

Biased Allostery

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ABSTRACT G-protein-coupled receptors (GPCRs) constitute a large group of integral membrane proteins that transduce extracellular signals from a wide range of agonists into targeted intracellular responses. Although the responses can vary depending on the category of G-proteins activated by a particular receptor, responses were also found to be triggered by interactions of the receptor with β -arrestins. It was subsequently discovered that for the same receptor molecule (e.g., the β -adrenergic receptor), some agonists have a propensity to specifically favor responses by G-proteins, others by β -arrestins, as has now been extensively studied. This feature of the GPCR system is known as biased agonism and is subject to various interpretations, including agonist-induced conformational change versus selective stabilization of preexisting active conformations. Here, we explore a complete allosteric framework for biased agonism based on alternative preexisting conformations that bind more strongly, but nonexclusively, either G-proteins or β -arrestins. The framework incorporates reciprocal effects among all interacting molecules. As a result, G-proteins and β -arrestins are in steric competition for binding to the cytoplasmic surface of either the G-protein-favoring or β -arrestin-favoring GPCR conformation. Moreover, through linkage relations, the strength of the interactions of G-proteins or β -arrestins with the corresponding active conformation potentiates the apparent affinity for the agonist, effectively equating these two proteins to allosteric modulators. The balance between response alternatives can also be influenced by the physiological concentrations of either G-proteins or β -arrestins, as well as by phosphorylation or interactions with positive or negative allosteric modulators. The nature of the interactions in the simulations presented suggests novel experimental tests to distinguish more fully among alternative mechanisms.

GPCRs are a large and diverse family of seven transmembrane helical receptors to which agonist binding triggers downstream cellular responses via G-proteins or β -arrestin (1–3). First identified for their role in desensitization, internalization, and recycling (4), β -arrestins were subsequently shown to participate directly in several intracellular signaling pathways (2). Although GPCRs have been studied for many years, with numerous agonists and many active pharmaceutical agents, only relatively recently has attention focused on the different agonists for a particular GPCR molecule that favor either G-protein or β -arrestin signaling (1). Such “biased agonism” reinforces the concept of multiple active conformations for GPCRs (5,6). However, a fundamental distinction can be made by asking whether their formation reflects “conformations induced” by the particular biased agonist (2) or stabilization of a small number of discrete preexisting conformations consistent with the Monod-Wyman-Changeux (MWC) allosteric model (7). Although particular features of GPCRs have been inter-

preted using allosteric concepts (8–11), a global allosteric formalism that explores the full range of allosteric linkages in the original MWC framework (7,12) is considered here. This formalism includes local steric competition between G-proteins and β -arrestin, as well as reciprocal effects that govern the potentiation of these two proteins on the corresponding agonist affinities. An earlier allosteric model involving two active states was applied to biased agonism, but reciprocal effects associated with binding of G-proteins and β -arrestins were not included (8).

The global allosteric formalism builds on recent developments involving both equilibrium and dynamic formulations of multiple conformational states in proteins (13,14). The ensemble nature of the states has been emphasized (15–17), including entropy-driven differences among states showing little or no overt structural changes (18,19). Changes in quaternary stoichiometry can also play a role, as, for example, in the passage from monomeric to dimeric states for GPCRs (20,21) and tyrosine kinases (22). Discrete conformational states are stabilized by noncovalent binding interactions of all molecular species (23). These include agonists and antagonists that bind to orthosteric sites, as well as by positive and negative modulators that bind to allosteric modulatory sites.

Submitted May 3, 2016, and accepted for publication July 20, 2016.

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Editor: Brian Salzberg.

<http://dx.doi.org/10.1016/j.bpj.2016.07.044>

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For GPCRs, the critical G-proteins and β -arrestins that transfer signals to downstream pathways upon binding to the specialized transfer site also influence the distribution of conformations (see Fig. 1). For a conformation that favors binding by a G-protein, the G-protein will also favor binding of the biased agonist. Covalent phosphorylation reactions also modify the stability among conformational states, with particular importance for biased agonism, since phosphorylation by G-protein receptor kinases (GPKs) at critical

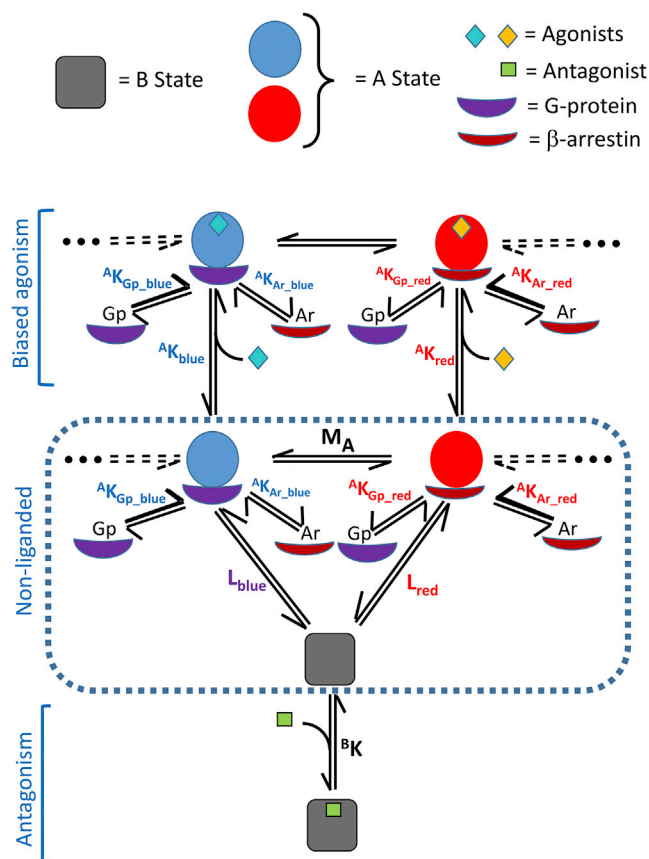


FIGURE 1 Minimal reactions for a GPCR molecule with two active states. The two active conformational A states are indicated by red and blue circles, with the basal B state as a gray square. (For the print version, the red and blue colors are replaced by shades of gray.) The central rectangular box presents the GPCR molecules in conformational equilibrium in the absence of agonists or transfer molecules. Above the rectangle are presented reactions involving binding by agonists, G-proteins, and β -arrestins. The agonists are indicated by turquoise or orange diamonds that preferentially bind to the A_{blue} or A_{red} states, respectively. All of these molecules can bind to both the A_{blue} and A_{red} states (as well as the B state) according to the principle of nonexclusive binding, but for simplicity, only binding to the A state with the highest affinity is shown. Similarly, for the two classes of agonists depicted, binding to the less favored state is not shown. Antagonists may also bind to each state, but are only shown for the B state. Allosteric modulators (not shown, but see Fig. 2 D) can also bind to each state. The conformational equilibrium constants are indicated by L_{blue} and L_{red} for the interactions of the respective states with the B state. The constant M_A defines the equilibrium between the A_{blue} and A_{red} states. Other constants are defined in Table 1. To see this figure in color, go online.

C-terminal residues of GPCRs can regulate the interactions with β -arrestins (2). Phosphorylation may also bias the coupling to transfer proteins as in the case of 5-HT₆ receptors for which Cdk5 phosphorylation changes from a ligand-dependent coupling to G_s to a ligand-independent coupling to Cdc42 (24). Specific agonists may be full or partial, in addition to showing a bias for interactions with G-proteins or β -arrestins. In some cases, ligands can be antagonists, including antagonists with negative intrinsic activity (inverse agonists) for either the G-protein or β -arrestin pathway, but agonists for the other (25,26). In addition, some antagonists upon close examination may be categorized as very weak partial agonists (27). Constitutively active receptors are a hallmark of preexisting conformational equilibria (12). Mutations that change the side chains of critical amino acid residues can also influence bias for G-proteins versus β -arrestins (28), as well as bias between G_q and G_s proteins (29). Pathologies such as the Kallmann Syndrome can arise from natural missense mutations that alter bias (30). Allosteric modulators also alter the specificity of agonist bias (31), reminiscent of allosteric modulation of enzyme substrate specificity, as in the case of ribonucleotide reductase (32). Complex interactions between agonists and allosteric modulators may occur particularly when their respective binding sites are in close proximity (33).

Recent progress in structural studies on GPCRs and rhodopsins have provided considerable insights into functional features of these proteins (34,35), including complexes with arrestin (36,37). The structural studies reinforce the evidence that G-proteins and β -arrestin cannot bind simultaneously and their binding is competitive. Other structural studies have addressed the issues of conformational switching (6,38), as well as allosteric modifications, including “bi-topic” ligands active at both agonist and allosteric sites (39). Insights into the structure and dynamics of heterotrimeric G-proteins trimers have also been recently obtained (40).

Historically, modeling of GPCRs has been placed in the classical context of the empirical approach of Black and Leff for competitive steric antagonism (41). The critical factors in their model are the apparent agonist affinity for the receptor (K_A) and the operational “transducer ratio” (τ), where $\tau = [R_0]/K_E$ (41,42). Here, R_0 is defined as the total receptor concentration and K_E is a virtual equilibrium constant for the physiological response, defined as the concentration of the agonist-receptor complex that elicits half-maximal response. Hence, τ is a composite constant with two components that cannot readily be determined independently, but τ remains a popular parameter for characterizing GPCR (43). In practice, τ is related to a given response for a specific GPCR agonist by the relationship $EC_{50} = K_A/(1 + \tau)$. When normalized to a reference full agonist, a particular agonist under consideration is characterized by its value of $\Delta \log(\tau/K_A)$. Although the Black-Leff model is not based on intrinsic parameters of chemical reactions, it

has endured as the favored model for quantifying biased agonism. For two agonists (Ag1 and Ag2) with bias toward G-protein or β -arrestin signaling, their comparative bias is given by $\Delta\Delta\log(\tau/K_A)_{Ag1-Ag2}$ (26).

In contrast to the phenomenological formulation of Black and Leff (41), we develop an explicit molecular mechanism based on defined chemical reactions within an allosteric context of multiple conformational transitions. The same principles were applied to extended networks of conformations and specific interactions (27), but for simplicity, here we consider only two preexisting active GPCR conformations (represented schematically as red and blue in Figs. 1 and 2, or with shades of gray in the black-and-white print versions). Both conformations can bind G-proteins and β -arrestin molecules nonexclusively at their transfer site, but where their affinities are not identical, we assign the blue conformation to the higher affinity for G-proteins and the red conformation to the higher affinity for β -arrestin. In the minimal form of this model, as examined here, we assume a single discrete active conformation stabilized by agonist binding and available for G-protein or β -arrestin binding without additional conformational change. As a result, under all conditions, reciprocal effects arise from competition between G-protein and β -arrestin binding, as well as their synergistic linkage effects on the apparent agonist affinities.

A global allosteric formalism for GPCR signaling

The critical parameter for conformational equilibria is the allosteric constant, L , defined by the ratio of the resting or basal (B) and active (A) conformational states and their multiple reactions, as shown in Fig. 1, using the B-A nomenclature as previously defined for membrane receptors (44). Since the A state is subdivided into two classes, distinct L values are defined: L_{blue} and L_{red} . In relation to these two parameters, the partition between red and blue active states is defined by the parameter M_A , where $M_A = L_{blue}/L_{red}$. Following the primary division into two active conformations, all other parameters must be distinguished with respect to these conformations, including agonists, transfer molecules (G-proteins and β -arrestin), and allosteric modulators. The same principles could be applied to any number of additional discrete active states with other distinguishing properties. An antagonist is depicted binding to the B state only in Fig. 1, but such a molecule would also be expected to display a significant, but weaker, affinity for the A states, according to the principle of nonexclusive binding. Although not explicitly represented in Fig. 1, the same principles would apply to any allosteric modulation along the lines depicted for an agonist, in the case of binding more strongly to the A state (a positive effector or a full or partial agonist), or alternatively more strongly to the B state (a negative effector, antagonist, or inverse agonist). The various param-

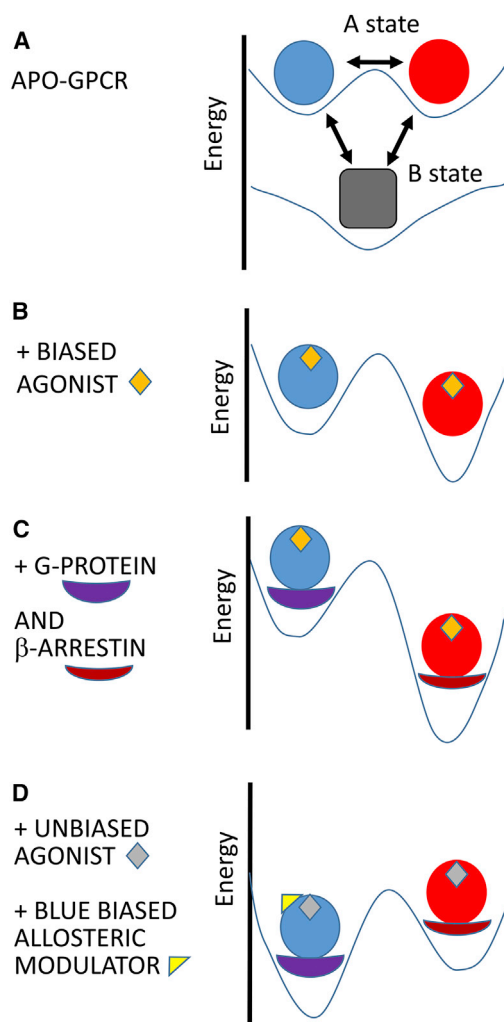


FIGURE 2 Energy relationships among the conformational states. (A) Intrinsic equilibrium among the conformations of the GPCR molecules in the absence of agonists, antagonists, transfer proteins, or allosteric modulators. Under these conditions the B state is more stable than the A states (shown here with equal stabilities). (B) The redistribution of A_{blue} and A_{red} states in the presence of a biased agonist (in this case favoring the A_{red} state). Both states are stabilized, but A_{red} is more so than A_{blue} . The B state is not shown. (C) Molecules are shown as in (B), with the addition of transfer proteins, G-protein and β -arrestin. Each transfer protein can bind to both the A_{blue} and A_{red} states, but only the preferential binding partners are presented here. An additional stabilization of the A_{red} state arises from the binding energy provided by β -arrestin. (D) The case of an unbiased agonist (gray diamond) bound in the presence of a biased positive allosteric modulator that favors the A_{blue} state. To see this figure in color, go online.

eters utilized in the modeling presented here are summarized in Table 1. Full details of the modeling equations are presented in the Supporting Material.

The effect of biased agonism according to the global allosteric formalism is schematically represented by the energy diagram in Fig. 2. In the absence of other components, the blue and red active conformations are of equivalent stability to, but less energetically favorable than, the weakly active B state (Fig. 2 A). Addition of an agonist biased toward the

TABLE 1 Parameters for the GPCR Model

Conformational Transition Parameters		Initial Values
L_{blue}	Intrinsic allosteric constant for the intrinsic equilibrium between resting and G-protein-favored state in the absence of agonist: $L_{\text{blue}} = [B]/[A_{\text{blue}}]$	100
L_{red}	Intrinsic allosteric constant for the intrinsic equilibrium between resting and G-protein favored state in the absence of agonist. $L_{\text{red}} = [B]/[A_{\text{red}}]$	100
M_A	Ratio of intrinsic stabilities of A_{blue} and A_{red} states: $M_A = [A_{\text{blue}}]/[A_{\text{red}}]$	1
A_{blue} State Parameters		
$^A K_{\text{Gp_blue}}$	Dissociation equilibrium constant of the complex between a GPCR molecule in the A_{blue} state and a G-protein	2×10^{-6} M
$^A K_{\text{Ar_blue}}$	Dissociation equilibrium constant of the complex between a GPCR molecule in the A_{blue} state and a β -arrestin	10^{-4} M
$^A K_{\text{Ag_blue}}$	Dissociation equilibrium constant of the complex between a GPCR molecule in the A_{blue} state and an agonist	5.5×10^{-8} M
$^A K_{\text{Al_blue}}$	Dissociation equilibrium constant of the complex between a GPCR molecule in the A_{blue} state and an allosteric modulator	NA
A_{red} State Parameters		
$^A K_{\text{Gp_red}}$	Dissociation equilibrium constant of the complex between a GPCR molecule in the A_{red} state and a G-protein	10^{-4} M
$^A K_{\text{Ar_red}}$	Dissociation equilibrium constant of the complex between a GPCR molecule in the A_{red} state and a β -arrestin	2×10^{-6} M
$^A K_{\text{Ag_red}}$	Dissociation equilibrium constant of the complex between a GPCR molecule in the A_{red} state and an agonist	3.5×10^{-8} M
$^A K_{\text{Al_red}}$	Dissociation equilibrium constant of the complex between a GPCR molecule in the A_{red} state and an allosteric modulator	NA
B State Parameters		
$^B K_{\text{Gp}}$	Dissociation equilibrium constant of the complex between a GPCR molecule in the B state and a G-protein	10^{-4} M
$^B K_{\text{Ar}}$	Dissociation equilibrium constant of the complex between a GPCR molecule in the B state and a β -arrestin	10^{-4} M
$^B K_{\text{Ag}}$	Dissociation equilibrium constant of the complex between a GPCR molecule in the B state and an agonist	10^{-4} M
$^B K_{\text{Al}}$	Dissociation equilibrium constant of the complex between a GPCR molecule in the B state and an allosteric modulator	NA

NA, not applicable.

A_{red} state is represented in Fig. 2 B. The binding of an agonist enhances the stability of all states, but preferentially one of the A states for a full or partial agonist. In contrast, preferential stabilization of a B state would arise from binding of an antagonist with higher affinity for a B state than for

an A state (also referred to as an inverse agonist). In the case of biased agonism, an agonist preferentially enhances the stability of the favored A state, the A_{red} state in Fig. 2 (for simplicity, the B state is not presented). Additional stability is accrued by binding of the transfer proteins, G-protein or β -arrestin, with quantitative differences related to the A_{blue} conformation with higher affinity for G-proteins versus the A_{red} conformation with higher affinity for β -arrestin (Fig. 2 C). An allosteric modulator with nonexclusive binding would enhance the stability of all states, but preferentially A or B, depending on whether the effector is positive or negative. For a nonbiased agonist, an allosteric modulator can produce biased agonism, as presented in Fig. 2 D for a modulator that favors the A_{blue} state. Weaker binding of the modulator to the A_{red} state is implicit.

The global allosteric formalism represents a system of fully linked reactions, such that the concentrations and affinities of each component influence the distribution of all other components. If a GPCR molecule is considered in isolation, the fundamental conformational equilibrium is defined by the allosteric constant L, as applied to the relative stability of two states (7). With two preexisting active states, two intrinsic constants apply, L_{blue} and L_{red} , but operationally the presence of G-proteins and β -arrestins will necessarily diminish these values due to preferential binding to the active states. Since these interactions are competitive due to their binding to the same region on the intracellular surface of the GPCRs, their relative balance will clearly influence the downstream signaling under physiological conditions. Moreover, the exact shape of the physiological response curve corresponds to the state function for which the shape and dependence on the allosteric constant are distinct from the agonist-binding function (45,46).

Quantitative features of the global allosteric model can be examined to explore the interactions between all of the components. An example is illustrated in Fig. 3 for clenbuterol, a biased agonist for β 1-adrenoceptors (47). In the data reported, clenbuterol produces partial signaling levels compared to the reference agonist for the G-protein response and ~50% higher for β -arrestin (47). Starting with the simple initial assumption of equal intrinsic stabilities of the red and blue conformations ($L_{\text{blue}} = L_{\text{red}} = 100$), the first consequence of the global allostery formulation is that stronger interactions of G-proteins and β -arrestin molecules with the A conformations compared to the B conformation necessarily reduce the effective L values by preferentially stabilizing the active state. In this simulation in Fig. 3 A, the experimentally observed bias in favor of β -arrestin can be accounted for by 1.5-fold stronger binding of clenbuterol to the red conformation. Therefore, as agonist binding increases (present as the \bar{Y} curve), the G-protein (*blue*) and β -arrestin (*red*) response curves (initially at the same low but nonzero value due to the effective L values of <100) diverge in favor of the β -arrestin response. However, the A_{red} and A_{blue} molecules are only partially

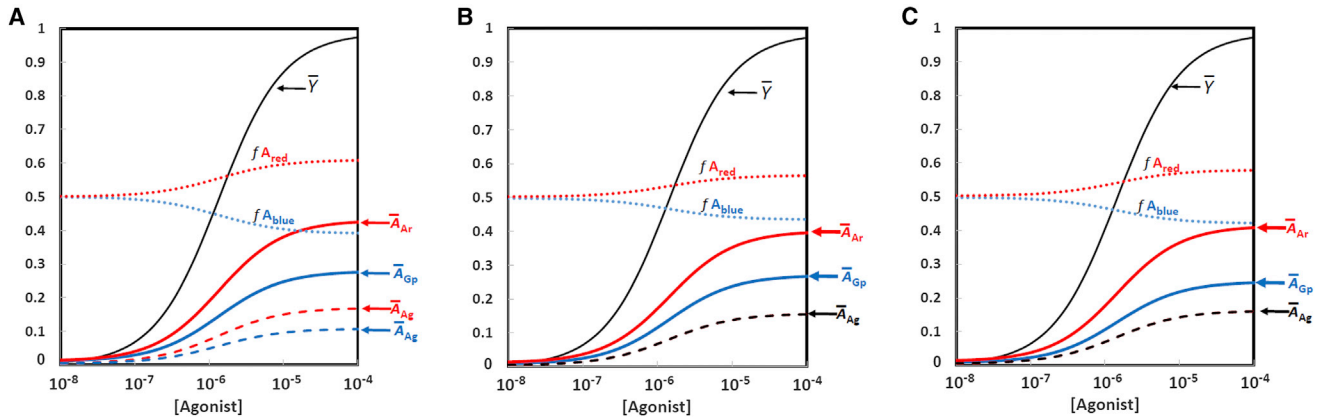


FIGURE 3 Simulated dose-response curves to represent clenbuterol. Each graph shows the fractional separation of the A state into red and blue components, given by $f_{A_{red}}$ and $f_{A_{blue}}$, respectively, along with the binding function, \bar{Y} . (A) Ratio of β -arrestin/G-protein = 1 (concentration of 5.0×10^{-6} M for both), but with stronger binding of the agonist to the A_{red} state corresponding to the parameter values in Table 1. The \bar{A} curves are separated for A-state molecules with agonist and G-protein (A_{blue}), agonist and β -arrestin (A_{red}), or agonist alone (A_{Ag}) shown separately for the A_{blue} and A_{red} states. Not shown are the fraction of A states that are fully nonliganded (negligible, $\sim 10^{-4}$) and the B state in its various liganded forms ($\sim 2\%$ at $[\text{agonist}] = 10^{-4}$ M). (B) Ratio of β -arrestin/G-protein = 1, with stronger binding for β -arrestin to the A_{red} state than for G-protein to the A_{blue} state. Parameter values are $[\text{G-protein}] = [\beta\text{-arrestin}] = 2.0 \times 10^{-5}$ M; other values are as in Table 1, except for $^A K_{Gp_blue} = 3.0 \times 10^{-6}$ M and $^A K_{Ar_red} = 2.0 \times 10^{-6}$ M, agonist affinity for both A_{red} and $A_{blue} = 4.0 \times 10^{-8}$. For these conditions agonist alone (A_{Ag}) is shown in black since the curves for the A_{blue} and A_{red} states overlap. (C) Ratio of β -arrestin/G-protein > 1: $[\text{G-protein}] = 3.0 \times 10^{-6}$ M, and $[\beta\text{-arrestin}] = 5.0 \times 10^{-6}$ M, with $^A K_{Gp_blue} = ^A K_{Ar_red} = 2.0 \times 10^{-6}$ M. Other details are as in (B). To see this figure in color, go online.

saturated with G-proteins or β -arrestin, as presented by the dashed red and blue lines for receptor-agonist complexes in the two states. Concomitantly, the fraction of receptor molecules in the A_{blue} conformation, $f_{A_{blue}}$, diminishes as the corresponding value for the A_{red} conformation, $f_{A_{red}}$, increases. The principle of nonexclusive binding implies that low levels of response could also be generated by binding of G-protein or β -arrestin molecules to the B state, but these responses would be considered background effects and were not included in the simulations presented here.

An important consequence of placing the GPCR system in a global allosteric context is to emphasize that biased responses can arise from factors other than biased agonism, hence the more appropriate designation “biased allostery.” A schematic example is presented in Fig. 2 D. In addition, under specific physiological conditions, the degree of bias can be affected by differences in the affinities of concentrations of G-proteins and β -arrestins for each active conformation. As shown in Fig. 3 B, a biased response can be achieved with equal affinities of the agonist for the blue and red conformations, but with slightly stronger (1.5-fold) binding by β -arrestin. Biased response can be influenced also by differences in their respective concentrations (Fig. 3 C), with equal affinities of the agonist, as well as G-proteins and β -arrestin, for the red and blue states but a slightly higher (1.7-fold) concentration of β -arrestin (Fig. 3 C).

General considerations

Exploring biased agonism with a global allosteric model involving all components interacting in the complete system

reveals that response-specific preexisting active states can readily accommodate the basic observations generated by biased agonists. In addition, incorporating G-proteins and β -arrestins directly into the allosteric framework reveals the reciprocal effects related to the concentrations and affinities of the G-proteins and β -arrestins, as well as their competition for binding to the cytoplasmic surface of GPCRs. Were all components to be manipulated quantitatively in an experimental setting, the global allosteric formalism would predict specific reciprocal effects. For example, increasing concentrations of G-proteins or β -arrestins should produce precise shifts in the agonist dose-response curves to the left.

With respect to the shape of dose-response curves, cooperativity in the binding of agonists may occur, as indicated by a sigmoidal character, although, as noted, the classical Hill coefficient cannot be interpreted in precisely the same manner as applied to ligand-binding equilibria (46). The issue of cooperativity of ligand binding can readily be incorporated into the global allosteric framework by incorporating multiple sites into the basic equations, as needed for a more comprehensive evaluation for oligomeric GPCRs including both dimeric and tetrameric heterooligomers (20,21,48). Where data are available, differences between binding curves and response curves (which follow the conformational state function) can provide additional insights, especially with respect to cooperativity (46). In addition, instances of negative cooperativity may reveal indications of homooligomerization, notably for $\beta 1$ -adrenoceptors (49). The kinetic context of various steps in the signal transduction pathway may also influence the impact of agonist bias depending on the timescale of

the measurements (50,51). Ultimately, the distinctions between red and blue conformations as presented here, without considerations of the time domain, may require specific adjustments for nonidentical kinetic properties of the signaling pathways. More generally, models based on equilibrium considerations cannot capture the full dynamics of the GPCR signaling system, particularly the quasi-irreversible step involving GTP hydrolysis, and future modeling efforts should take these features into account.

Information on the exact stereochemical basis for conformational equilibria and the alterations provoked by various interacting components is emerging from studies combining structural observations with dynamic measurement using powerful optical methods (38,52–54). These approaches bring a new level of precision to the classical concept of isosterism concerning the potential of small differences in pharmacological agents to exert novel effects (55). Recent developments now permit characterization at the level of single molecules (56,57). The equilibrium equations presented here are readily extended to single molecules with a stochastic kinetic model, as developed for nicotinic receptors (58). Experimental observations may therefore be applied to testing the basic assumptions of the general allosteric formalism, particularly the reciprocal effects of G-proteins or β -arrestins on agonist affinities, as well as the minimalist assumption that discrete, relatively rigid active conformations are effectively unchanged by binding of agonist or G-proteins or β -arrestins. There is considerable evidence in the scientific literature for reciprocal effects that involve enhancement of agonist affinity by G-proteins (59–61), although they have not as yet been interpreted with a quantitative functional model along the lines of that presented here. Evidence has also been presented to indicate that β -arrestin binding favors a conformational state with high agonist affinity (37,62).

More broadly, new experimental approaches may also examine the question of whether the transfer molecules, G-proteins or β -arrestins, can be formally represented as allosteric modulators, a role previously assigned to smaller druggable molecules (63). Finally, the rapidly expanding field of DREADDs (Designer Receptors Exclusively Activated by Designer Drugs) is clarifying the consequences of conformational equilibria among discrete states of GPCRs, including the presence of constitutively active states (64). Since current progress is in a rapidly accelerating phase, all of these issues merit revisiting in the near future. The general allosteric framework provides a context for potentially integrating various experimental approaches while at the same time subjecting the assumptions of the model to new experimental tests.

SUPPORTING MATERIAL

Supporting Materials and Methods are available at [http://www.biophysj.org/biophysj/supplemental/S0006-3495\(16\)30656-7](http://www.biophysj.org/biophysj/supplemental/S0006-3495(16)30656-7).

AUTHOR CONTRIBUTIONS

S.J.E. and J.-P.C. designed research; S.J.E. performed research; and S.L.E. and J.-P.C. wrote the manuscript.

ACKNOWLEDGMENTS

We thank the three anonymous reviewers for helpful suggestions and Jean-Philippe Pin for valuable discussions. We dedicate this article to the memory of Howard K. Schachman (December 5, 1918–August 5, 2016).

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Biophysical Journal, Volume 111

Supplemental Information

Biased Allostery

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SUPPORTING MATERIAL

Biased Allostery

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As originally developed the allosteric model described the properties of feedback-inhibited enzymes (1) based on two conformational states, T and R. For receptors the states were renamed B and R, respectively (2)—see below. The original model distinguished between homotropic and heterotropic interactions. For homotropic interactions, cooperative binding of the substrate or a substrate analog was characterized in terms of the fractional occupancy, \bar{Y} :

$$\bar{Y} = \frac{\alpha(1+\alpha)^{N-1} + Lc\alpha(1+c\alpha)^{N-1}}{(1+\alpha)^N + L(1+c\alpha)^N} \quad (1)$$

along with the state function to give the fraction of molecules in the R state, \bar{R} :

$$\bar{R} = \frac{(1+\alpha)^N}{(1+\alpha)^N + L(1+c\alpha)^N} \quad (2)$$

These equations are expressed in terms of the affinity of the substrate or other ligand, X, normalized to the R state, $\alpha = [X]/K_R$. The allosteric constant, L , specifies the relative stability of the T and R states in the absence of ligand, $L = [T]/[R]$. The ratio of the affinities of the two states for the ligand is given by c , where $c = K_R/K_T$. For heterotropic interactions the modulation of L by binding of positive or negative allosteric modulators is defined by L' (3):

$$L' = L \left[\frac{(1+d\beta)(1+e\gamma)}{(1+\beta)(1+\gamma)} \right]^N \quad (3)$$

with heterotropic modulators classified as either inhibitors ($d = K_R/K_T > 1$) or activators ($e = K_R/K_T < 1$) present at normalized concentrations of β or γ , respectively, relative to the corresponding value of K_R .

An important assumption for the above analysis is distinct allosteric sites for the positive and negative modulators. For example, the above equations were applied to the enzyme aspartate transcarbamylase to describe the regulatory effects in the presence of both the allosteric inhibitor CTP and the allosteric activator ATP (4). However, subsequent crystallographic studies revealed that the two families of compounds bind to the same regulator site on the enzyme (5). When two different effector ligands bind competitively, the equations must be reformulated to take into

account competitive binding between different effector ligands to the same site. The competition of two ligands for the same site modifies the basic allosteric equations. For two ligands X and Z that bind to separate sites on an allosteric protein with relative affinities β and γ , all possible species, S_i for binding to a particular conformational state are given by the expansion of the equation:

$$\sum S_i = (1 + \beta)^N (1 + \gamma)^N \quad (4)$$

whereas for competition of the two ligands for the same sites, the various species are given by:

$$\sum S_i = (1 + \beta + \gamma)^N \quad (5)$$

As a result, competitive allostery changes the equations \bar{Y} and \bar{R} . For example, in the case of two competitive ligands X and Z the competitive forms of equations 1-3 are modified to give:

$$\bar{Y} = \frac{(\beta + \gamma)(1 + \beta + \delta)^{N-1} + L(d\beta + e\gamma)(1 + d\beta + e\gamma)^{N-1}}{(1 + \beta + \gamma)^N + L(1 + d\beta + e\gamma)^N} \quad (6)$$

$$\bar{R} = \frac{(1 + \beta + \delta)^N}{(1 + \beta + \gamma)^N + L(1 + d\beta + e\gamma)^N} \quad (7)$$

$$L' = \left[\frac{(1 + d\beta + e\gamma)}{(1 + \beta + \gamma)} \right]^N \quad (8)$$

Following the principles described above for competitive allostery, the mathematical representation of biased allostery for GPCRs can be presented in terms of a global partition function for receptors in basal state B, active states A_{blue} and A_{red} , that interact with transfer molecules G-protein and β -arrestin, and potentially biased agonists. In this case the sum of all states, $\sum S_i$, is given by the equation below with fixed equilibrium constants defined in Table 1 and variable concentrations of agonist, [Ag]; G-protein [Gp]; and β -arrestin [Ar]. Where $\sum S_i = \sum S_A + \sum S_B$, the two latter terms calculated separately for the A and B states are:

$$\sum S_A = \left(1 + \frac{[Ag]}{A_{K_{Ag_red}}}\right) \left\{ \left(1 + \frac{[Gp]}{A_{K_{Gp_red}}} + \frac{[Ar]}{A_{K_{Ar_red}}}\right) \right\} + M_A \left(1 + \frac{[Ag]}{A_{K_{Ag_blue}}}\right) \left\{ \left(1 + \frac{[Gp]}{A_{K_{Gp_blue}}} + \frac{[Ar]}{A_{K_{Ar_blue}}}\right) \right\} \quad (9)$$

$$\sum S_B = L_{red} \left(1 + \frac{[Ag]}{B_{K_{Ag_red}}}\right) \left\{ \left(1 + \frac{[Gp]}{B_{K_{Gp_red}}} + \frac{[Ar]}{B_{K_{Ar_red}}}\right) \right\} + M_B \left(1 + \frac{[Ag]}{B_{K_{Ag_blue}}}\right) \left\{ \left(1 + \frac{[Gp]}{B_{K_{Gp_blue}}} + \frac{[Ar]}{A_{K_{Ar_blue}}}\right) \right\} \quad (10)$$

For the simulations in the present study a single B state was considered, but in principle discrete B_{blue} and B_{red} could be present in parallel with the A_{blue} and A_{red} states, as would be encountered for biased inverse agonism. In order to accommodate this hypothetical situation, equation (10) for $\sum S_B$ allows for B_{blue} and B_{red} , (where $M_B = [B_{\text{blue}}]/[B_{\text{red}}]$). For the simulations presented here the two potential B states were combined by using identical values for the relevant parameters and only the combined constants are presented in Table 1.

For agonist binding to the full system, the equation for the agonist binding function \bar{Y} is obtain by calculating the appropriate numerator (Y_N) and dividing by $\sum S_i$ to yield $\bar{Y} = Y_N/\sum S_i$, where $Y_N = Y_{N_A} + Y_{N_B}$.

$$Y_{N_A} = \left(\frac{[Ag]}{A K_{Ag_red}}\right) \left\{ \left(1 + \frac{[Gp]}{A K_{Gp_red}} + \frac{[Ar]}{A K_{Ar_red}}\right) \right\} + M_A \left(\frac{[Ag]}{A K_{Ag_blue}}\right) \left\{ \left(1 + \frac{[Gp]}{A K_{Gp_blue}} + \frac{[Ar]}{A K_{Ar_blue}}\right) \right\} \quad (11)$$

$$Y_{N_B} = \left(\frac{[Ag]}{B K_{Ag_red}}\right) \left\{ \left(1 + \frac{[Gp]}{B K_{Gp_red}} + \frac{[Ar]}{B K_{Ar_red}}\right) \right\} + M_B \left(\frac{[Ag]}{B K_{Ag_blue}}\right) \left\{ \left(1 + \frac{[Gp]}{B K_{Gp_blue}} + \frac{[Ar]}{B K_{Ar_blue}}\right) \right\} \quad (12)$$

For the A state, the \bar{A} functions are defined separately with respect to A state complexes with either G-proteins or β -arrestins:

$$\bar{A}_{Gp} = \left[\left(1 + \frac{[Ag]}{A K_{Ag_red}}\right) \left\{ \left(\frac{[Gp]}{A K_{Gp_red}}\right) \right\} + M_A \left(1 + \frac{[Ag]}{A K_{Ag_blue}}\right) \left\{ \left(\frac{[Gp]}{A K_{Gp_blue}}\right) \right\} \right] / \sum S_i \quad (13)$$

$$\bar{A}_{Ar} = \left[\left(1 + \frac{[Ag]}{A K_{Ag_red}}\right) \left\{ \left(\frac{[Ar]}{A K_{Ar_red}}\right) \right\} + M_A \left(1 + \frac{[Ag]}{A K_{Ag_blue}}\right) \left\{ \left(\frac{[Ar]}{A K_{Ar_blue}}\right) \right\} \right] / \sum S_i \quad (14)$$

An additional contribution to the total \bar{A} is made by A-state molecules with agonist bound, but neither G-protein nor β -arrestin bound.

$$\bar{A}_{Ag_red} = \left[\left(1 + \frac{[Ag]}{A K_{Ag_red}}\right) + M_A \left(1 + \frac{[Ag]}{A K_{Ag_blue}}\right) \right] / \sum S_i \quad (15)$$

$$\bar{A}_{Ag_blue} = \left[\left(1 + \frac{[Ag]}{A K_{Ag_red}}\right) + M_A \left(1 + \frac{[Ag]}{A K_{Ag_blue}}\right) \right] / \sum S_i \quad (16)$$

Fractions of the molecular population in red and blue states, f_{A_red} and f_{A_blue} , respectively, calculated from the $\sum S_i = \sum S_A + \sum S_B$ in equations (11) and (12) by separating terms to give $\sum S_{Red}$ and $\sum S_{Blue}$ to yield $f_{A_red} = \sum S_{Red}/\sum S_i$ and $f_{A_blue} = \sum S_{Blue}/\sum S_i$. Equations (9) – (16) apply to monomeric receptors, but appropriate versions bases on equations (1) – (8) may be readily derived for oligomeric receptors with integer values of $N > 1$.