bias. These studies could have provided structural insights into the development of novel biased ligands for the D_2 receptor (Chen *et al.*, 2012; Shonberg *et al.*, 2013). Using site-directed mutagenesis, Bock *et al.* (2012) have found that Trp422$^{7.35}$ of ACh M_2 receptor, which lines the passage between the allosteric vestibule and the orthosteric binding cavity, is critical for the ligand bias of dualsteric agonists (which bind to both the allosteric and the orthosteric sites) to activate a G_i over G_s signalling as compared with the orthosteric agonists. It is postulated that conformational rearrangement around Trp422$^{7.35}$ as a result of agonist binding is transmitted to the cytoplasmic end and consequently leads to differential G-protein coupling.

Ligand–receptor interactions determine biased signalling

Recently, we have investigated the molecular mechanism of functional selectivity at β_2-adrenoceptor in cardiomyocytes (Woo *et al.*, 2014). Like many other GPCRs, the β_2-adrenoceptor couples to two different G-proteins (G_s and G_i) (Xiao *et al.*, 1995; 1999). While most β_2-adrenoceptor agonists stimulate the receptor to activate both G_s and G_i, fenoterol preferentially activates the β_2-adrenoceptor-G_s signalling (Xiao *et al.*, 2003). However, the mechanism of G_s-biased β_2-adrenoceptor signalling was unknown. Using site-directed mutagenesis and biophysical assays on adult cardiomyocytes, we found that Tyr308$^{7.35}$ of the β_2-adrenoceptor is necessary for ligand-directed G_s-biased signalling. A structure–functional selectivity relationship study using a cohort of fenoterol analogues (Beigi *et al.*, 2006; Jozwiak *et al.*, 2007; 2010) (Figure 2) has revealed that stereochemistry and the substituent at the amine end of the fenoterol compound determine G_s-biased β_2-adrenoceptor signalling. Firstly, the compound should be in an (*R,R'*) rather than in an (*S,R'*) configuration (Woo *et al.*, 2009). Secondly, the aminoalkyl substituent should contain a benzyl, but not a naphthyl moiety, and the benzyl moiety must contain a 4'-OH, 4'-OCH_3 or 4'-NH_2 group (Figure 2). In computer simulation, the phenyl hydroxyl group of β_2-adrenoceptor-Tyr308$^{7.35}$ forms a hydrogen bond with each of these functional groups (Plazinska *et al.*, 2013; Woo *et al.*, 2014). This hydrogen bond is specific to G_s-biased signalling because its absence in the simulated interactions with the β_2-adrenoceptor-Y308F (deletion of the phenolic-OH group) mutant or compounds lacking a 4'-(O/N)-benzyl moiety is correlated with G_s and G_i dual signalling. Thus, our data have provided the evidence confirming the identification of a ligand–receptor interaction important for G_s-biased β_2-adrenoceptor signalling.

Several lines of evidence suggest ligand-specific receptor conformation to be the major player in the mechanistic process leading to G_s-biased β_2-adrenoceptor signalling.

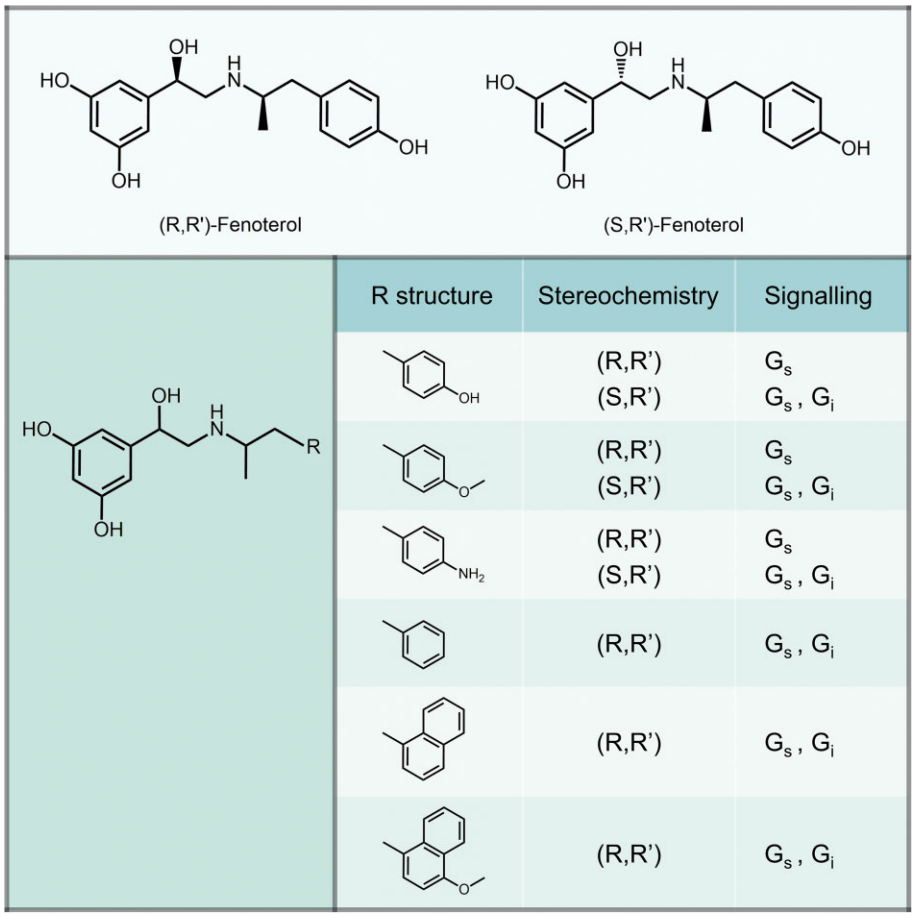

Figure 2

Structure–functional selectivity relationship of fenoterol derivatives for differential β_2-adrenoceptor-G-protein signalling. Fenoterol contains two chiral centres in its molecule and can exists as the (*R,R'*), (*R,S'*), (*S,R'*) and (*S,S'*) stereoisomers. The structures of (*R,R'*)-fenoterol and (*S,R'*)-fenoterol are shown at the top. Different fenoterol derivatives stimulate the β_2-adrenoceptor to either couple to G_s or couple dually to G_s and G_i. Signalling induced by selective G_s or promiscuous G_s and G_i activation depends on both the stereochemistry and the substituent on the aminoalkyl portion of the fenoterol compound as shown. (Data are extracted from Woo *et al.*, 2009; 2014.)

Firstly, the G_s-biased signalling is independent of the phosphorylation status of the β_2-adrenoceptor, contrasting a previous 'G-protein switch' hypothesis in β_2-adrenoceptor-G_i protein coupling (Daaka *et al.*, 1997). Secondly, the signalling bias phenomena of the fenoterol analogues are preserved in human embryonic kidney cells as well as cardiomyocytes isolated from mouse or rat. Also, a study in a canine heart failure model (Chakir *et al.*, 2011) has independently confirmed our previous finding in spontaneous hypertensive rats (Xiao *et al.*, 2003) that fenoterol is a G_s-biased β_2-adrenoceptor agonist. These data collectively suggest that the induction of G_s-biased β_2-adrenoceptor signalling by fenoterol is a species-independent phenomenon. Thirdly, consistent results have been obtained in experiments using cells expressing different levels of β_2-adrenoceptor (from basal to 100-fold over basal), suggesting that the G_s-biased signalling is independent of receptor expression level. In addition, the 7.35 residue has also been suggested to play a role in ligand bias at other GPCRs (Bock *et al.*, 2012; Fowler *et al.*, 2012). Overall, these findings suggests that the hydrogen bond interaction between the G_s-biased agonist and β_2-adrenoceptor-Tyr308$^{7.35}$ induces or stabilizes receptor conformation(s) that lead to preferential β_2-adrenoceptor-G_s signalling (Wang, 2014).

Future prospects

While understanding the molecular and structural mechanism of functional selectivity is crucial for a rational approach to develop novel functionally selective GPCR ligands, translation of this concept into novel treatments requires the development of new computational tools and biological assays that can predict or identify highly (patho)-physiologically relevant drug candidates. In addition, to clearly define which specific signal transduction pathway is clinically beneficial and which one should be avoided from our understanding based on cellular assays is clearly a big leap of faith. Factors such as species difference and the expression levels of receptors and effector proteins have to be considered in accessing the appropriateness of an assay system. As far as the mechanism of functional selectivity is concerned, recent

advances in biophysical methodology have already set a stage to the identification of ligand-specific functionally selective conformations in GPCRs. The resolving of the three-dimensional structures of, for example, the β_2-adrenoceptor-G_i complex and the β_2-adrenoceptor-β-arrestin 2 complex will probably advance our understanding of how functionally selective GPCR conformations induce different changes in the conformations of its partners with important impacts on their functions. During the preparation of the present paper, a report on a complex between the BI167101-bound β_2-adrenoceptor-V_2 receptor chimera and β-arrestin 1 characterized by HDX-MS and electron microscopy was published (Shukla *et al.*, 2014). With the maturation of these techniques, it is likely that many more breakthrough studies will appear in the near future. Another recent development is the use of total internal reflection fluorescent microscopy to characterize the interaction dynamics of single molecules of fluorescently labelled GPCRs with their partners in the membrane of a living cell (Calebiro *et al.*, 2013). As this technique is clearly useful in the spatiotemporal characterization of receptor dimerization and oligomerization, it may be innovatively used to study the interaction of GPCRs with G-proteins (Yao *et al.*, 2009) and other interacting partners, which have been shown to be spatially compartmentalized in cardiomyocytes (Head *et al.*, 2005). Research in this direction could improve our understanding of the mechanistic process of functional selectivity *in situ*. As most of our present understanding of functional selectivity is based on *in vitro* experiments using recombinant cellular systems, demonstration of ligand-directed signalling in native cells ideally in intact animals merits future investigation. This approach is likely to identify biased ligands efficacious in more physiologically relevant settings and speed up the process of translation.

Recent development of the G_s-biased β_2-adrenoceptor agonist fenoterol (Xiao *et al.*, 2003; Ahmet *et al.*, 2004; 2005; 2008; 2009; Chakir *et al.*, 2011) has demonstrated how studies on native cells have aided the translation of biased ligands into novel therapies for major human diseases such as heart failure (Woo *et al.*, 2015). These studies have substantiated the concept that long-term combination treatment with a G_s-biased β_2-adrenoceptor agonist and a β_1-adrenoceptor antagonist improves the structure and function of the failing hearts in rodent (Ahmet *et al.*, 2008; 2009). Further studies and clinical evaluation are underway to translate this concept into therapy.

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Conflict of interest

The authors declare no conflicts of interests.

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