[1] Considerations in the Evaluation of Inverse Agonism and Protean Agonism at G Protein-Coupled Receptors

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Introduction

Over the past decade it has become accepted that G protein-coupled receptors (GPCRs) can activate G proteins and thus initiate signaling in the absence of agonist; this *spontaneous receptor activity* can be inhibited to varying degrees by antagonists, a phenomenon termed *inverse agonism*. The modulation of spontaneous GPCR activity by the binding of specific ligands implies that receptors can interconvert between active (R^*) and inactive forms (R), with agonists promoting the former and inverse agonists the latter. Thus, inverse agonists produce biochemical effects opposite to those of agonists. This stands in contrast to earlier theories that antagonists lack intrinsic activity and merely preclude the binding of agonists to receptors.

Inverse agonism has been observed in a wide variety of systems, with both endogenously and heterologously expressed GPCRs.[1] Notwithstanding these repeated observations *in vitro*, however, the contribution of inverse agonism to the overall therapeutic effects of antagonists is difficult to determine due to the continual presence of endogenous agonists under normal physiological conditions. Proof of the therapeutic relevance of inverse agonism awaits evidence that spontaneous receptor signaling is significant *in vivo* and/or that changes in receptor density,[2] distribution,[3,4] or posttranslational modification,[5] attributable to inverse agonist effects at the cellular and subcellular levels, also occur in intact organisms.

An emerging concept related to inverse agonism is that of *protean agonism*,[6] wherein some ligands display both agonist and inverse agonist properties at a single GPCR. Although this phenomenon has only been observed at a handful of GPCRs and is poorly understood at present, it may ultimately provide a key to understanding how all ligands modulate GPCR behavior.

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Experimental Detection of Inverse Agonist Activity

In theory, any preparation used to measure GPCR stimulation by agonists *in vitro* can also be used to study the inhibitory effects of inverse agonists. Because both agonists and inverse agonists appear to produce their effects by modulating the balance between active and inactive receptors, some level of spontaneous receptor activity presumably is needed to measure the effects of either class of ligand. The detection of inverse agonism additionally requires that spontaneous receptor activity be clearly distinguishable from background noise, which in practice frequently is not the case. Measuring inverse agonism thus is intrinsically more difficult, and the best conditions for doing so will not necessarily be the same as for measuring receptor activation by agonists. Optimizing protein expression levels, buffer components, and so on may help reveal spontaneous receptor activity. If a ligand is found to have a negative effect on agonist-independent GPCR signaling, the experimenter must verify that it is genuine. Thus, the possible presence of endogenous activating ligands needs to be ruled out; if that cannot be done, one should at least demonstrate that the effects of strong inverse agonists are competitively inhibited by neutral antagonists or weak inverse agonists.[7] Also, the possible confounding effects of related receptor subtypes, if any are present, should be considered. Inverse agonism can be assessed at the level of the receptor, G protein, effector, or events further downstream. Which technique works best may depend on the system under investigation. All end points will not necessarily yield equivalent results, and therefore it is worthwhile to assay ligand activity at multiple levels.

At the receptor, the binding of inverse agonists tends to be increased by guanine nucleotides, whereas that of agonists is decreased.[8] Thus, in competition experiments with radiolabeled antagonists, inverse agonist binding profiles are left-shifted by the inclusion of GTP analogues, implying a nucleotide-associated increase in affinity, whereas agonist binding profiles are rightshifted, implying a decrease.[9] Using fluorescently labeled β_2-adrenergic receptors, Gether and coworkers[10] have shown that agonists and inverse agonists appear to favor different receptor conformations, with the change in fluorescence induced by a given ligand corresponding to its ability to modulate receptor activity. GPCR conformation is also important for recognition by GPCR-selective kinases (GRKs), which preferentially phosphorylate agonist-bound receptors and thus appear to favor the active state. Accordingly, basal phosphorylation of the CB2 cannabinoid receptor is decreased by inverse agonists.[5] Finally, receptor localization within cells is

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sensitive to agonists, which typically promote internalization upon prolonged cell exposure. Emerging evidence indicates that inverse agonists can have the opposite effect, promoting GPCR movement from intracellular compartments to the plasma membrane.[3,4]

At the level of the G protein, activated GPCRs promote the dissociation of GDP; inverse agonists thus are expected to decrease GDP off rates, although this approach is not used commonly. In the presence of micromolar concentrations of unlabeled GDP, the binding of [^{35}S]GTPγS to G proteins is decreased by inverse agonists, opposite to the effect of agonists.[11,12] (It should be noted that receptor-related changes in [^{35}S]GTPγS binding are typically GDP dependent and thus reflect changes in the affinity of GDP but not necessarily that of [^{35}S]GTPγS, as is frequently assumed.) GPCR effects on G proteins are also manifested as changes in GTP turnover, with agonists increasing and inverse agonists decreasing the rate of steady-state GTP hydrolysis.[13] While measurements of G protein activity usually are made using cell membranes, inverse agonism has also been observed in GTPase assays with purified receptors and G proteins coreconstituted into phospholipid vesicles,[14] although that approach is technically challenging. In addition to their effects on nucleotide binding and hydrolysis, inverse agonists in whole cells may also influence G protein synthesis and/or degradation, as exposure of cells expressing the CB2 cannabinoid receptor to an inverse agonist upregulates G_i, the target G protein.[15]

At the level of effector proteins, inverse agonism can be observed via changes in second messenger production, both in intact cells and in membrane-based assays. The production of cyclic AMP by adenylyl cyclase is decreased by inverse agonists acting on G_s-coupled receptors[7] and is increased by inverse agonists acting on G_i-coupled receptors. Particularly in the latter case, the ability to detect inverse agonism may be enhanced in the presence of the adenylyl cyclase-stimulating diterpene forskolin.[11,16] Also, IP_3 production by phospholipase Cβ is inhibited by inverse agonists to receptors coupled to that effector system, via G_q.[18,19] Inhibition

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of phospholipase Cβ activity by inverse agonists has also been implied by ligand-induced decreases in intracellular calcium.[20] Other downstream events subject to inhibition by inverse agonists include GPCR-dependent MAP kinase activity[21] and agonist-independent, β-adrenergic receptor-mediated inotropic effects in isolated myocardium.[22,23]

Factors Affecting the Measurement of Inverse Agonism

Multiple factors influence the measurement of inverse agonism, most importantly the levels of receptor and G protein present, the G protein-effector pathways available, and the end point being measured. In addition, ligand effects can be influenced by other variables, such as assay buffer components, receptor mutations, receptor heterogeneity, and the presence of auxiliary proteins. Note that the same combination of receptor and ligand may yield differing results depending on the experimental context. Indeed, protean agonists were first identified by their ability to stimulate receptor activity in one assay and inhibit in another.[7]

Typically, inverse agonism is detected in systems in which GPCRs are expressed at high levels (>100 fmol/mg of membrane protein). Agonist-independent signal tends to increase proportionally as receptor expression level increases.[7] Therefore, maximizing receptor expression increases the likelihood of being able to detect inverse agonism, although other signaling components, such as G protein or effector, might become limiting as the receptor is increased. With overexpressed receptors it may be difficult to detect guanine nucleotide-induced changes in agonist and inverse agonist binding, but this can be remedied by the coexpression of an appropriate G protein. Increasing G protein levels may also make inverse agonism easier to detect[12,24]; however the increased spontaneous receptor signal associated with higher G protein expression sometimes is accompanied by increased receptor-independent background noise (e.g., second messenger production or [^{35}S]GTPγS binding). Many receptors are capable of activating multiple G protein subtypes and sometimes even different families of G proteins, and many G proteins in turn can activate multiple effector pathways.[25] The types and subtypes of G poteins and effectors available, either endogenously expressed or cotransfected

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with a receptor, can affect receptor–G protein coupling efficiency,[26,27] as well as events downstream. Note also that the same receptor, when coupled to different G protein-effector pathways, may exhibit different rank orders of ligand potency or efficacy.[28]

In addition to the densities and subtypes of receptors, G proteins, and effectors contained in an experimental preparation, the presence of auxiliary proteins, such as RGS proteins, scaffolding proteins, phosducin, and arrestins, all can influence the final readout of receptor–ligand interactions. The organization and availability of these auxiliary proteins may be altered by manipulations such as cell lysis and membrane preparation, which in turn can modulate GPCR responsiveness. Disruption of the cytoskeleton, for example, can increase G_s-dependent cAMP production in S49 cells via several different pathways.[29] Finally, spontaneous receptor activity can be highly sensitive to the ingredients of the experimental buffer. For example, NaCl decreases the agonist-independent GTPase activity of G_i coupled to 5HT1-serotonergic[30] and δ-opioid receptors in membrane-based assays,[13] whereas KCl has the opposite effect at the δ-opioid receptor.[13] NaCl thus decreases one's ability to detect spontaneous receptor activity in these systems.

Whereas ionic conditions and receptor expression levels influence primarily the magnitude of GPCR stimulatory and inhibitory responses, other factors presumably exist that can switch the activity of protean agonists between stimulatory and inhibitory modes. Labetalol increases intracellular cAMP in Sf9 cells expressing the β_2-adrenergic receptor, but conversely inhibits adenylyl cyclase activity in membranes derived from those same cells.[7] Although the determinants of protean activity are poorly understood, some chemical modifications of the receptor itself, such as the phosphorylation or substitution of key amino acid residues, appear to be important. Prolonged agonist stimulation of GPCRs in intact cells leads to desensitization, characterized by decreased agonist responsiveness and increased GPCR phosphorylation.[31] In contrast to the loss of stimulation with agonists, the ability of labetalol to inhibit spontaneous β_2-adrenergic receptor activity in Sf9 membranes is *increased* by GPCR desensitization.[32] Moreover, dichloroisoproterenol, which is strongly stimulatory in whole cells (~50% of isoproterenol activity),[7] variably acts as a weak agonist or weak inverse agonist in nondesensitized membranes (±20% of ligand-independent adenylyl cyclase activity) from Sf9 cells

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expressing β_2-adrenergic receptors, but consistently causes a 40% decrease in activity in membranes from desensitized cells.[32] Receptor phosphorylation thus appears to act as a switch for these protean ligands.

Similar to the effects of posttranslational modifications, mutations in GPCR amino acid sequences can significantly alter GPCR responsiveness to ligands. The most striking examples of this are perhaps the single amino acid substitutions that result in highly elevated spontaneous receptor activity.[33] These mutants allow a larger window through which to view the effects of inverse agonists. Mirroring the effect of desensitization on weak β_2AR partial agonists described in the preceding paragraph, activating receptor mutations at the B_2 bradykinin receptor cause drugs that are weak inverse agonists at wild-type receptors to behave as partial agonists.[34,35] In contrast, a spontaneously active form of the secretin receptor containing two point mutations was found to be inhibited by secretin, the natural activating ligand.[36]

While they occasionally occur in humans, it should be kept in mind that with constitutively activated GPCRs one is no longer dealing with the normal physiological target. Any alteration in GPCR structure can potentially influence both isomerization and affinity. Thus, the spontaneous activity of a receptor, the selectivity of ligands among receptor states, or both may be affected; note that it may be possible to misinterpret a change in isomerization as a change in affinity, as Colquhoun[37] has pointed out. Changes in GPCR responsiveness to ligand binding therefore need to be interpreted with care. Furthermore, compared to their wild-type counterparts, constitutively activated mutants are likely to be more highly phosphorylated because they may be better substrates for G protein receptor kinases (GRKs),[33] cause increased activation of second messenger-dependent kinases, or both.

Quantifying Inverse Agonist Activity

The fraction of spontaneous receptor activity that can be inhibited by an inverse agonist can be taken as an empirical measure of its activity in an experiment. A full inverse agonist would have an activity of 1.0, corresponding to 100% inhibition, whereas a partial inverse agonist would have an activity between 0 and 1.0, and a neutral antagonist lacking intrinsic activity would fail to inhibit and thus yield a value of 0. This is roughly analogous to assigning values of 1.0 or less for intrinsic

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agonist activity, as proposed originally by Ariens. Note that in both cases the parameter is system dependent.

To calculate inverse agonist activity, one must know the level of spontaneous receptor activity, which can be determined by subtracting the ligand-independent signal measured in the absence of receptor from that measured in the presence of receptor. While straightforward to do with heterologously expressed receptors, where cells transfected (or infected) with empty vector can be used to get an approximation of receptor-free signal,[7] this may not be feasible with endogenously expressed receptors (although specific alkylating ligands are available for some).

Figure 1 shows a hypothetical experiment with increasing concentrations of an inverse agonist: *A* represents the observed signal in the presence of spontaneously

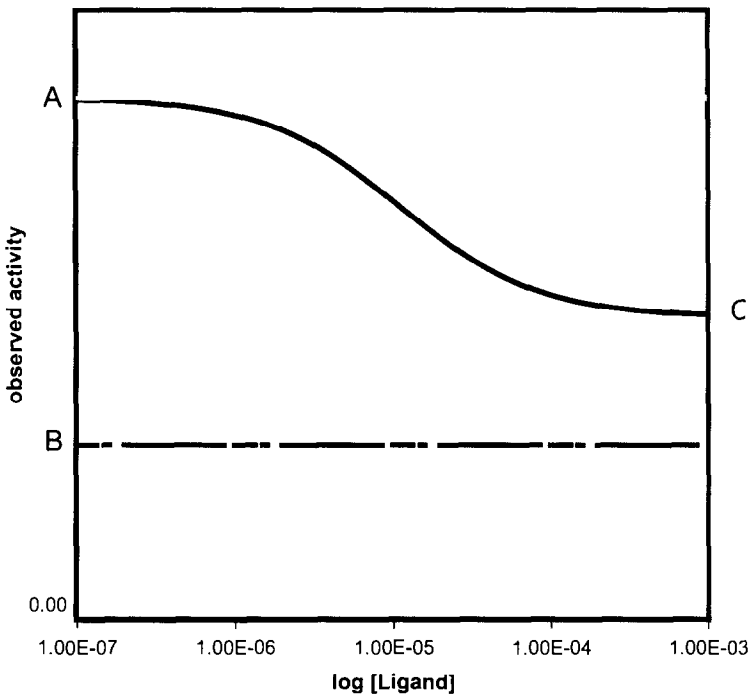

FIG. 1. Quantification of inverse agonist activity. Two hypothetical concentration dependence profiles with the same ligand in two equivalent preparations are shown: one in the absence and the other in the presence of a spontaneously active receptor. *A* represents observed activity in the presence of the receptor but in the absence of ligand, *B* represents the basal activity of the system in the absence of the receptor, and *C* represents the maximally inhibited signal at a saturating concentration of ligand. Maximal inverse agonist activity is defined as $(A-C)/(A-B)$, which equals the fraction of spontaneous receptor activity that can be inhibited by the ligand.

active receptor but in the absence of ligand, *B* represents the signal in the absence of receptor, and *C* represents the signal in the presence of receptor and a saturating concentration of inverse agonist. Spontaneous receptor activity equals the difference between *A* and *B*. Maximal inverse agonist activity equals *(A−C)/(A−B)*, corresponding to the fraction of spontaneous receptor activity that can be inhibited. Findings from various laboratories show that the same ligand may vary in maximal activity depending on the experimental conditions used to measure receptor function. For example, at the β_2-adrenergic receptor, propranolol has an inverse agonist activity of up to 0.8 in some systems[7] but is essentially inactive (i.e., zero) in others.[38]

The apparent equilibrium binding affinity (K_d) of an inverse agonist can be measured using standard agonist equilibrium-binding techniques, i.e., either by the direct binding to a receptor of increasing concentrations of a radiolabeled form of the inverse agonist or via competition between the unlabeled inverse agonist and a radioligand that binds to the same receptor (sometimes referred to as K_i). K_d is taken as equivalent to the half-saturating concentration of inverse agonist in a direct binding experiment (assuming mass-action behavior), or calculated from competition binding results, accounting for the rightward shift in the inverse agonist-binding profile caused by the presence of the radioligand (e.g., Cheng–Prusoff). Note that for both agonists and inverse agonists, K_d actually represents an amalgam of the individual affinities of the various receptor states.[37,39]

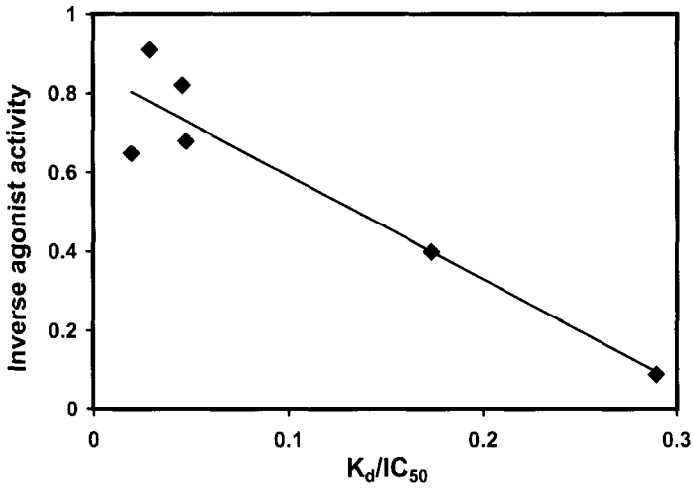

FIG. 2. Negative relationship between inverse agonist activity and K_d/IC_{50}. Apparent binding affinity, potency, and inverse agonist activity were measured with a variety of ligands in membranes prepared from Sf9 cells expressing the β_2-adrenergic receptor. Values of IC_{50} were higher than the corresponding K_d values, and this discrepancy between affinity and potency decreased with maximal inverse agonist activity. Plotted from data in Ref. 7.

The IC_{50} of an inverse agonist, i.e., the concentration required to produce 50% of its maximal inhibitory effect on spontaneous receptor activity, can be taken as an indication of its potency. Interestingly, IC_{50} values for inverse agonists sometimes tend to be *higher* than their K_d values. As shown in Fig. 2, this discrepancy is greater for strong inverse agonists than for weak inverse agonists at the β_2-adrenergic receptor. Although reports of such observations are limited, this trend is the reverse of what is found with GPCR agonists. Disproportionately high agonist activities at partial receptor occupancies and the analogous discrepancy found with inverse agonists may represent different manifestations of the same underlying phenomenon, which in the case of agonists is believed to reflect the existence of spare receptors.

Models of GPCR Activity

The simplest scheme for describing spontaneous receptor activity and inverse agonism is the two-state model:

$$\begin{array}{ccc} & K_I & \\ L + R & \leftrightarrows & L + R^* \\ K_L \downarrow\uparrow & & \downarrow\uparrow \alpha K_L \\ LR & \leftrightarrows & LR^* \\ & \alpha K_I & \end{array}$$

In this model, the receptor spontaneously isomerizes between inactive (R) and active (R*) conformations or states, and the ratio of [R]/[R*] is described by the unimolecular equilibrium constant K_I. A ligand (L) binds to the inactive state with an affinity $K_L = [L][R]/[LR]$ (equilibrium dissociation constant) and to the active state with an affinity $\alpha K_L = [L][R^*]/[LR^*]$. The selectivity factor α describes the mutual effect of the ligand on isomerization to R* and of that isomerization on ligand affinity. If $\alpha < 1$, the ligand will bind with higher affinity to R* and also promote the isomerization of R to R*, and therefore will act as an agonist; analogously, if $\alpha > 1$, the ligand will favor R and act as an inverse agonist. Ligands with only a weak binding preference will fail to drive the receptor completely into either state, and thus will act as partial agonists or partial inverse agonists. Ligands for which $\alpha = 1$ (sometimes referred to as neutral or true antagonists) will have no effect on receptor activity, but will competitively inhibit the effects of both agonists and inverse agonists.

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The two-state model is formally analogous to the *basic ternary complex model*[40] provided that G protein is not limiting. In that model, the receptor is considered to alternate between a free form (R) and a G protein-bound (RG) form, rather than between two conformations with different activities (i.e., R* and R). Samama and co-workers[33] combined the two-state model and the ternary complex model together to create the *extended ternary complex model* based on the idea that a constitutively active mutant form of the β_2AR could exhibit high-affinity agonist binding when free from G protein. Krumins and Barber[41] have argued that the extended ternary complex model reduces to a form of the two-state model wherein the receptor has two binding sites, one for ligands and one for G proteins, but still isomerizes between only two states, R and R*.

Some observations suggest that the two-state model may be too simplistic. For example, the activity of LR* can differ from one ligand to the next,[41] suggesting that ligands may distinguish between multiple active and inactive receptor conformations and/or induce distinct conformational changes on binding. Analogously, the observation of dissimilar agonist rank orders for the coupling of a single receptor to two different G proteins suggests that the latter also may recognize different active receptor states.[6] Still, it has been proposed that "R" and "R*" can be taken as representing two clusters of microstates drawn within the entire conformational space of a GPCR,[42] in which case differences among ligands and G proteins may be viewed as essentially consistent with the two-state model, although there might be preferences for different microstates within those clusters.

While the individual effects of agonists and inverse agonists are arguably consistent with the simple two-state model, some data remain difficult to explain in that context. Specifically, the phenomenon of protean agonism, wherein a single ligand can act as an agonist or inverse agonist at a single receptor, is problematic. It is difficult, indeed, to understand how one ligand can both increase and decrease GPCR activity. The *cubic ternary complex model* (CTCM)[6] is capable of accounting for protean agonism; for instance, if a ligand can promote the isomerization of R to R* but simultaneously disfavor the binding of receptor to G protein, then its net activity will depend on how these two effects balance out under a given set of experimental conditions. Protean agonism can also be accounted for in terms of the three-state model,[28] wherein a single receptor can couple to two different G proteins (termed G1 and G2), both of which are freely available to interact with the receptor, but which recognize different conformations (e.g., R* and R**). In the context of the three-state model, if an activating ligand promotes the formation of R*G1 more than R**G2, then assays based on an end point dependent on R**G2 will show agonism if no G1 is present, but increasing the availability of G1 will

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[41] A. M. Krumins and R. Barber, *Mol. Pharmacol.* **52,** 144 (1997).
[42] H. O. Onaran and T. Costa, *Ann. N.Y. Acad. Sci.* **812,** 98 (1997).

create a sink for the receptor, leading to a diminution of the R**G2 response and ultimately manifesting as inverse agonism (provided that sufficient spontaneous receptor activity is present). Hence, the same ligand can appear either stimulatory or inhibitory, depending on the available G protein complement.

The models discussed up to this point are based on the premise that a single receptor can assume two or more conformations that differ in affinity for ligands and/or G proteins, with the underlying assumption that the population of receptors under consideration is homogeneous. While it is clear that cloned receptors can be expressed as single gene products in cultured cells, a uniform amino acid sequence does not guarantee a homogeneous population of proteins. Thus, a GPCR may normally exist as a mixture of posttranslationtially modified forms of a common primary structure. This would be significant if more than one structurally modified form were to contribute to the experimental readout of receptor function. As noted earlier, relatively minor changes in the structure of a GPCR can profoundly affect its basal activity and regulation by ligands. In particular, it appears that the phosphorylation that accompanies receptor desensitization does not really turn off receptors but rather serves to alter their responsiveness to agonists and inverse agonists.[32]

The idea that desensitized receptors can still interact productively with their G proteins runs counter to the prevailing notion that phosphorylation causes the functional uncoupling of receptor and G protein.[43] Receptor phosphorylation precedes or accompanies internalization from the plasma membrane; however, blocking that sequestration does not prevent desensitization *per se*.[31] The notion that receptor phosphorylation prevents coupling to G proteins presumably stems from the observed similarity of the changes in the concentration dependence of agonist activity that occur after either receptor alkylation (i.e., loss of spare receptors) or receptor desensitization. In both cases, agonist potency and subsequently maximal activity decrease in a characteristic pattern[44]; interestingly, the same pattern can be produced using the two-state model shown earlier (Fig. 3), indicating that desensitization is superficially consistent with at least two possible mechanisms, namely a decrease in receptor number and a loss of agonist selectivity for R*.

Equating desensitization to a decrease in the number of functional receptors dictates that there should be a proportional decrease in spontaneous receptor activity, but such a relationship has seldom, if ever, been described. Alternatively, desensitization can be modeled as a decrease in the ability of agonists to distinguish between R and R*. The simulations in Fig. 3 show that decreasing the selectivity of an agonist for R* (i.e., increasing α) mimics observed patterns of agonist desensitization, without having to assume a decrease in receptor density.

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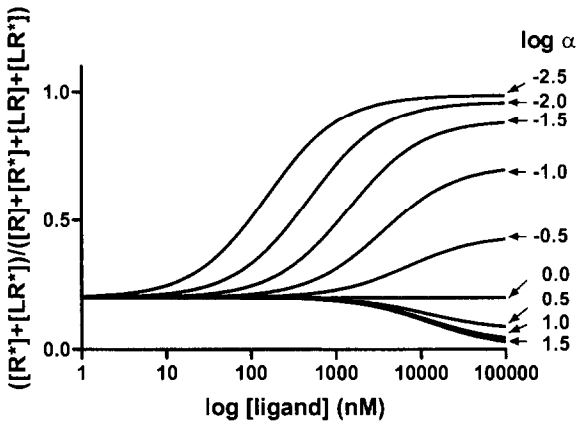

FIG. 3. Effect of varying α on ligand behavior. In the simulations shown, which were carried out according to the two-state model, one in five free receptors is assumed to be in the active conformation (i.e., $K_I = 4$), and K_L, the affinity for R, is set to 10 μM throughout. Agonist activity is modeled by setting the selectivity factor α to values of less than one so that the ligand binds with higher affinity to R* than to R. When the preference for R* is 300-fold or greater (i.e., $\alpha < 0.003$), the ligand is able to fully activate the receptor, with smaller values of α shifting the curve increasingly to the left. As α approaches unity, there is a rightward shift in the ligand concentration dependence, and eventually a complete loss of agonist activity. Inverse agonist activity analogously is modeled by setting α to values of greater than one. Maximal inhibition increases with α; however, once inhibition is complete (i.e., $\alpha \sim 30$), there is no further effect of increasing α.

This interpretation is preferable to the prevailing "functional uncoupling" hypothesis because it allows spontaneous receptor activity to be maintained. Another advantage of the two-state model of course is that it accounts for inverse agonism; moreover, desensitization-induced changes in inverse agonism are consistent with a decrease in ligand affinity for R* relative to R. The simulations in Fig. 3 show that an increase in α increases the maximal effect of a partial inverse agonist but has no effect on a full inverse agonist, again recalling the results seen with desensitization of the β_2AR.[32] For both agonists and inverse agonists, a 10-fold or lower decrease in ligand affinity for R* with no change in affinity for R appears consistent with the effects of receptor desensitization. In addition, a similar change in α can account for protean agonism; for example, in Fig. 3, increasing α from 0.32 to 3.2 (i.e., changing log α from -0.5 to 0.5) shifts the ligand from being a partial agonist to being a partial inverse agonist, recalling experiments with dichloroisoproterenol before and after desensitization at the β_2AR.[32]

The effects of phosphorylation (and presumably other posttranslational modifications) on GPCR behavior argue that each posttranslationally modified form of a GPCR should be considered as a distinct protein capable of isomerizing between

active and inactive conformations. It follows that any measurement of receptor activity would represent the summation of the activities of all of these related structures. The relative amount of each differentially modified form of the receptor would be in constant flux in whole cells, where phosphorylation, dephosphorylation, palmitoylation, and so on occur continuously; subcellular preparations and purified proteins would tend to have fixed receptor subpopulations, as the factors governing their interconversion would largely be lost or inactivated. From this perspective, the two-state model is clearly too simplistic; following the same basic principle, the minimum number of receptor states would be twice the number of structural forms, in essence begetting a multi two-state model. Briefly, the observed response to a ligand would be equal to the sum of its effects on each structural variant of the target receptor. For protean agonists, where the changes in receptor activity would occur simultaneously in two opposite directions, the predominating effect would dictate whether the ligand appeared to be stimulatory or inhibitory. Thus, such ligands could appear to function as agonists or inverse agonists, depending on the relative amount of each posttranslationally modified form available in a given experiment. For an illustration of this concept, the reader is referred to previously published simulations carried out assuming a mixture of two subpopulations of receptor with "normal" and "desensitized" ligand-binding properties.[32]

$$
\begin{array}{ccccccc}
 & K_I & & K_I' & & K_I'' & \\
L + R & \leftrightarrows & L + R^* & L + R' \leftrightarrows L + R'^* & & L + R'' \leftrightarrows L + R''^* & \\
K_L \downarrow\uparrow & & \downarrow\uparrow \alpha K_L & + \quad K_L' \downarrow\uparrow & \downarrow\uparrow \alpha' K_L' & + \quad K_L'' \downarrow\uparrow & \downarrow\uparrow \alpha'' K_L'' + \cdots \\
LR & \leftrightarrows & LR^* & LR' \leftrightarrows & LR'^* & LR'' \leftrightarrows & LR''^* \\
 & \alpha K_I & & \alpha' K_I' & & \alpha'' K_I'' &
\end{array}
$$

multi two-state model

The multi two-state model offers an alternative explanation for the phenomenon of protean agonism that has some advantages over the three-state and cubic ternary complex models. For example, the three-state model requires that the receptor can interact freely with multiple pools of G protein, a situation that has been difficult to verify experimentally.[45] Also, the CTCM may be unable to account for protean agonism in cases where receptors and G proteins are precoupled or form stable complexes. The basic premise of the multi two-state model is that altering the structure of a receptor changes its function; the same idea could be incorporated into modified three-state or cubic ternary models, as these are essentially extensions of the two-state model. The implied number of parameters might become unwieldy, however, especially in the case of a multi-CTCM.

[45] R. R. Neubig, *FASEB J.* **8,** 939 (1994).

The implications of the multi two-state model go beyond its ability to account for protean agonism and the effects of receptor desensitization on agonist and inverse agonist activities. Indeed, several studies have found that for receptors that couple to multiple G proteins, the phosphorylation state of the receptor influences which G protein pathway will be activated.[46,47] A logical extension of this is that the inconsistencies in ligand rank orders of potency and activity found when one receptor couples to two different G proteins may reflect the existence of receptor populations with different posttranslational modifications.

Conclusions

The discovery of spontaneous receptor activity and inverse agonism has brought about significant advances in our understanding of how GPCR activity can be modulated by the binding of stimulatory and inhibitory drugs. Because agonists and inverse agonists appear to bind to different receptor conformations, it may be possible to design improved therapeutics by targeting them to specific receptor states. Moreover, the sensitivity of inverse agonists and protean agonists to receptor desensitization implies an additional level of complexity in structure–activity relationships. While inverse agonism and protean agonism reveal unforseen complexities in GPCR regulation, the elucidation of the molecular events underlying these phenomena ultimately should increase our understanding of GPCR-related diseases as well as our ability to design more selective and more efficacious therapeutics.

Acknowledgments

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