Inverse, protean, and ligand-selective agonism: matters of receptor conformation

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ABSTRACT **Concepts regarding the mechanisms by which drugs activate receptors to produce physiological response have progressed beyond considering the receptor as a simple on-off switch. Current evidence suggests that the idea that agonists produce only varying degrees of receptor activation is obsolete and must be reconciled with data to show that agonist efficacy has texture as well as magnitude. Thus, agonists can block system constitutive response (inverse agonists), behave as positive and inverse agonists on the same receptor (protean agonists), and differ in the stimulus pattern they produce in physiological systems (ligand-selective agonists). The molecular mechanism for this seemingly diverse array of activities is the same, namely, the selective microaffinity of ligands for different conformational states of the receptor. This paper reviews evidence for the existence of the various types of agonism and the potential therapeutic utility of different agonist types.—Kenakin, T. Inverse, protean, and ligand-selective agonism: matters of receptor conformation. *FASEB J.* 15, 598–611 (2001)**

Key Words: G-protein-coupled receptors · receptor activation · inverse agonism · receptor theory · constitutive receptor activity

NEW MOLECULAR TARGETS FOR DRUG DISCOVERY

The past decade has brought an explosion of new information about G-protein-coupled receptors (GPCRs), their nature, and how they behave. With the sequencing of the human genome will come a plethora of new GPCR targets. This alliance of information is leading to a revolution in the drug discovery process. Similarly, there is a burgeoning number of chemical targets for therapeutic advantage; the list of drug targets for receptors has grown considerably (see **Fig. 1**). Before 1995, the major targets for drug development were full and partial agonists and antagonists. Since the principal mode of high-throughput screening has been radioligand binding, orthosteric ligands (those that sterically hinder the access of the radiolabel to the receptor binding site) primarily were discovered. Allosteric ligands (those that affect receptor function through binding to their own binding site separate from that of the endogenous ligand) were detected only if the allosteric interaction resulted in an alteration of the affinity of the receptor for the radiolabel. With the technological advances enabling high-throughput *functional* receptor screens should come an increase in the types of GPCR ligands in the new millennium. This will result in an increase in the number of allosteric ligands (modulators, agonists, enhancers) that modify receptor function without necessarily modifying steric access of the endogenous ligands to the receptor. Therefore, the changing mode of high-throughput screening can be predicted to lead to an increase in the texture of drug types for GPCRs. Another reason for the increasing number of drug targets is increased knowledge of GPCR behavior in cellular systems. This has led to the discovery of inverse agonism. This review will concentrate on a subset of these new chemical targets, namely, those that possess efficacy, either positive or negative, and these will be discussed in terms of their mechanisms of action and possible relevance to therapy of disease.

Drugs with 'efficacy'

Drugs can be thought of as having two properties with respect to biological systems: affinity for the receptor and intrinsic efficacy. A common usage of the word efficacy in clinical pharmacology is 'therapeutically useful activity'. Thus, a drug is considered 'efficacious' if it alleviates the symptoms of a disease in a patient. Within this context, even a competitive antagonist would have 'efficacy'. This review will discuss efficacy in terms of its formal definition in pharmacological receptor theory, that is, the property of a molecule that causes it to produce some observable physiological response. In terms of GPCRs, a useful working definition of receptor is the property of a molecule that causes the receptor to change its behavior toward the host system (1).

Three types of efficacious drugs will be discussed. Inverse agonists are an established drug class and possess what is termed 'negative efficacy'. Protean agonists are a theoretical class that produce receptor

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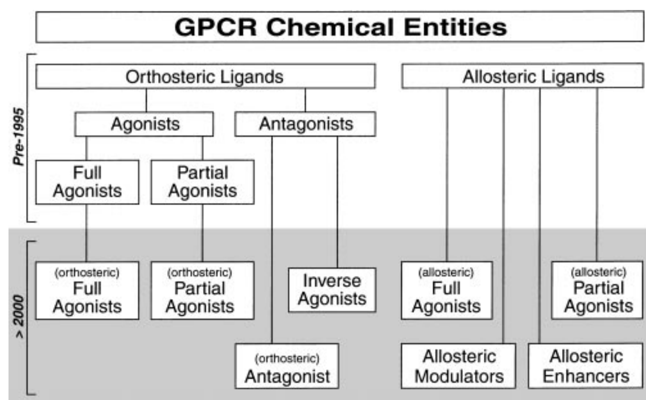


Figure 1. Chemical targets for GPCRs. Prior to 1995, the principal targets were full and partial agonists and antagonists. It is predicted that the types of ligands discovered will increase with increased screening technology. Full agonist: produces full receptor activation leading to production of the system maximal response. Partial agonist: produces submaximal receptor activation leading to production of submaximal system response and possible blockade of full agonist activation. Antagonist: produces no physiological response but rather blocks the response to endogenous or exogenous agonists. Inverse agonist: functions as an antagonist in nonconstitutively active systems, but has the added property of actively reducing receptor-mediated constitutive activity of GPCR systems (response not resulting from agonist activation but rather spontaneously emanating from the system itself). Allosteric agonist: functions as an agonist but activates the receptor through interaction at a site distinct from that of the endogenous agonist (usually a nonpeptide ligand for a peptide receptor). Allosteric modulator (antagonist): blocks receptor function but does not necessarily interfere with ligand receptor interaction (receptor occupancy). Allosteric enhancer: potentiates the effects of agonists on the receptor.

activation of lower magnitude than that emanating from spontaneous receptor constitutive activity. The predicted behavior for this class would be the observation of positive agonism in some GPCR systems and inverse agonism in others. Although this has been observed experimentally, an explanation of the effect in terms of receptor conformation is still theoretical. Finally, 'ligand-specific' agonism, which considers that some agonists have a different quality as well as quantity of efficacy for a given GPCR, will be considered. All of these classes will be discussed in terms of the evidence for their classification and their possible therapeutic relevance. As a preface to discussion of these drug entities, it is useful to discuss the dynamics of the GPCR systems with which they interact.

GPCR systems

G-protein-coupled receptors are allosteric proteins designed by nature to respond to small 'drug-like' molecules (i.e., neurotransmitters) to affect changes in large protein–protein interaction (receptors and G-proteins). The common currency of this translation of information is receptor protein conformation. It is essential to understand three particular properties of GPCR systems in order to understand how ligands can function as inverse, protean, and structure-specific agonists. The first is that, like all proteins, receptors can exist in various conformations. However, in the case of GPCRs, some of these conformations reveal sequences in their cytosolic loops, which can then activate G-proteins to initiate response. These conformations are referred to as the 'active state' (Ra) of the receptor; correspondingly, the conformation(s) that do not activate G-proteins are referred to as the 'inactive state' (Ri). In the simplest case, one single conformation of each will be assumed with the two conformations existing in an equilibrium defined by an 'allosteric constant' (denoted L and defined as [Ra]/[Ri]).

A second property of GPCR systems is that they are synoptic and interactive. Therefore, it is incorrect to describe GPCR function simply in terms of the receptor (two-state theory). Rather, the G-protein is an interactive and essential part of the system. The G-protein influences the receptor in ways that modify the behavior of the receptor and vice versa. Of particular relevance is the fact that a receptor can spontaneously interact with G-proteins in the absence of agonist ligands. Thus, if the affinity of Ri for a G-protein is denoted Kg (equilibrium association constant), the affinity of the active state Ra for the same protein is denoted βKg where $\beta > 1$. Response emanates from the hydrolysis of GTP by the G-protein resulting from activation by Ra. From these elements the simplest version of a GPCR system can be constructed:

$$\mathrm{Ri} \overset{\mathrm{L}}{=} \mathrm{Ra} + \mathrm{G} \overset{\beta\mathrm{Kg}}{=} \mathrm{RaG} \quad (1)$$

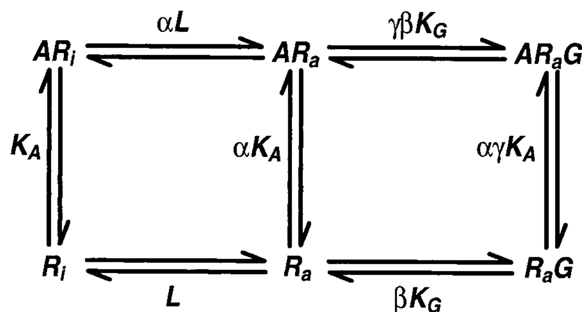
It can be seen that such a system defines the possibility of constitutive activity whereby a response can be produced by the GPCR system in the absence of an agonist. The system can be made to produce a response through stoichiometry of the reactants, namely, Ri and G. Thus, the constitutive activity (as defined by elevated levels of [RaG]) can be increased by raising the receptor concentration:

$$\text{Constitutive Activity} = \frac{\beta L[\mathrm{Ri}]/K_G}{1 + \beta L[\mathrm{Ri}]/K_G} \quad (2)$$

where K_G is the equilibrium dissociation constant of the receptor/G-protein complex (K_G=1/Kg), or by increasing the concentration of G-protein:

$$\text{Constitutive Activity} = \frac{\beta L[\mathrm{G}]/K_G}{1 + L(1 + \beta[\mathrm{G}]/K_G)} \quad (3)$$

Another way in which constitutive activity can be produced is through alteration of L, the allosteric constant. Under normal circumstances, L is a unique molecular constant for a given receptor (i.e., the energy barrier to formation of spontaneous active states for some receptors is lower than it is for others), but experimental methods such as the removal of sodium ions (2, 3) or point mutation (4–10) can affect L and make receptors more constitutively active.



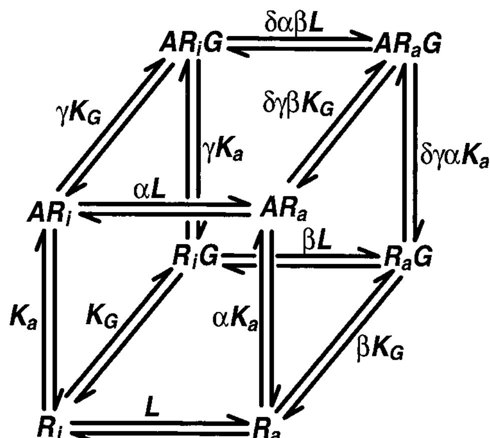


Figure 2. Two models for GPCR systems. The extended ternary complex (ETC) model (5) assumes that only the active-state receptor (Ra) can interact with the G-protein either spontaneously (to form RaG) or through ligand binding (to form ARaG). The association constants are K (ligand to receptor) and βK_g (receptor to G-protein). L is the allosteric constant and α, γ the modifiers of affinity once the receptor is active or ligand bound, respectively. The cubic ternary complex is very similar except it allows the inactive-state receptor Ri to interact with the G-protein as well (13–15).

The simple model for GPCR systems can be completed by adding the interaction of ligand (designated [A]) to the system to produce a corresponding array of species ARi, ARa, and ARaG. When the ligand-bound ensemble is added to the scheme shown in Equation 1 (see also **Fig. 2*A***), the extended ternary complex model (ETC model) for GPCR systems results (5). A more thermodynamically complete version of the system allows the inactive receptor Ri to interact with the G-protein. In terms of thermodynamic modeling, this must be allowed to occur (11). However, the existence of an inactive ternary complex comprising ARiG is largely theoretical. Some examples of this complex can be found for some receptors (see ref 12 for a review); however, the thermodynamically complete model for GPCR systems, termed the cubic ternary complex model (CTC model; 13–15), requires a greater number of microaffinity constants than the ETC model and generally is more complex (see Fig. 2*B*). The ETC model can be regarded as a subset of the CTC model and adequate for GPCR systems for which the interaction of Ri with G-protein is thought to be minimal. For the purposes of this review, both models yield similar predictions for GPCR behavior with some minor exceptions.

The third relevant property of GPCR systems is an extension of the first: the production of multiple *active* receptor states (that go on to produce response through interaction with G-proteins). The minimal requirement for a GPCR model is that one receptor active state be formed. Thus, in principle, agonists can induce response by causing enrichment of that single receptor active state. Under these circumstances, efficacy would then be a matter of the quantity of the active state produced by the agonist. However, there is no theoretical constraint on the number of receptor active states. Even though the ETC and CTC model have both been referred to as ‘two-state’ models, this is a misnomer in that there is the capability within both to be multi-state models. The two-state aspect of these models refers only to the unliganded species Ri and Ra. In principle, the microaffinity constant of the liganded receptor could be specific for the ligand (through the values α and γ for the ETC model and α, γ, and δ for the CTC model; see Fig. 2), i.e., the affinity of the ligand-bound receptor for G-protein (ARaG) could be different from the unbound form (RaG). Under these circumstances, both the ETC and CTC models can accommodate an infinite number of receptor active states for agonism.

It is clear that proteins, including GPCRs, can adopt numerous conformations according to thermal energy (16, 17). What is not clear is what proportion of these conformations are capable of activating G-proteins, i.e., how many are receptor active states? Amino acid sequences have been identified in the intracellular loops of GPCRs that, when exposed to G-proteins, activate them (18–20). In fact, small oligopeptide isolated sequences have been found to activate G-proteins on their own (21, 22). With this model in mind, it would suggest that the inactive form of the receptor prevents access of G-proteins to these sequences, thereby precluding receptor activation of G-proteins. The corollary to this is that any disruption of the tertiary structure of the receptor could expose these activating amino acid sequences to initiate G-protein activation. On theoretical grounds, it might be expected that there could be numerous tertiary conformations of the receptor capable of exposing these intracellular sequences, i.e., there could be numerous active state conformations of the receptor. Mutation studies support this idea. For example, the substitution of 20 amino acids in position 293 of the α_{1A}-adrenoreceptor produces a constitutively active receptor—essentially 20 different active state similar forms of the α_{1A}-adrenoreceptor (4). The production of constitutive activity (whereby the receptor spontaneously adopts an active state and produces

G-protein activation) through such mutations for receptors indicates that disruption of receptor tertiary conformation can expose activating sequences to G-proteins (10, 23, 24). A general message from these studies is the possibility of the existence of numerous active state conformations of GPCR able to initiate physiological response. The apparent ligand-specific production of receptor conformations that interact differently toward other membrane proteins (including G-proteins), to be discussed later in the context of ligand-specific agonist efficacy, further suggest the existence of multiple receptor active states for GPCRs.

The previous discussion has described essentially three characteristic behaviors of GPCR systems: the capability to exist in multiple states, the ability of these states to spontaneously interact with other membrane proteins, and the possible existence of multiple states capable of inducing physiological response. To explore the interaction of ligands with such systems, it is useful first to discuss the mechanism by which ligands can influence receptor/G-protein ensembles.

The influence of ligands on GPCR systems

The relative quantities of various protein species existing in equilibria with each other are governed by the equilibrium dissociation constants that define their ratio. Thus, the allosteric constant is defined as [Ra]/[Ri]. The nature of L is controlled by the molecular nature of the receptor; thus, for any quantity of Ri there will be a quantity of Ra governed by the magnitude of L. However, this can be changed if external forces perturb the quantity of either one of the species. For example, if a ligand binds selectively to the Ra species to form ARa, then the quantity of free Ra is depleted and the magnitude of L will dictate that more Ra must be formed at the expense of existing Ri (see Scheme 1).

This can be shown mathematically within the constraints of either the ETC or CTC model (Fig. 2). For example, the concentration of response producing species (RaG and ARaG) in the presence of a ligand A in terms of the ETC model is given by Kenakin et al. (12):

$$\rho = \frac{\beta L[G]/K_G(1 + \alpha\gamma[A]/K_A)}{[A]/K_A(1 + \alpha L(1 + \gamma\beta[G]/K_G)) + 1 + L(1 + \beta[G]/K_G)} \quad (4)$$

In the absence of agonist ([A]=0)

$$\rho_0 = \frac{\beta L[G]/K_G}{1 + L(1 + \beta[G]/K_G)} \quad (5)$$

In the presence and absence of a maximal concentration of ligand (saturating the receptors; $[A] \rightarrow \infty$)

$$\rho_\infty = \frac{\alpha\gamma\beta L[G]/K_G}{1 + \alpha L(1 + \gamma\beta[G]/K_G)} \quad (6)$$

The ratio of response producing species in the presence and absence of ligand is given by:

$$\frac{\rho_\infty}{\rho_0} = \frac{\alpha\gamma(1 + L(1 + \beta[G]/K_G))}{(1 + \alpha L(1 + \gamma\beta[G]/K_G))} \quad (7)$$

As depicted in Fig. 2, α and γ reflect modifiers of the affinity constant of the receptor for the G-protein when the receptor is activated and occupied by ligand, respectively. For example, a value of $\alpha > 1$ indicates a greater affinity of the ligand for the active-state receptor Ra. It can be seen from Equation 7 that only one condition will enable a ligand to bind to the GPCR species in the system and *not* cause a redistribution of receptor species. That is if $\alpha = \gamma = 1$ (the presence of the ligand on the receptor does not in any way affect the affinity of the receptor for G-proteins, i.e., the ligand has no efficacy). If α or $\gamma \neq 1$, then the ratio of active-state species will change in the presence of A: when A is added to the system, the concentrations of the various species will redistribute. Therefore, the selective affinity of ligands for various receptor conformations will change the overall distribution of species in GPCR receptor ensembles and thus, either induce or inhibit response. This is the basic mechanism of ligand efficacy and the basis for the molecular nature of inverse, protean, and ligand-selective agonism.

INVERSE AGONISTS

Inverse agonists were discovered only after the tools with which they could be detected were created, namely, constitutively active GPCR systems. Whereas ligands that depress the basal benzodiazepine receptor (a non-GPCR) activity had been studied a decade before (25, 26), true GPCR constitutive activity was first quantified in recombinant receptor systems where experimental conditions could be manipulated to produce constitutive activity. As noted, the ability to spontaneously produce an active receptor conformation (the conformation that activates G-proteins) is defined by the allosteric constant, a receptor-specific constant defining the energy barriers to the production of tertiary conformations. Thus, in natural systems with defined receptor/G-protein stoichiometry, the amount of spontaneously formed active-state receptor species may not be sufficient to demonstrate visible constitutive receptor activity. This constraint was eliminated by the introduction of recombinant GPCR systems, which could experimentally manipulate the relative stoichiometry of receptors and G-proteins.

A classic study by Costa and Herz (2) of NG108–15 cells recombinantly expressing opioid receptors was instrumental in defining constitutive GPCR activity and inverse agonism. Costa and Herz (2) produced a system that responded to the classic opioid agonist (i.e., [D-Ala^2), D-Leu]enkephalin), but also had an elevated basal response and demonstrated a depression of basal activity with the peptide ICI 174864 ([N,N′-diallyl-Tyr^1,Aib^{2,3}]Leu^5-enkephalin). In this constitutively active GPCR system, ICI 174864 depressed the ligand-independent elevated basal responses and was thus

defined as an inverse agonist. The simplest mechanism by which inverse agonism could occur is the selective affinity of the ligand for the inactive state of the receptor. Thus, as the ligand binds selectively to Ri, the receptor species in the system will redistribute. If the system has RaG present (constitutive activity), then this species will be depleted as more receptor transforms into ligand-bound Ri; the result will be a decrease in constitutive activity.

Inverse agonism is a fairly newly discovered phenomenon for GPCR systems. The effect was initially met with some skepticism since it required the reclassification of established antagonists as inverse agonists. Also, in some systems the trace presence of endogenous agonists leads to an apparent constitutive activity, which could then be depressed by simple competitive antagonists, i.e., inverse agonism could be an artifact in some systems. However, the lack of depression of basal responses to some antagonists (i.e., neutral antagonists) and the use of such neutral antagonists to block the effects of inverse agonists clearly indicate that the phenomenon is real. For example, Costa and Herz (2) used the neutral antagonist MR 2266 to block the effects of the positive agonist DADLE and the inverse agonist ICI 174864, and showed that the potency for the inhibition of both effects was the same.

After the initial discovery, there was a period when there was a paucity of data available to judge the prevalence of inverse agonists in pharmacology. However, with time has come an increasing number of reports describing previously classified antagonists as inverse agonists. This rise coincided with the increased availability of recombinant and constitutively active GPCR systems, a prerequisite for the observation of inverse agonism. Thus, now that more laboratories have eyes to see inverse agonism, the more it has been seen.

It is still premature to judge the prevalence of inverse agonism in chemical space. In theoretical terms, there is reason to believe that all ligands should not possess efficacy. As described above, for a ligand *not* to cause redistribution of GPCR species it must recognize at least two receptor conformational species as being identical: Ra and Ri. In a constitutively active system, this is increased to three species by the presence of RaG. As shown in Equation 7, the ligand-specific constants α and γ must be unity in terms of the ETC model (and α, γ, δ in the CTC model) for redistribution not to occur (i.e., for a ligand to have no efficacy). The question then is: How often, in thermodynamic terms, is this likely to occur? Although some studies appear to support the prediction that most antagonists are inverse agonists (i.e., of 23 α_1-adrenoreceptor antagonists of varying structure, all were inverse agonists) (27), there are clear examples of neutral antagonists in the literature. The degree of inverse agonism observed depends on the relative affinity of the inverse agonist for the various receptor species and the degree of constitutive activity in the system. Thus, ligands that only slightly differentiate receptor conformations will essentially appear to be neutral antagonists, especially in systems with low levels of constitutive activity.

Although the existence of inverse agonists has been substantiated in experimental systems, the therapeutic relevance of this drug class is as yet unknown. It also is not clear whether negative efficacy would be a desirable or undesirable property to have in an antagonist molecule. In the absence of constitutive receptor activity, an inverse agonist behaves exactly as a simple competitive antagonist. However, if there is constitutive activity present in the therapeutic system, then, unlike a simple competitive antagonist, an inverse agonist will depress the resulting elevated basal response. There are physiological scenarios where this may or may not be advantageous.

Adverse effects of inverse agonists

Inverse agonism has been associated with receptor up-regulation leading to tolerance to chronic antagonism. For example, in treating an ulcer, tolerance to some histamine H2 receptor antagonists has been observed (28–30). It has been postulated that chronic treatment with histamine antagonists results in increased levels of membrane histamine receptors (31). The ligands shown to cause increases in histamine H2 receptor density—cimetidine and ranitidine—are inverse agonists but there is no concomitant increase in receptor density observed with the neutral antagonist burimamide (32). In that membrane receptor populations are not static, but rather are a series of steady states resulting from receptor synthesis, transport to the surface, internalization, and degradation, any ligand that perturbs receptor states theoretically can affect the steady-state level of the receptor density. For example, activation by agonists increases phosphorylation of many receptors and subsequent internalization (33–35). It has been shown that spontaneous formation of receptor active states (constitutive activity) leads to eventual internalization of receptor as well (33, 36). Possessing equal affinity for both the inactive and active receptor states, a neutral agonist would not alter flow of receptor to and from the membrane surface. However, an inverse agonist could halt the spontaneous cycle of receptor synthesis, transport, internalization, and degradation at the membrane by selectively stabilizing the inactive state of the receptor. If this state is more resistant to phosphorylation and subsequent internalization, then receptor degradation would be slowed in the face of unaltered receptor synthesis. The extent of change of steady-state membrane receptors would be a function of the rates of the various processes synthesizing, transporting, and internalizing them (37), but under appropriate conditions elevations of receptor could occur leading to increased agonist response. This, in turn, would result in a decrease in the effectiveness of the antagonist. Thus, in this scenario, inverse agonism would be an undesirable property (38). Receptor up-regulation by inverse agonists has been shown to occur with inverse agonists for histamine H2

receptors (32), β_2-adrenoreceptors (39), and α_1-adrenoreceptors (40). In addition to changes in receptor density, inverse agonists have also been found to alter levels of G-protein. Thus, up-regulation of levels of $G_{q/11\alpha}$ through 5-HT_{2C} receptors (41) and $G_{s\alpha}$ through β_2-adrenoreceptors (42) has been obtained with inverse agonists for the respective receptors. Presumably the changes in receptor stimulation of these pathways leads to secondary effects on G-proteins.

Therapeutic application of inverse agonists

The extent to which inverse agonism could be a therapeutic advantage depends on the role of constitutive GPCR activity in pathology. One potential therapeutic area where this might have relevance is cancer. It has been shown that chronic elevation of second messengers in cells produced by constitutive G-protein activity can lead to cell transformation (43–45). For example, receptors such as the α_1-adrenoreceptor have been shown to be agonist-independent proto-oncogenes (46). Constitutive GPCR activity leading to chronic elevation of cell metabolism may also have a role in promoting the growth of tumors. There are examples of high levels of expression of specific GPCRs in tumor cells; it has been shown that endogenous ligands for these receptors are present at high levels in the tumor cells (self-regulation) and that they have proliferative properties. There also is evidence to show that inhibition of the cellular effects of these ligands can inhibit tumor growth.

One such receptor is vasoactive intestinal peptide receptor (VIP). Receptors for VIP are found in high density in a number of tumors (47–55); see **Table 1**. In fact, these high levels of VIP receptors can be used to image tumors through binding of ^{123}I-VIP (55) and ^{123}I-labeled octreotide (VIP ligand; 57, 58) binding.

The relevance of high levels of VIP GPCR activity on tumors relates to the fact that this peptide promotes growth and proliferation of normal and malignant cells (59–63). Inhibition of VIP function in these cells leads to a decrease in cancer growth (64, 65); see **Fig. 3**. The relevant question for inverse agonism is, to what extent can the VIP-mediated proliferation be attributed to constitutive VIP GPCR activity? Many of these tumors have high levels of VIP, and it has been suggested that VIP secretion from these tumors regulates VIP receptor expression on the same cells (66). Certainly the high levels of VIP receptor present on the tumor cell membrane would make them extremely sensitive to low levels of released VIP. However, the sheer magnitude of the receptor expression suggests that constitutive receptor activity may also play a role in the pathology.

TABLE 1. *VIP receptors in tumors and normal cells*

Cells	B_{max} (sites/cell)[a]	Multiple of platelet cell density
Platelets	$2.1 \pm 0.3 \times 10^3$	1
A431[b]	$1.6 \pm 0.3 \times 10^6$ (high-affinity sites)	800×
	$9.7 \pm 0.4 \times 10^6$ (low-affinity sites)	4620×
COLO 320[c]	$1.9 \pm 0.4 \times 10^8$ (high-affinity sites)	90,500×
	$7.3 \pm 0.8 \times 10^6$ (low-affinity sites)	347,600×
HT29[c]	$1.2 \pm 0.5 \times 10^8$ (high-affinity sites)	5700×
	$6.9 \pm 0.9 \times 10^6$ (low-affinity sites)	32,850×
PANC1[d]	$2.1 \pm 0.4 \times 10^8$ (high-affinity sites)	100,000×
	$6.9 \pm 0.8 \times 10^6$ (low-affinity sites)	32,850×

[a] Binding of ^{125}I-labeled VIP. [b] Epidermoid mammary carcinoma. [c] Adrenocarcinoma. [d] Pancreatic epitheloid carcinoma. From ref 56.

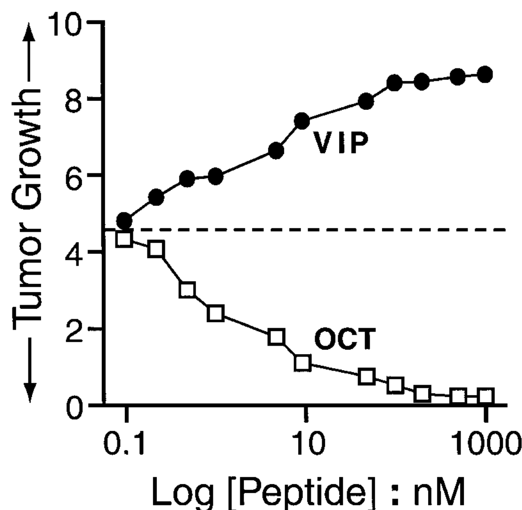


Figure 3. Effects of VIP and peptide fragment octreotide on tumor growth as measured by ^{3}H-thymidine-incorporation (cpm × 10^3). Abscissae are logarithms of molar concentration of vasoactive intestinal peptide (VIP) or octreotide. Redrawn from ref 56 with permission.

There are differences in the proclivity with which different GPCRs spontaneously produce an active-state receptor (with corresponding constitutive activity). Some receptors have a low-energy barrier for the formation of Ra (i.e., human calcitonin, chemokine CCR5, neuropeptide Y types 2 and 4) whereas others, such as NPY1, do not readily produce constitutive activity (67); the difference lies in the magnitude of the allosteric constant, L. However, since the definition of L is the ratio of Ra to Ri ($L=[Ra]/[Ri]$), then irrespective of the magnitude of L, a 1000- to 10,000-fold increase in the number of receptors will lead to a corresponding 1000- to 10,000-fold increase in the number of spontaneously existing active-state receptors. Thus, the magnitude of L for VIP would need to be exceedingly small to prevent such high levels of receptor from producing constitutive activity.

Another peptide of interest in cancer is bombesin. Bombesin, gastrin-releasing peptide, and VIP are related in that VIP may induce the release of bombesin/GRP in small cell lung cancer (65, 68). Bombesin-like peptides are potent mitogens, and a role has been proposed for them in oncogenesis and/or proliferation of malignant cells (69). Bombesin/gastrin-releasing peptides are found in high levels in small cell lung

carcinomas, suggesting that these could be autocrine factors for cancer growth (70–72). As with VIP, blockade of bombesin activity through monoclonal antibodies attenuates cancer growth (69).

Inverse agonists would both block the effects of humoral activation of these receptors on cancer cells (i.e., secreted VIP, bombesin) and constitutive activity in the tumor due to either receptor over-expression and/or mutation. Whereas the effect would be cytostatic rather than a cytocidal (tumor death would not be achieved), a reduction in tumor cell metabolic activity could be a useful adjunct to chemotherapy.

Certain disease states may be treated effectively *only* with inverse agonists. These are instances where the pathological entity is a constitutively active GPCR, which produces physiological response in the absence of endogenous agonists. For example, certain pathological mutations lead to constitutively active GPCRs, which in turn result in diseases such as retinitis pigmentosa and hyperthyroidism (see review by Spiegel, ref 73). Constitutively active GPCRs may also be important in autoimmune diseases (see review by de Ligt et al., ref 74). Viral infection also can lead to constitutively active GPCR pathology. For example, infection with Kaposi's sarcoma-associated herpes virus leads to expression of a constitutive chemokine receptor, which in turn elevates IP3 to lead to cell proliferation and continued viral replication (75, 76).

In general, it still is not clear to what extent GPCR constitutive activity plays a role in pathology. However, it is known that receptors and enzymes levels change in conditions of trauma (hypoxia, ischemia, physical damage), disease (inflammation, viral or bacterial infection), or development (78, 79). Although, in general, solid examples of constitutive receptor activity playing a role in disease are sparse, with the classification of clinically used inverse agonists, the relationship between negative efficacy and therapeutic utility should become clearer. Along with clarification of the role of constitutively active GPCRs in pathophysiology will come a measure of the value of inverse agonists in therapy.

PROTEAN AGONISTS

A unique reversal of drug activity, based on the notion that some agonists may produce an active receptor conformation of lower efficacy than the spontaneously formed one has been predicted on theoretical grounds (80, 81). These kinds of ligands were given the name protean agonists after Proteus, the Greek god who could change shape and appearance at will. In this case, the reversal from positive to negative agonism is protean. If a given agonist produces a receptor active state that is less efficacious (to be denoted [Ra′]) than the spontaneously formed one (denoted [Ra]), then in systems that are quiescent (no constitutive activity), the ligand would produce excitation by virtue of changing the predominant Ri into Ra′. However, if the system were constitutively active (significant amount of Ra), then the ligand would reduce the activity by changing Ra to Ra′. Therefore, in quiescent systems the ligand would be a positive agonist and in constitutively active systems it would be an inverse agonist. Presently it is not clear what therapeutic relevance such an agonist would have except perhaps to set the level of stimulation of a given system to a constant level. Thus, if pathology produced constitutive activity to create an overstimulation of the system or if the endogenous stimulus to the system were to be diminished by pathology, then a protean agonist would be useful if the maximal effect of the agonist was appropriate.

On the other hand, there is a considerable theoretical interest in protean ligands since they can act as a looking glass into agonist-specific receptor active states. Thus, the observation of protean agonism would be presumptive evidence that the ligand in question produces a receptor active state of lower intrinsic efficacy than the naturally occurring constitutively active state. It is worth considering the experimental conditions under which such protean agonism would be observed.

The starting point is to have a ligand that produces a positive agonist response in a quiescent (nonconstitutively active) receptor system. It might be supposed that the agonism should be partial (in keeping with a less efficacious ligand-bound active state). However, saturation of system stimulus-response mechanisms might allow low efficacy agonists to produce the full system response; therefore, partial agonism may not be a prerequisite. The next step is to observe the effect of ligand in a system where the receptor is made to spontaneously form the natural active state. For example, **Fig. 4*A*** shows the effect of increasing the magnitude of the allosteric constant L (as might be produced by removal of sodium ions) in a hypothetical GPCR system. The ligand is a theoretical drug that promotes the formation of the natural active state ($\alpha=100$) but forms a ligand bound species that has a lower affinity for the G-protein than the natural active state (ARa has a lower affinity for G than does Ra; $\gamma=0.01$). Calculations with the CTC model show that in the quiescent system ($L=0.01$), the ligand is a positive agonist. Changing L from 0.01 to 0.3 elevates the basal response of the system and causes the ligand to demonstrate inverse agonism. Another way to produce constitutive activity is by increasing the amount of G-protein available to interact with the receptor (Equation 3). Under these circumstances, a similar ligand ($\gamma=0.03$) will demonstrate protean agonism as well (Fig. 4*B*). Another condition that may yield protean agonism is when the receptor reactivity to the G-protein changes. For example, Fig. 4*C* shows that if the affinity of both Ri and Ra is reduced for the G-protein (K_G increases), as might be produced by desensitization, an inversion of agonism for the same ligand would be observed. Note how in this case the basal activity is not altered.

There have been experimentally observed instances of protean agonism for β_2-adrenoreceptor ligands. For example, dichloroisoproterenol (DCI) is a positive par-

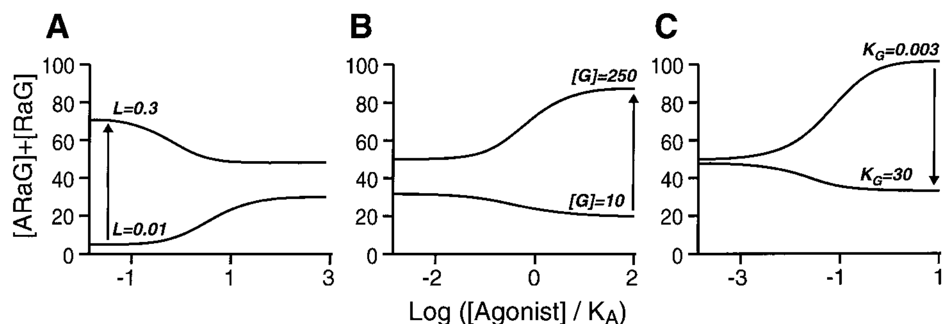


Figure 4. Three theoretical conditions that promote protean agonism. Simulation made with cubic ternary complex model (Fig. 2*B*) for a ligand with α = 100, γ = 0.01, δ = 0.1. [R] = 100. *A)* [G] = 100, K_G = 30, β = 10. The system ranges from quiescent (L=0.01) to constitutively active (L=0.3). *B)* Ligand with α = 100, γ = 0.03; L = 0.1; [G] increased from a value of 10 to 250. *C)* Ligand with α = 100, γ = 0.03, L = 0.1; receptor 'desensitized'; [G] = 500, interaction of receptor and G-protein efficient (K_G=0.003) to inefficient (K_G=30).

tial agonist for β_2-adrenoreceptors transfected into sf9 cells. Upon desensitization of the system through prolonged treatment with the full agonist isoproterenol (as depicted in the simulation Fig. 4*C*), DCI produces inverse agonism (82). **Figure 5** shows the effects of three β_2-adrenoreceptor ligands on transfected sf9 whole cells; DCI, labetalol, and pindolol all produce increases in cyclic AMP (positive agonism). However, when membranes were made from the same cells, the system became constitutively active (due to removal of GTP) and, under these circumstances, these same ligands produced depression of basal cyclic AMP levels (inverse agonism) (83). It is not clear to what extent low efficacy receptor conformations are responsible for the experimentally observed protean agonism. However, observation of the phenomenon is suggestive of selective receptor states and this may be a useful tool for discovery of ligand-specific receptor active-states.

LIGAND-SPECIFIC RECEPTOR ACTIVE STATES

Numerous lines of experimental evidence indicate that all agonists do not produce the same active state of

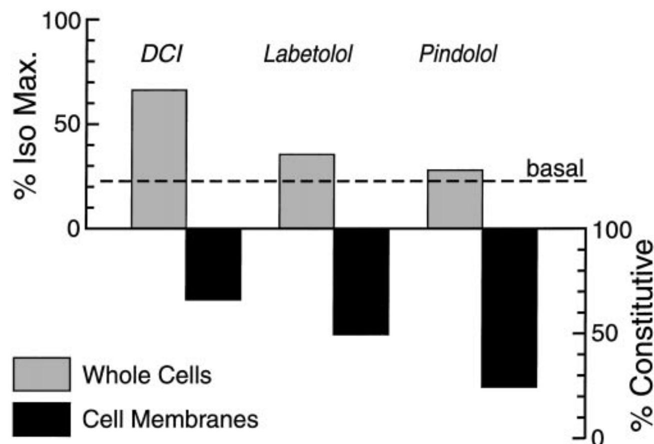


Figure 5. Experimentally observed protean agonism. Sf9 cells transfected with β_2-adrenoreceptor. Gray bars represent whole cells (not constitutively active due to presence of intracellular GTP); ligands produce stimulation of cyclic AMP. Membranes from same cells are constitutively active; the same ligands produce inverse agonism (filled bars). Data from ref 83 with permission; figure from ref 1 with permission.

GPCRs. One of the most compelling findings is the reversal of relative potency of agonists for receptors that activate more than one stimulus-response element. For example, the human $5\text{-}HT_{2C}$ receptor is coupled to two separate response pathways in CHO cells: phospholipase C-mediated inositol phosphate accumulation (IP accumulation) and phospholipase A2-mediated arachidonic acid release (AA release). The agonist (±)-1-(2,5-dimethoxy-4-iodophenyl)-2-aminopropane (DOI) produces a higher maximal stimulation than the 5-HT agonist quipazine for arachidonic acid release (77). Since maximal response is dependent only on efficacy, this indicates that DOI has a greater efficacy than quipazine for AA release. In contrast, the efficacies of the two agonists are reversed for the IP accumulation where quipazine has the greater efficacy. This cannot be explained by a uniform active-state receptor interacting with the two pathways identically for the two agonists, but rather it suggests that the active state formed by DOI (arachidonic release-preferring) is different from that produced by quipazine (IP accumulation-preferring). Similar reversals of efficacy have been reported for PACAP (pituitary adenylate cyclase-activating polypeptide) receptors (84), dopamine D_2 receptors (85) and *Drosophila* tyramine receptors (86). A striking reversal of relative potency of substance P analogs on neurokinin-1 receptors has been reported (87). Thus, whereas substance P is 2.1× more potent than the analog $[P_3{}^{E}met(O_2)$ (11)SP for producing cyclic AMP through NK-1 receptor activation, it is 0.11× *less* potent than the analog for producing phosphoinositol hydrolysis through activation of the same receptor. Whereas no reversals in relative efficacy for agonists was found in a study of CB1 cannabinoid receptors (known to activate both Gs and Gi protein), marked discontinuities in the activity of agonists were observed indicating that some agonists produced conformations that favored one of the two G-proteins while others did not (88).

Differential activation of G-proteins by receptors (referred to as stimulus trafficking; refs 89–91) cannot be accommodated by a mechanism whereby one single receptor active state produced by all agonists interacts with G-proteins. Although differential stimulus pathway activation can occur through strength of signal type of mechanism (i.e., a highly efficacious agonist may activate two pathways whereas a weaker agonist may acti-

vate only the more sensitive one), reversal of relative activity cannot be explained in this manner. Rather, the two G-proteins involved must see different conformations. It would be expected that different conformations of the receptor would have differential activation reactivities to different G-proteins since it is known that different areas of the cytosolic loops on receptors activate different G-proteins (92, 93). It would not be expected that different tertiary conformations of the receptor would expose these different G-protein-activating sequences in an identical manner.

Stimulus trafficking can be detected in specially designed recombinant GPCR systems. Referred to as stimulus-biased assay systems (94), these are hosts with identical cellular backgrounds except for the enrichment of a single Gα subunit. For example, human calcitonin receptors are pleiotropic with respect to the G-proteins with which they can interact (Gs, Gq, Gi; ref 95). Transfection of human calcitonin receptors (type 2, denoted hCTR2) into wild-type HEK 293 cells and HEK cells stably transfected with enriched populations of Gα subunits show striking differences in *relative* agonist potencies. **Figure 6*A*** shows that not only does the relative potency of eight calcitonin agonists on hCTR2, transfected in wild-type cells, and HEK cells stably enriched with Gαs subunit change, but so does their rank order of potency. Figure 6*B, C* shows dose-response curves to rat amylin and porcine calcitonin in wild-type cells and Gαs-enriched cells, respectively. It can be seen that the relative potency of the agonists changes from 4.6 to 84 with Gαs-enrichment, a finding that cannot be accommodated by the assumption that both agonists produce the same receptor active state (94). Rather ,it suggests that porcine calcitonin produces a conformation more conducive to using Gs than does amylin.

Other experimental approaches have furnished data to indicate differential G-protein activation by different agonists produced by agonist-specific receptor conformations. For example, the kinetics of adenylate cyclase activity in the presence of limiting GTP concentrations indicates a differential rate of heterotrimer dissociation for different β_2-adrenoreceptor agonists (96). Similarly, whereas the efficacy of β_2-adrenoreceptor agonists for promoting GTP hydrolysis correlates well for the efficacy of the agonists for stimulating adenylate cyclase, the same is not true for the hydrolysis of inosine triphosphate. The differences in the ability of different agonists to hydrolyze GTP vs. ITP suggest that different receptor active states are produced (97).

There are still other lines of evidence to suggest that agonists produce ligand-specific receptor conformations. Selective mutations of dopamine D_2 receptors caused selective abolition of receptor/G-protein activation by dopamine but not other dopamine agonists. This suggests that these agonists produce different receptor conformations interacting with G-protein (98). Studies of the receptor desensitizing effects of different agonists also indicate the production of ligand-specific receptor conformations. For example, it

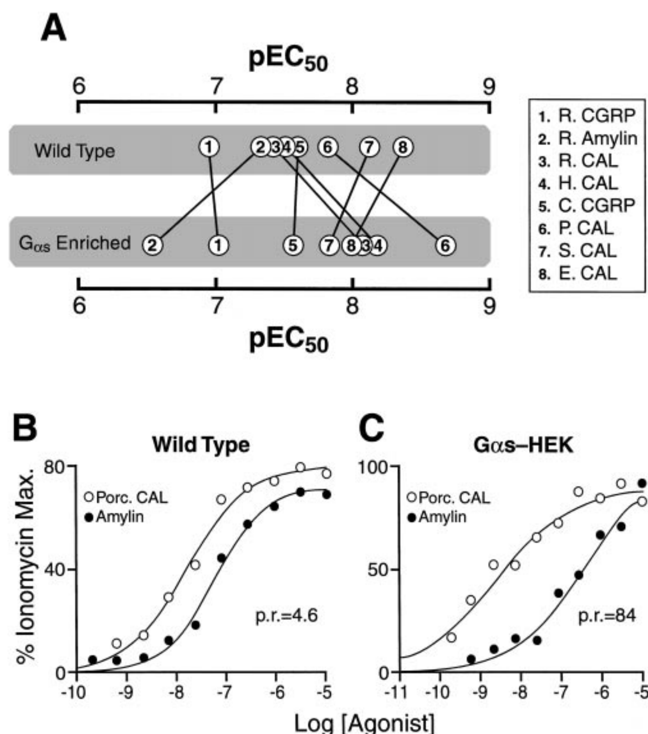


Figure 6. Relative potency of calcitonin receptor agonists in wild-type and stimulus-biased GPCR systems. *A)* pEC_{50} (−log of the molar concentration producing half maximal stimulation) for calcium mobilization (n=3) for 8 agonists (code shown in box: CAL=calcitonin, R=rat, H=human, E=eel, P=porcine, C=chicken, S=salmon) on calcitonin receptors (hCTR2) transfected into wild-type HEK293 cells (top bar) and HEK293 cells stably enriched with Gαs subunit (bottom bar). Change in pEC_{50} shown for each agonist by lines joining the points. *B)* Dose-response curves (calcium mobilization as measured by fluorescence) to porcine calcitonin (open circles) and rat amylin (filled circles) in wild-type HEK293 cells. The relative potency of calcitonin to amylin is 4.6. *C)* Relative potency of the same agonists as shown in panel *B* in Gαs-enriched stimulus-biased host cells. The relative potency is now 84. Data for panel *A* previously unpublished by Chris Watson, GlaxoWellcome Research, panels *B* and *C* from ref 1 with permission.

would be expected that the relative propensity of agonists to induce desensitization would parallel their relative efficacies. This was shown to be generally true for μ opioid receptor agonists, with the notable exception of methadone and L-α-acetyl methadone. These latter agonists produced disproportionate desensitization and receptor phosphorylation, suggesting different receptor conformational changes (99). Similarly, methadone and buprenorphine have been shown to demonstrate different desensitizing properties from morphine on μ opioid receptors (100). In other studies of recovery from desensitization, it has been shown that agonists appear to produce different conformations. Thus, whereas the recovery from prolonged activation of 5-HT_3 receptor with partial agonists is mono-exponential, it is sigmoidal (indicating 3 steps and 4 states) with full agonists (101).

The effects of agonists on receptor internalization also have furnished interesting data regarding ligand-

specific receptor conformation. Here it can clearly be shown that the simple strength of receptor stimulation can be differentiated from the ability of ligands to induce receptor internalization. For example, the cholecystokinin (CCK) receptor antagonist D-Tyr-Gly-[(Nle^{28,31},D-Trp^{30})cholecystokinin-26–32]-phenethyl ester does not produce receptor stimulation but rather blocks CCK responses. This antagonist also produces profound receptor internalization (102). Similarly, whereas enkephalins and morphine produce stimulation of δ and μ opioid receptors, enkephalins induce rapid receptor internalization whereas morphine does not (103). These data indicate that the conformations that lead to response are not necessarily the same as those that induce receptor internalization. It also suggests that different agonists produce receptor conformations with differential propensity to internalize.

In conclusion, diverse experimental approaches have provided evidence that ligands can stabilize different receptor conformations. Some of these conformations relate to receptor signaling, whereas others may relate to receptor sensitivity to endogenous agonist or presence on the cell membrane. The challenge is to exploit this behavior for therapeutic advantage.

Ligand-selective conformations and therapeutic utility: the quality of efficacy

Historically, receptors have been thought of operationally in terms of 'on-off' switches. In this context, efficacy was considered to be the 'on' position and the only gradation available in this scheme was degree of strength. With the possibility of agonist-selective activation of receptors and the definition of efficacy as a change in the behavior of receptors to their hosts comes the capacity to control the 'quality' of efficacy as well.

In terms of signaling, a common quest in drug discovery is to obtain ligands with a subset of activity for a given endogenous ligand receptor system. Historically, the method for doing this was through discovery of receptor subtypes. Thus, whereas epinephrine has a plethora of metabolic activities in the body mediated by β-adrenoreceptors, selective agonist stimulation of only the β_2-adrenoreceptor subtype provides useful therapy for asthma. Stimulation of the receptor subtype reduces the spectrum of metabolic responses produced by the general receptor family. If it is accepted that different receptor conformations most likely reveal different portions of the intracellular cytosolic loops of GPCRs, then ligand selective receptor conformations can lead to further selective directing of activation to G-proteins (trafficking of receptor stimulus). Such trafficking has been shown in natural and recombinant systems. For receptors that produce pleiotropic activation of multiple G-proteins, this would limit the signaling pathway activated by the particular ligand and thus confer further selectivity to the agonist (see **Fig. 7**).

It is not obvious how knowledge of ligand selective efficacy would be applied to drug discovery. However, it

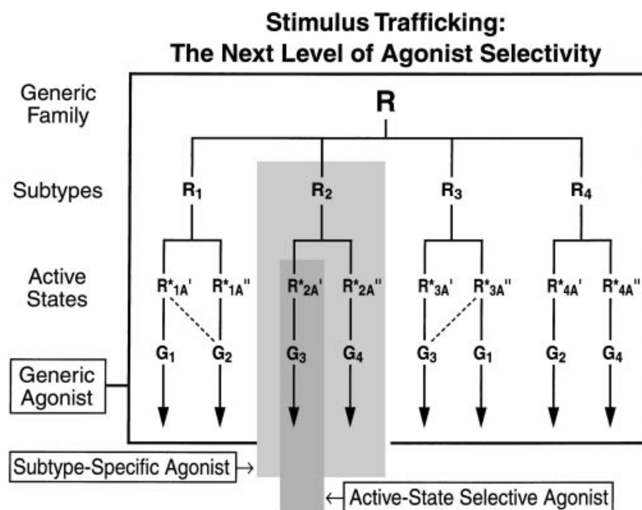


Figure 7. Schematic representation of the relative selection of stimulus pathways for receptors as a generic family is divided into subtypes and then each subtype is allowed to produce different active conformations that preferentially interact with different G-proteins; stimulus pathways are restricted. From ref 104 with permission.

could be useful to classify agonists on the basis of stimulus-response coupling as knowledge for retrospective analysis. Currently, agonists are all assumed uniformly to stimulate receptors and differ only on a spectrum of strength of signal. Separating agonists, in terms of the stimulus-pathways that they preferentially activate, may offer insights into preferred profiles of agonism as compounds are progressed from screening assays into therapeutically oriented secondary assays (104).

There are other realms of ligand-selective receptor conformation selection that may have therapeutic utility. For example, ligands that selectively induce receptor internalization may have great utility in the prevention of HIV-1 infection through chemokine receptor fusion. Ligands that cause internalization of CXCR4 (105, 106) or CCR5 (107, 108) have been shown to protect against HIV-1 infection *in vitro*. The selective removal of chemokine receptor from the cell surface could be superior to blocking chemokine receptor interaction with HIV viral coat proteins because it would circumvent possible rapid emergence of resistant HIV variants through therapeutic pressure and mutation (109–111).

There are other realms where differential conformations leading to differences in receptor disposition could be useful therapeutically. For example, ligand-selective bias in the production of receptor desensitization could be beneficial in treatment of tolerance (99, 100). Similarly, receptor dimerization may be implicated in numerous areas including HIV-1 infection (112, 113) and the function of cannabinoid receptors (114), $GABA_B$ receptors (115–118), adenosine A1 receptors (119), δ-opioid receptors (120), β_2-adrenoreceptors (121), and calcium-sensing receptors (122–124). Ligands that induce selective conformations

affecting dimerization may produce unique effects not necessarily associated with direct receptor signaling.

Finally, it is becoming evident that GPCRs can associate with other membrane proteins to change their affinities to ligands and reactivities toward G-proteins. For example, receptor activity-modifying proteins can change the phenotype of calcitonin gene-related peptide, adrenomedullin receptors, and calcitonin receptors (125–129). Similarly, GPCRs are known to interact with other accessory proteins such as PDZ domain-containing proteins. Thus, β_2-adrenoreceptors interact with Na^+/H^+-exchanger regulatory factor (130) and 5-HT_{2C} receptors with MUPP1 (a multi-PDZ domain protein with no currently known function (131). Again, as with desensitization, dimerization, and internalization, these receptor functions could, in theory, be regulated differentially by different ligand-induced receptor conformations to change receptor function. This could lead to another dimension in control of the quality of ligand efficacy.

CONCLUSIONS

This review describes three apparently separate phenomena—inverse agonism, protean agonism, and different types of positive agonism—in terms of a single mechanism of action, namely, the interaction of different receptor conformations (some spontaneously formed and some ligand directed) with G-proteins. It can be seen that such a system has a vastly increased range of adjustment over one in which a single activated receptor interacts with G-proteins on a scale of strength of signal. In this scheme, the stoichiometries of cellular components can adjust GPCR system set points and sensitivities; ligands theoretically can bias such systems in a multitude of ways. The challenge for the next millennium in drug discovery and receptor pharmacology will be to exploit ligand bias in these complex systems for therapeutic advantage. FJ

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