MINIREVIEW

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

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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 (Daeflfler 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 agonist 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

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.
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.

<table>
<thead>
<tr>
<th>Generic Name (Trade Name)</th>
<th>Therapeutic Area</th>
<th>Receptor Target</th>
<th>Inverse Agonist?</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Olanzapine Antipsychotic</td>
<td>5-HT2C/5-HT2A/others</td>
<td>Yes</td>
<td>Herrick-Davis et al., 2000</td>
<td></td>
</tr>
<tr>
<td>Losartan Cardiovascular</td>
<td>AT1</td>
<td>Yes</td>
<td>Groblewski et al., 1997, Miserey-Lenkei et al., 2002</td>
<td></td>
</tr>
<tr>
<td>Risperidone Antipsychotic</td>
<td>5-HT2/dopamine D2,D3</td>
<td>Yes</td>
<td>Vanhauwe et al., 1999; Herrick-Davis et al., 2000</td>
<td></td>
</tr>
<tr>
<td>Fexofenadine Respiratory</td>
<td>histamine H1</td>
<td>Probably</td>
<td>Leurs et al., 2002</td>
<td></td>
</tr>
<tr>
<td>Clopidogrel Thrombosis</td>
<td>P2Y12</td>
<td>Unclear</td>
<td>Conley and Delaney, 2003</td>
<td></td>
</tr>
<tr>
<td>Valsartan Hypertension</td>
<td>At1</td>
<td>Unclear</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Montelukast Respiratory</td>
<td>CysLT1</td>
<td>Unclear</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Loratidine Respiratory</td>
<td>Histamine H1</td>
<td>Probably</td>
<td>Leurs et al., 2002</td>
<td></td>
</tr>
<tr>
<td>Quetiapine Anti-Psychotic</td>
<td>Dopamine D2/5-HT2C/5HT2A</td>
<td>Unclear</td>
<td>Rauser et al., 2001</td>
<td></td>
</tr>
<tr>
<td>Cetirizine Respiratory</td>
<td>Histamine H1</td>
<td>Probably</td>
<td>Leurs et al., 2002</td>
<td></td>
</tr>
<tr>
<td>Metoprolol Cardiovascular</td>
<td>1-Adrenoceptor</td>
<td>Yes</td>
<td>Engelhardt et al., 2001; Levin et al., 2002</td>
<td></td>
</tr>
<tr>
<td>Tolterodine Genitourinary</td>
<td>Muscarinic M3/M2</td>
<td>Unclear</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Famotidine Gastrointestinal</td>
<td>Histamine H2</td>
<td>Yes</td>
<td>Alewijnse et al., 1998</td>
<td></td>
</tr>
</tbody>
</table>

See also, Cropley et al. (2002) for 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. (see below).

Both the GPCR of interest to the total high-affinity GTPase can be measured 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 had been utilized or to the agonist-protein interaction. 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 β2-adrenoceptor compared with the β1-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 β2-adrenoceptor to a high degree via a heart-specific promoter. Isolated atria from the transgenic mice displayed isotropic 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 β2-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 adenyl 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 that 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 malfunction 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 (Pleikoff et al., 1997). Both the US28 and US33 GPCRs also display constitutive activity when expressed in heterologous systems, and a nonpeptidic 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 case 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 [3H]arachidonic acid release, whereas thioperoxamide 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 [3H]histamine release or basal and ligand-regulated guanosine 5′-O-(3-[35S]thio)triphosphate binding in rat brain membranes. In both assays, proxyfan reversed both the agonist effects of imetit and the inverse agonist effects of thioperoxamide 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 (Anu 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 homr 3 but not other members of the homr family (Anu et al., 2001). After treatment of such cells with antisense oligonucleotides targeting homr 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 (Anu 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 β1- and β2-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 receptors, involving Homer proteins, and the constitutive activity of their cognate GPCRs. This suggests a novel mechanism for regulating the extent of constitutive activity of these receptors in vivo.
Constitutive Activity and Inverse Agonists of GPCRs

Partial 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.

References


Becamel C, Alonso G, Galeotti N, Demey E, Verzijl D, Timmerman H, and Leurs R (2003) β2-adrenoceptor is significantly more constitutively active in primary rat ventricular myocytes, compared with the coexpressed β1-adrenoceptor (Rynin 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 internalization of these two adrenoceptors. There are varying views on whether secretion 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 β2-adrenoceptor compared with the β1-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 β1- and the β2-adrenoceptor, with the long isoform of Gα12 (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 β2-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

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