0026-895X/03/6406-1271–1276$7.00
MOLECULAR PHARMACOLOGY
Vol. 64, No. 6
2756/1109668
Mol Pharmacol 64:1271–1276, 2003
Printed in U.S.A.

MINIREVIEW

Constitutive Activity and Inverse Agonists of G Protein-Coupled Receptors: a Current Perspective

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Received June 20, 2003; accepted August 29, 2003

This article is available online at http://molpharm.aspetjournals.org

ABSTRACT

Over the last decade, the ability to detect agonist-independent signal transduction by G protein-coupled receptors has in turn resulted in the detection and study of ligands able to block this activity. Such ligands are generically described as inverse agonists. Considerable attention has recently been devoted to the presence and roles of endogenous antagonist/inverse agonists and the concept that inverse agonists may have specific therapeutic benefits compared with neutral antagonists.

Although now appreciated to represent a substantial oversimplification (Milligan and IJzerman, 2000; Strange, 2002), the two-state model of GPCR function (Samama et al., 1993) remains an extremely useful concept. Agonist ligands stabilize or increase the fraction of the active state of a GPCR such that it can interact with and activate a G protein. As such, basic thermodynamics define that there must be a finite probability that this active state also occurs in the absence of the agonist. Equally, if agonists enrich such active states, then it should be possible to identify ligands (inverse agonists) that stabilize or enrich the inactive state. With hindsight, it is easy to argue that compounds (neutral antagonists) that bind to GPCRs without altering the equilibrium between active and inactive states of the receptor are likely to be rather rare. However, early cartoons that specifically illustrated this point (Milligan et al., 1995) were contentious because they did not reflect pharmacological experience in which 'antagonists' were common reagents. However, within such models, the scale of ligand efficacy ranged from 1 (full agonist) to −1 (full inverse agonist) and neutral antagonists were defined very precisely as possessing 0 efficacy. Heterologous expression of many GPCRs resulted in the detection of ligand-independent signal transduction that increased in an essentially linear fashion with increasing levels of GPCR expression (Tiberi and Caron, 1994). After clear demonstration that the constitutive activity of the GPCR was not associated with the presence of low concentrations of endogenous agonists, the ability of many traditional 'antagonists' to block the constitutive activity of expressed GPCRs rapidly saw these compounds reclassified as inverse agonists (Milligan et al., 1995). This process continues to the present with many studies still reporting the inverse agonist activity of ligands at a wide range of both native and mutated GPCRs (Daeffler and Landry, 2000). Compounds with close to zero efficacy are vital tools in ligand classification because any compound with this characteristic that binds in a competitive manner will act as a functional antagonist to compounds with agonist or inverse agonist properties. They can thus be used to exclude apparent inverse agonism that derives from competition with an endogenous ligand rather than via suppression of receptor constitutive activity.

GPCRs Display Varying Levels of Constitutive Activity

A key question for the potential importance of inverse agonists and whether they may have inherent benefits as therapeutic agents relates to the level of constitutive activity of individual GPCRs. Many clinically important medicines have been demonstrated to behave as inverse agonists when tested against either wild-type or mutated GPCRs (Table 1). In most cases, these studies have used GPCRs expressed recombinantly in cell lines, but in certain cases, significant

This work is based on a presentation to the American Society for Pharmacology and Experimental Therapeutics meeting in San Diego on 12 April 2003.

ABBREVIATIONS: GPCR, G protein–coupled receptor; RGS, regulator of G protein signaling; MSH, melanocyte-stimulating hormone; AGRP, agouti-related peptide; mGluR, metabotropic glutamate receptor; PDZ, postsynaptic density 95/disc-large/ZO-1.

TABLE 1
Are clinically effective medicines inverse agonists?
Based on sales in the USA in 2002, a significant number of the top one hundred selling medicines target GPCRs. Those that are antagonists/inverse agonists are listed along with some examples where they have been assessed as potential inverse agonists at either the wild-type or constitutively active mutants of GPCRs.

Generic Name (Trade Name)	Therapeutic Area	Receptor Target	Inverse Agonist?	Reference
Olanzapine	Antipsychotic	5-HT2C/5-HT2A/others	Yes	Herrick-Davis et al., 2000
Losartan	Cardiovascular	AT1	Yes	Groblewski et al., 1997, Miserey-Lenkei et al., 2002
Risperidone	Antipsychotic	5-HT2/dopamine D2,D3	Yes	Vanhauwe et al., 1999; Herrick-Davis et al., 2000
Fexofenadine	Respiratory	histamine H1	Probably	Leurs et al., 2002.
Clopidogrel	Thrombosis	P2Y12	Unclear	Conley and Delaney, 2003
Valsartan	Hypertension	At1	Unclear	
Montelukast	Respiratory	CysLT1	Unclear	
Loratidine	Respiratory	Histamine H1	Probably	Leurs et al., 2002.
Quetiapine	Anti-Psychotic	Dopamine D2/5-HT2C/5HT2A	Unclear	Rauser et al., 2001
Cetirizine	Respiratory	Histamine H1	Probably	Leurs et al., 2002
Metoprolol	Cardiovascular	β1-Adrenoceptor	Yes	Engelhardt et al., 2001; Levin et al., 2002
Tolterodine	Genitourinary	Muscarinic M3/M2	Unclear	
Famotidine	Gastrointestinal	Histamine H2	Yes	Alewijnse et al., 1998.

overexpression of GPCRs in tissues of transgenic animals has been employed to produce measurable levels of constitutive activity. Although virtually all GPCRs can be modified to display constitutive activity (Chalmers and Behan, 2002), many GPCRs display rather low levels of constitutive activity when expressed in recombinant systems and display greatly increased activity in the presence of an agonist. A classic example is the hamster α_{1b}-adrenoceptor. In this GPCR, mutation of Ala^{293} to any other amino acid increased constitutive activity (Kjelsberg et al., 1992). This indicates a strong evolutionary pressure to maintain the wild-type form of the receptor in a nearly silent state. Indeed, for many GPCRs, mutational changes in the region near the the interface at the end of the third intracellular loop and the start of transmembrane helix VI result in elevated levels of constitutive activity (Pauwels and Wurch, 1998; Seifert and Wenzel-Seifert, 2002). This may relate to the well-appreciated movement of the bottom of transmembrane helix VI in response to the binding of agonist ligands (Hubbell et al., 2003). It is also noteworthy that mutations in this region of a number of GPCRs with endocrine ligands have been associated with relatively rare but extremely well studied, hormone-independent, human diseases (Parnot et al., 2002). These diseases would certainly be treated more effectively with inverse agonists compared with neutral antagonists. Enhanced levels of constitutive activity can also be produced by judicious mutation in many other locations in GPCRs (Pauwels and Wurch, 1998; Seifert and Wenzel-Seifert, 2002). Indeed, a recent random mutagenesis study employing the DOP opioid receptor used the development of constitutive activity to trace pathways of GPCR activation (Decaillot et al., 2003). These studies further indicate significant evolutionary pressure to maintain low levels of agonist-independent activity. In the case of rhodopsin, it is vital that levels of constitutive activity are virtually nil, otherwise the sensitivity and amplification of the visual pathway would result in permanent photobleaching. This is achieved by the covalent association of the ligand retinal in the binding pocket of the GPCR. In the 11-*cis* configuration, retinal holds rhodopsin in an inactive state, and this has resulted in 11-*cis* retinal being described as an endogenous inverse agonist (see below). Spontaneous isomerization of 11-*cis* retinal to the agonist all-*trans* retinal is an extremely rare event. However, the rapidity and efficiency of the photon-induced switch of 11-*cis* retinal to the all-*trans* configuration results in highly effective signal transduction.

Except for the very specialized example of rhodopsin noted above, measurable constitutive activity can be recorded, without mutation, for virtually all GPCRs. However, in assays for constitutive activity that monitor GPCR-mediated GTPase activity in membrane fractions, the contribution of the GPCR of interest to the total high-affinity GTPase can be relatively poor. This can be improved by addition of regulator of G protein signaling (RGS) proteins to the assay (Welsby et al., 2002). These proteins function to accelerate the intrinsic GTPase activity of many heterotrimeric G proteins (Neubig and Siderovski, 2002). It is of little significance to the RGS whether GTP was loaded onto the G protein by an agonist-mediated or agonist-independent event. Thus, the presence of sufficient RGS increases the fraction of basal GTPase activity contributed by the constitutive activity of a GPCR and provides a greater level of activity that can be inhibited

by inverse agonists and hence measured (Welsby et al., 2002). In intact cells, RGS proteins and other modulators of G protein function will regulate the constitutive activity of GPCRs and thus the likely therapeutic effectiveness of inverse agonists.

Closely related GPCRs frequently display significantly different levels of constitutive activity when expressed at equal levels. For example, a number of studies have noted higher levels of constitutive activity of the β_2-adrenoceptor compared with the β_1-adrenoceptor (Engelhardt et al., 2001; Zhou et al., 2000) and of the dopamine D5 receptor compared with the dopamine D1 receptor (Tiberi and Caron, 1994). Possible reasons for such differences are discussed later. Tissue-targeted transgenic expression of wild-type GPCRs also results in constitutive signaling, which can result in physiological function largely independent of requirement for agonist. One of the earliest examples was the generation of transgenic mice overexpressing the β_2-adrenoceptor to a high degree via a heart-specific promoter. Isolated atria from the transgenic mice displayed isometric tension as high in the absence of agonist as that produced by a maximal concentration of isoprenaline in atria of control mice. Basal heart rate was also much higher in the transgenic animals and not further increased by infusion of isoprenaline (Milano et al., 1994). Interestingly, this phenotype was not observed by transgenic expression of a constitutively active mutant of the β_2-adrenoceptor (Samama et al., 1997). This reflected the now well appreciated instability of such mutants that frequently results in low steady-state levels of expression. Treatment of the animals with β-blockers that are inverse agonists up-regulated the mutant protein and uncovered the constitutively active phenotype (Samama et al., 1997). In contrast to examples noted earlier, a number of GPCRs do seem to have significant levels of constitutive activity when expressed in cell lines; in some cases, ligand-induced stimulation of activity is relatively small compared with the signal in the absence of ligand. Three families of GPCRs that cluster closely together in sequence similarity plots are the sphingolipid receptors, the melanocortin receptors, and the cannabinoid receptors. These tend to display high levels of agonist-independent activity, and it is noteworthy that unlike the vast majority of the rhodopsin-like class A GPCRs, they do not have the possibility to form a disulfide bond between cysteine residues positioned adjacently in extracellular loops II and III. However, it is not a simple issue to test whether lack of this disulfide bond enhances the constitutive activity of a range of GPCRs, because mutational alteration of these residues frequently prevents transport of the modified GPCR to the cell surface (Ai and Liao, 2002; Kuwasako et al., 2003).

Endogenous Inverse Agonists

For GPCRs with high constitutive activity, an intriguing idea is that endogenous antagonists/inverse agonists are produced to dampen this activity. There is one system that provides well-documented examples of such a phenomenon. This is the family of melanocortin receptors (Adan and Kas, 2003). Of the melanocortin receptors, the MC1 receptor is expressed in melanophores in the skin. In mice, α-MSH acts as agonist at the MC1 receptor and via stimulation of cAMP levels promotes production of melanin and dark coat color. The polypeptide ligand agouti acts as an antagonist at this receptor and this results in yellow coat color (Wolff, 2003). The MC3 and MC4 receptors are expressed in the brain and play key roles in obesity and cachexia (Goodfellow and Saunders, 2003; Zimanyi and Pelleymounter, 2003). Here, α-MSH acts as agonist to regulate feeding, whereas agouti or agouti-related peptide (AGRP) can act as functional antagonists. However, AGRP and smaller fragments of this ligand function as endogenously produced inverse agonists (Haskell-Luevano and Monck, 2001; Nijenhuis et al., 2001). After heterologous expression of the human MC4 receptor, both basal and forskolin-stimulated adenylyl cyclase activity was inhibited by the 83 to 132 fragment of AGRP, and the extent of inhibition was both dependent on the level of MC4 receptor expression and not observed in cells that did not express the receptor. Importantly, the small molecule SHU9119 seems to be a nearly neutral antagonist for this receptor and is able to block both the agonist effect of α-MSH and the inverse agonist effect of AGRP 83–132 (Nijenhuis et al., 2001). Ectopic overexpression of AGRP in mice results in an obese phenotype, presumably by interacting with the MC3/MC4, whereas ectopic over-expression of agouti results in both an obese phenotype and light coat color via the added effect at the MC1 receptor. Although the numbers are relatively small, a range of nonsynonomous single nucleotide polymorphisms that result in single amino acid alterations in the MC4 receptor have been reported in morbidly obese persons (Vaisse et al., 2000; Dubern et al., 2001). When expressed in heterologous systems, many of these mutants are poorly delivered to the cell surface (Lubrano-Berthelier et al., 2003; Nijenhuis et al., 2003; Yeo et al., 2003). It remains to be explored whether these alterations produce a distinct modulation of constitutive signal transduction. Because malfunction of the MC4 receptor may be implicated in some severe genetically controlled examples of obesity, it is also interesting to note than a correlation has been observed between a polymorphic variation in AGRP and the development of anorexia nervosa (Lu, 2001; Vink et al., 2001). Poor suppression of constitutive MC4 receptor signaling and function by a malfunctional or poorly expressed form of AGRP would be consistent with poor food intake, but more studies need to be performed to investigate the implications of such studies. Although there is currently no evidence to support the idea, it may be worthwhile to examine whether endogenous antagonists/inverse agonists are produced that act at the sphingolipid and/or cannabinoid receptors.

Virally Encoded GPCRs

A number of viruses encode combinations of chemokine-like GPCRs and GPCR ligands in their genome (Rosenkilde et al., 2001). These include ORF74 of human herpes virus-8 (also called Kaposi sarcoma herpes virus) and US28 and US33, both encoded by human cytomegalovirus. Presumably, these were pirated from mammalian cells infected by the ancestors of these viruses. ORF74 displays homology to the CXCR2 receptor and was shown to possess constitutive activity when expressed in heterologous systems. Furthermore, ORF74 transfected NIH-3T3 cells can induced tumor formation in nude mice (Bais et al., 1998) and mice transgenic for ORF74 expression develop Kaposi sarcoma-like symptoms (Yang et al., 2000; Guo et al., 2003). The majority of chemokine receptors can be regulated by a range of chemokine

ligands; indeed, in certain cases, chemokines seem to act as endogenous inverse agonists of these virally encoded GPCRs. Both human interferon-γ-inducible protein 10 and stromal cell-derived factor-1α have been shown to inhibit the constitutive signaling of ORF74 (Rosenkilde et al., 1999). The US28 gene product is distantly related to the human CCR5 and CXCR4 chemokine receptors. Like these, US28 allows infection of CD4-positive human cell lines by primary isolates of HIV-1 and HIV-2 (Pleskoff et al., 1997). Both the US28 and US33 GPCRs also display constitutive activity when expressed in heterologous systems, and a nonpeptidergic ligand, VUF2274, has been shown to act as a relatively low-affinity inverse agonist of US28 without affecting the constitutive activity of either US33 or ORF74 (Casarosa et al., 2003). This is of considerable interest because VUF2274 was also shown to be able to partially inhibit HIV-1 entry into US28-expressing, CD4-positive cells (Casarosa et al., 2003). This viral GPCR demonstrates high levels of constitutive endocytosis (Waldhoer et al., 2003) but studies with variants of US28 lacking elements of the C-terminal tail have dissociated constitutive internalization and constitutive signaling (Waldhoer et al., 2003), implying that high levels of constitutive activity does not directly induce endocytosis. This might have been expected to be the case if the constitutive activity were associated with ligand-independent-phosphorylation and translocation of β-arrestins. However, there are potentially conflicting recent data on the likely importance of interactions with β-arrestins in the constitutive internalization of this GPCR (Fraile-Ramos et al., 2003; Miller et al., 2003).

Constitutive Activity of GPCRs in Native Systems

As noted above, in many situations, the inability to clearly exclude a contribution from endogenous ligands to basal activity has made unambiguous scoring of GPCR constitutive activity in native systems difficult. As such, a thorny issue is whether significant levels of constitutive activity are seen only in recombinant systems in which GPCR expression levels can be manipulated and are often relatively high. Early data to suggest that this is not the explanation in all cases has been reviewed elegantly and extensively by de Ligt et al. (2000).

The availability of ligands with close to zero efficacy is required to allow detailed study in native tissues. After expression of splice variants of the rat histamine H3 receptor in Chinese hamster ovary cells, both histamine and imetit were able to elevate [^{3}H]arachidonic acid release, whereas thioperamide and a range of other histamine H3 blockers were able to inhibit basal release. By contrast, proxyfan was without effect. Importantly, the effects of both agonists and inverse agonists were blocked by proxyfan in a concentration-dependent fashion (Morisset et al., 2000). Based on this definition of proxyfan as a neutral antagonist for the rat histamine H3 receptor, this compound was used to examine the relevance of constitutive activity of this receptor on either autoreceptor-mediated [^{3}H]histamine release or basal and ligand-regulated guanosine 5′-*O*-(3-[^{35}S]thio)triphosphate binding in rat brain membranes. In both assays, proxyfan reversed both the agonist effects of imetit and the inverse agonist effects of thioperamide without producing significant effects itself (Morisset et al., 2000). This remains one of the most impressive examples of constitutive activity in a native setting and is consistent with significant tonic regulation via ligand-independent activity of a GPCR. In a clinical content, these results have led to the suggestion that inverse agonists might be preferred to neutral antagonists as 'cognitive enhancers' (Schwartz et al., 2003).

Most studies on constitutive activity and inverse agonism continue to rely on heterologous expression of GPCRs. A further complication for the use of transfected cell systems as a means to gauge the potential for constitutive activity of GPCRs in native tissues is that it is now clear that GPCRs generally do not exist in isolation but may have (many) potential binding partners. Interactions between intracellular regions of GPCRs, particularly the C-terminal tail (Bockaert et al., 2003), and other polypeptides has become an intensely studied topic (Kreienkamp, 2002; Premont and Hall, 2002). In many cases, such interactions serve to regulate the cellular location, scaffolding and/or trafficking of a GPCR. Such interactions can, however, also regulate the degree of constitutive activity observed. If the heterologous cell system selected for expression of a GPCR lacks expression of a specific interacting protein, this can modulate the observed degree of constitutive activity. For example, when expressing either the mGluR 1a or mGluR 5 receptor in HEK293 cells, Fagni and colleagues observed constitutive inositol phosphate generation (Ango et al., 2001). This was not observed, however, in neurons. Although differences in expression levels in the two host systems might have provided a trivial explanation for these findings, a more interesting one emerged. The C-terminal tail of the mGluR1a and mGluR 5 but not other members of this GPCR subfamily family contain consensus regions for the binding of homer proteins. Homer proteins have been particularly well studied owing to their capacity to provide a bridge between cell surface receptors and the cortical actin system of the cytoskeleton and thus anchor receptors in specific locations (e.g., the synapse) (Thomas, 2002). Cultured cerebellar granule cells were shown to express homer 3 but not other members of the homer family (Ango et al., 2001). After treatment of such cells with antisense oligonucleotides targeting homer 3 expression, basal inositol phosphate production increased. This was unaffected by addition of the mGluR1 neutral antagonist CPCCOEt but reversed by addition of the inverse agonist BAY36. Such constitutive activity of the mGluR1a receptor was not uncovered when cells were treated with a scrambled oligonucleotide sequence based on the effective antisense (Agno et al., 2001).

Are There Cellular Constraints on Constitutive Activity of GPCRs?

As detailed above, closely related GPCR pairs, such as the dopamine D1 and D5 receptors and the β_1- and β_2-adrenoceptors, have been shown to display significantly different extents of constitutive activity. Although clearly not the only element contributing to the differences in the extent of constitutive activity of the dopamine D1 and D5 receptors, the C-terminal tail plays an important role (Tumova et al., 2003). Sequence variation between these two GPCRs is relatively high in this region. Elegant recent studies have demonstrated direct interactions between the *N*-methyl-D-aspartate

receptor and the dopamine D1 receptor but not the dopamine D5 receptor (Lee et al., 2002) and between the GABA-A receptor and the dopamine D5 receptor but not the dopamine D1 receptor (Liu et al., 2000). These protein-protein interactions are defined by elements within the C-terminal tail of the GPCRs. Although effects of these interactions on constitutive activity were not explored, it would not be surprising if it were modulated. The ongoing revolution in proteomics is uncovering many prospective interactions between GPCRs and other polypeptides. Forms of the 5-hydroxytryptamine 5-HT_{2C} receptor have been extensively studied as model GPCRs. These display significant constitutive activity at which a series of therapeutically relevant ligands have been shown to act as inverse agonists in both heterologous and native systems (Barker et al., 1994; Herrick-Davis et al., 2000; Rauser et al., 2001) (see also Table 1). However, proteomic detection of polypeptides that interact with the 5-HT_{2C} receptor has identified at least 13 distinct proteins (Becamel et al., 2002), and it will be interesting to see how these interactions alter agonist-independent signal transduction. The multi-PDZ domain-containing protein MUPP-1 is one of these (Becamel et al., 2001) and elimination of the PDZ binding domain at the C-terminal tail of the 5-HT_{2C} receptor alters phosphorylation and resensitization of this receptor (Backstrom et al., 2000). Recently, an interaction between the MOP1 and MOP1A variants of the human μ-opioid receptor and the intermediate filament binding protein periplakin was shown to disrupt agonist activation of G protein (Feng et al., 2003). This and similar protein-protein interactions may also limit constitutive activity. The application of antisense and particularly small interfering RNA based reagents (Chi et al., 2003; Semizarov et al., 2003) offers an attractive and selective means to 'knock-down' expression levels of GPCR interacting proteins in native tissues to explore whether this alters the extent of constitutive activity.

Signaling proteins are not evenly and randomly distributed at the plasma membrane of cells. Many, including the heterotrimeric G proteins, are concentrated selectivity in specialized regions known as lipid rafts (Foster et al., 2003). Although the distribution of GPCRs is in general significantly less clear, in rat ventricular myocytes, it has been reported that the β_2-adrenoceptor is significantly more concentrated in such rafts than the coexpressed β_1-adrenoceptor (Rybin et al., 2000). A range of other signaling proteins display differential distribution in myocytes (Steinberg and Brunton, 2001), and this may further contribute to the differential function of these two adrenoceptors. There are varying views on whether concentration of G proteins in lipid rafts is designed to enhance or restrict their signaling activity. However, one obvious scenario is that the higher constitutive activity of the β_2-adrenoceptor compared with the β_1-adrenoceptor may simply reflect greater access to G proteins and effector enzymes. Potentially in support of such a model are data generated by comparison of fusion proteins between both the β_1- and the β_2-adrenoceptor, with the long isoform of $G_s\alpha$ (Wenzel-Seifert et al., 2002). Here, when the ratio of GPCR to G protein is fixed, both GPCRs displayed similar characteristics of constitutive activity and indeed the β_1-adrenoceptor was the more effective at activating the G protein. Clearly, a great deal more information remains to be gleaned about the role of protein-protein interactions and cellular targeting to membrane subdomains in the generation of receptor constitutive activity and thus the potential practical importance of inverse agonists in therapeutic and pathophysiological settings.

Conclusions

Partially because of the relative simplicity of the assays, 'antagonist' ligands are now routinely assessed for inverse agonism. Many clinically effective medicines can be shown to be inverse agonists. However, the bulk of such studies still rely on heterologous expression of GPCRs in cell lines, and it remains unclear whether inverse agonists offer inherent advantages over neutral antagonists in the vast majority of clinical scenarios, or indeed whether the inverse agonism of some medicines contributes significantly to their effectiveness. Unraveling this issue remains a key challenge. The complexity of signal transduction scaffolds and the varying distribution of GPCR interacting proteins implies that simple heterologous expression systems may not provide a useful assessment of the significance of inverse agonism in a clinical setting.

Acknowledgments

I thank all the participants of the Esteve Foundation meeting on inverse agonism held in S'Agaro, Spain, 3–6 October 2002 for their insights into this topic.

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