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# Novel approaches to enhance the detection of receptor constitutive activity and inverse agonists

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**Abstract**

Many wild-type G-protein-coupled receptors display relatively low levels of constitutive activity and thus identification of ligands with inverse agonist activity can be difficult. Two assays are available that monitor receptor interaction with a G protein, GTPase activity assays and [$^{35}$S]GTPγS binding assays. GTPase assays are, however, frequently limited by high background. The fraction of membrane GTPase activity contributed by the constitutive activity of a receptor can be enhanced by addition of a recombinant regulator of G protein signalling protein to the assay. Detection of inverse agonists is then improved markedly. [$^{35}$S]GTPγS binding assays for the detection of inverse agonists have historically been limited to receptors that activate pertussis toxin-sensitive G proteins. Introduction of a selective immunoprecipitation step using a G protein subtype specific antiserum allows this approach to be used for all G protein families. Application of these novel approaches, combined with the use of receptor–G protein fusion proteins to optimise receptor to G protein information transfer allows quantitative analysis of the extent of enhancement of constitutive activity produced by mutation of receptor or G protein.


*Keywords:* Inverse agonist; G-protein-coupled receptor; G protein; Regulator of G protein signalling

## 1. Introduction

It is likely that very few compounds that bind to G-protein-coupled receptors (GPCRs) in a competitive fashion with the natural ligand(s) will be entirely lacking in efficacy.

*Abbreviations:* GAP, GTPase-activating protein; GPCR, G-protein-coupled receptor; RGS, regulator of G protein signalling.

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However, despite the current fascination to describe as 'inverse agonists' a great number of ligands that have historically been defined as 'antagonists', many systems are far from ideal for measurement of such inverse activity. There are a number of GPCRs that display a high degree of ligand-independent, or constitutive, activity. However, this is not the norm and the constitutive ability of many GPCRs to stimulate their cognate G proteins is generally modest compared to the level of activation achieved in the presence of the natural ligand. To overcome this, many researchers have resorted either to expressing high levels of a native GPCR or to mutating GPCRs to enhance the level of constitutive activity. In the former case, this has often been required to produce a level of signal that is enhanced sufficiently over background to allow detection of the effects of an inverse agonist. In the latter case, this aim is supplemented by a desire to identify regions that contribute to, and to understand conformational changes in the receptor associated with, agonism and information transfer from GPCR to G protein.

One approach to amplify the signal of a GPCR is to monitor its effects at a point distal to G protein activation and hence allow significant amplification of the constitutive signal. Although such measurements, whether at the level of second messenger production or the regulation of gene transcription, can provide high sensitivity, they are far removed from the initial GPCR–G protein interaction that inherently provides the most accurate and suitable reporter. Two direct measures of G protein activation can be made. As the exchange of GDP bound to the G protein α subunit for GTP is the rate-limiting step in the cycle of G protein stimulation and deactivation, then activation can be monitored either by direct assessment of the exchange process or at the subsequent hydrolysis of GTP. Both of these processes have been used to assess GPCR constitutive activity and the effects of inverse agonists. However, both have technical limitations. A series of improvements to each approach is suggested.

## 2. Materials and methods

### *2.1. GPCR–G protein α subunit fusions*

A major issue in pharmacology is that most of the models that usefully predict the effects of ligands on GPCR–G protein interactions are based on assumptions that the concentration of GPCR is at least equal to that of the G protein. Even when aspects of compartmentalisation of such polypeptides into specific membrane subdomains [1] are taken into account, it is clear that the levels of expression of G proteins are routinely far higher than any individual GPCR [2]. This may have marked benefits for amplification of signal transduction processes but is not ideal for testing various concepts derived from such models. Furthermore, details of features such as efficacy and potency of ligands are affected by varying stoichiometry of GPCR to G protein [3]. For a number of years, fusion proteins have been studied in which the N-terminus of a G protein α subunit is linked in frame to the C-terminal tail of a GPCR from which the stop codon has been eliminated [4,5]. Such constructs have been used to explore aspects of information transfer from GPCR to G protein under conditions in which the stoichiometry of the two polypeptides and their relative orientation was fixed [6]. This has allowed detailed analysis of the effects

of mutations in both receptors and G proteins. Importantly, such constructs are able to interact with and release the G protein β/γ complex [7] and accessory polypeptides such as regulators of G protein signalling (RGS) proteins [8,9].

### 2.2. GTPase assays

There are four distinct families of heterotrimeric G proteins. Certain GPCRs have the capacity to activate members of more than one family. However, in general, GPCRs display selectivity in G protein activation to ensure fidelity of signal transduction. Most mammalian cell membranes express a considerable range of the G proteins. Thus, although the concept of measuring the enhanced rate of GTP hydrolysis of such G proteins attributable to the constitutive activity of a single GPCR is simple [10], it is regularly frustrated by basal GTP hydrolysis by the G protein population and by the action of other polypeptides that can hydrolyse this nucleotide. As RGS proteins function to accelerate the hydrolysis of GTP by G protein α subunits [11], they should do so for GTP loaded by the constitutive activity of a GPCR as well as that produced by agonists. Basic enzyme kinetics defines that the action of polypeptides, such as RGS proteins, with GTPase-activating protein (GAP) activity, will increase the observed $K_m$ for GTP at the G protein as well as stimulate $V_{max}$ [8]. However, when measuring the action of agonists on GTP hydrolysis in many membrane preparations, enzyme kinetics indicate that agonists stimulate GTPase $V_{max}$ without altering the $K_m$ for GTP. The obvious conclusion from such results is that the membrane preparations are lacking in relevant GAP activity.

The extreme C-terminus of G protein α subunits is a key contact site for GPCRs [12]. All pertussis toxin-sensitive G proteins have a cysteine residue four amino acids from the C-terminus and this is the target for pertussis toxin-catalysed ADP ribosylation. We have constructed fusion proteins between either the 5-$HT_{1A}$ receptor [13–15] or the δ opioid receptor [16,17] with forms of $G_{i1}\alpha$ in which the pertussis toxin-sensitive cysteine was replaced with either isoleucine or glycine. We have noted that the receptors form a greater proportion of high affinity agonist ternary complexes with the form of $G_{i1}\alpha$ with isoleucine at this position [15,17]. For the 5-$HT_{1A}$ receptor, this is associated with enhanced constitutive information transfer from GPCR to G protein [13] and this results in elevated basal GTPase activity. Addition of recombinant forms of either RGS1 or RGS16 further enhances this activity [14]. As this enhanced activity reflects constitutive activity of the $5HT_{1A}$ receptor, this increases the amount of GTPase activity that can be inhibited by ligands possessing inverse agonism. Indeed, this provides a useful increase in sensitivity of detection of inverse agonists and their resolution from neutral antagonists and ligands with weak positive efficacy [14]. Even more usefully, when the 5-$HT_{1A}$ receptor was fused to a cysteine to isoleucine mutant of $G_{o1}\alpha$, basal GTPase activity in membranes expressing this construct was increased to a substantially greater extent by addition of recombinant RGS1 [14]. This allowed greatly improved sensitivity of detection of inverse agonists at the 5-$HT_{1A}$receptor (Fig. 1). There is evidence that RGS proteins only function as GAPs for G proteins activated by particular GPCRs [18,19]. However, it would appear that this basic alteration to the assay format can provide a simple means to enhance the sensitivity and precision of analysis of the action of inverse agonists at many GPCRs.

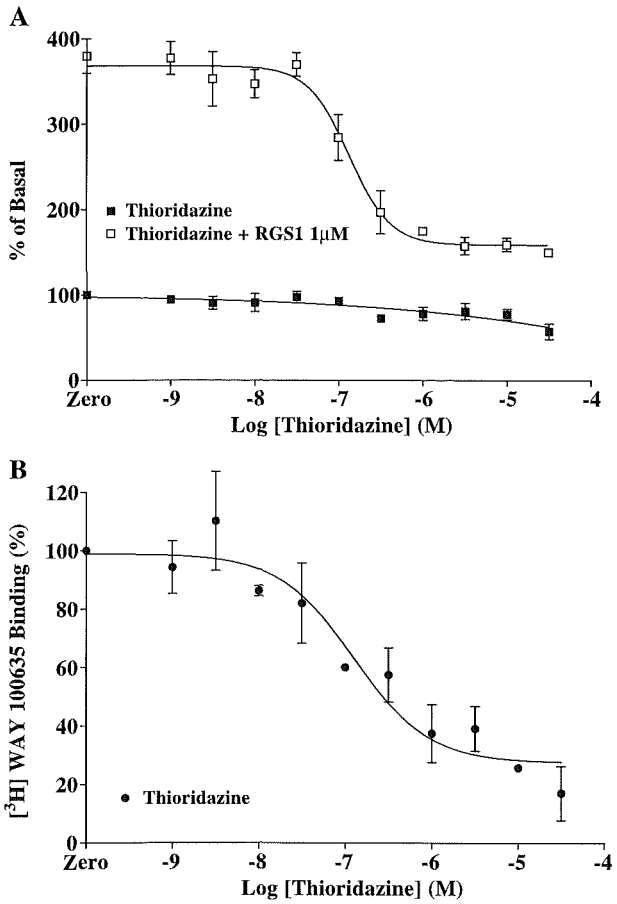


Fig. 1. Enhanced detection of the inverse agonist properties of thioridazine at the $5HT_{1A}$ receptor in the presence of regulator of G protein signalling 1. Membranes were generated from pertussis toxin-treated HEK293 cells stably expressing a $5HT_{1A}$ receptor–$(Cys^{351}Ile)G_{o1}\alpha$ fusion protein. (A) High affinity GTPase activity, presented as % of basal activity in the absence of RGS or ligand, was assessed in the absence (filled symbols) or presence (open symbols) of recombinant RGS1 (1 μM) and varying concentrations of thioridazine. (B) The ability of varying concentrations of thioridazine to compete with [$^3$H]WAY 100635 for binding to the $5HT_{1A}$ receptor–$(Cys^{351}Ile)G_{o1}\alpha$ fusion protein was assessed.

## 2.3. *[$^{35}$S]GTPγS binding studies*

By far, the most popular means to monitor guanine nucleotide exchange on G protein α subunits is to measure the binding of [$^{35}$S]GTPγS [20]. As an analogue of GTP that is resistant to GTPase activity, this nucleotide is generally added as a tracer because it is available radiolabelled to high specific activity. Although conceptually suited to analysis of the activation of all classes of heterotrimeric G proteins, in practise it has been restricted, until recently, to the $G_i$ family. As with GTPase assays, this reflects that the relatively high basal guanine nucleotide exchange rate of this group and their high levels

of expression swamps the signals from other G proteins. Thus, even a significant increase in nucleotide exchange on other G proteins can be difficult to observe above this background.

This has been overcome to a large degree by incorporating a selective immunoprecipitation step into the assay [21–23]. Selective antisera able to immunoprecipitate various G protein α subunits are available and these can be used to eliminate the binding of [$^{35}$S]GTPγS to other G proteins and unrelated polypeptides. Furthermore, combinations of the expression of GPCR–G protein fusions incorporating receptors containing mutations that enhance constitutive activity with selective immunoprecipitation can provide a solid platform for analysis of inverse agonists. For example, immunoprecipitation of a fusion protein between the $\alpha_{1b}$-adrenoceptor and the α subunit of $G_{11}$ following a [$^{35}$S]GTPγS binding assay results in very few counts being present in the immunoprecipitate [7,24,25]. This, however, is increased some 30-fold by inclusion of the $\alpha_1$-adrenoceptor agonist phenylephrine in the assay [24,25]. Such results provide an excellent means to monitor activation of the G protein by the GPCR but also indicate that the $\alpha_{1b}$-adrenoceptor has only low levels of constitutive activity. It is thus impractical to determine if ligands are inherently inverse agonists using this construct. A large number of mutations have been identified that increase the level of constitutive activity of this GPCR [26]. However, many of these mutations have large effects on steady state expression levels of the receptor. By incorporating such mutations into $\alpha_{1b}$-adrenoceptor–$G_{11}\alpha$ fusion proteins and then measuring levels of expression of each construct, we have been able to add the same amount of each construct to [$^{35}$S]GTPγS binding assays [25]. Previously characterised receptor mutations do indeed elevate levels of nucleotide binding in the absence of ligand. However, in general, these studies do not indicate that the extent of constitutive activity is as high as when analysed using a downstream measure, such as [$^{3}$H]inositol phosphate production [25]. The elevated levels of [$^{35}$S]GTPγS binding can be inhibited by the presence of an inverse agonist and the extent of reduction provides a measure of inverse efficacy [25].

The vast majority of commercially available anti-G protein antisera are targeted against the extreme C-terminal sequences. As mutation of the tyrosine located four amino acids from the C-terminus of $G_{11}$ and $G_q$ prevents effective recognition and immunoprecipitation by such antisera, it was impossible to analyse the effect of such mutations on the efficiency of information transfer between the $\alpha_{1b}$-adrenoceptor and $G_{11}$ without employing the fusion strategy. However, following fusion of the $\alpha_{1b}$-adrenoceptor to mutated forms of $G_{11}$, the polypeptides could be recovered subsequent to a [$^{35}$S]GTPγS binding assay by using an antiserum directed towards the N-terminus of the receptor [7]. Use of epitope-tagged GPCRs could extend this basic approach to any GPCR–G protein combination, even when an anti-G protein antiserum effective for immunoprecipitation is not available. As the stoichoimetry of GPCR to G protein remains 1:1 for any combination of mutation in the two polypeptides or for two distinct G proteins linked to the same receptor, it is then possible to analyse G protein selectivity for GPCRs. For example, we have shown a marked preference for the δ opioid receptor to activate $G_{i1}\alpha$ compared to $G_{o1}\alpha$ and that the activation of $G_{i1}\alpha$ by the δ and μ opioid receptors is equivalent [16].

We have also extended this basic strategy to allow analysis of the activation of $G_s\alpha$ by wild-type and mutated forms of the $\beta_1$- and $\beta_2$-adrenoceptors [27]. Again, effective

analysis of the inverse agonist properties of ligands at these GPCRs has required introduction of activating mutations into these receptors. These GPCRs have been of particular interest in the analysis of constitutive activity and inverse agonism because of their expression in cardiac tissue. A range of studies have overexpressed them in transgenic mice and related this to the development of disease and the use of β-blockers in the treatment of congestive heart failure.

## 3. Conclusions

Measures of guanine nucleotide exchange on G protein α subunits have been performed for many years as a means to assess their activation. Although historically these have concentrated on the $G_i$ family because of issues of sensitivity, variations in the approaches used have opened up use of these conceptually and technically simple assays to other classes of G proteins. It is likely that these modifications will be adopted widely and provide a useful addition to the suite of approaches used to detect and analyse inverse agonism.

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**Discussion 2**

G. Milligan

There is an area that is maybe worth opening up into the wider discussion, essentially clinical efficacy versus inverse agonism. Have we really just got correlations, or is it a requirement? Because clearly, there's some quite interesting big studies on the β-blocker field, which Martin Lohse may be able to comment on. How relevant inverse agonism has been versus the actual clinical efficacy of the β-blockers that have been into the clinic?

M. Lohse

I would love to say that it is important, but to be frank, I don't think so. That's because the $\beta_1$-receptors are not really an example of receptors where constitutive activity is the major effect. The constitutive activity is so small, that I think it doesn't matter whether a compound is an inverse agonist or not. What I think we do know is that partial agonism at this receptor is bad, and all the trials that have failed in using β-blockers for heart failure were trials with compounds that probably have some degree of partial agonist activity.

G. Milligan

But that then does extend the discussion slightly to almost ask the question: Are there any neutral ligands, or do all ligands possess some degree of efficacy, it's just our capacity to measure them effectively? As we've heard from Tommaso Costa's talk, in the old days, most of us would probably have thought of alprenolol as a purely neutral ligand. But as we've got better and better at measuring these things, those compounds just tend to come out slightly on the positive side of baseline. I mean, does anyone have any thoughts simply that they truly have a neutral ligand?, and if they do have, what it actually means for the binding characteristics of that ligand to the receptor?

R. Bond

I agree that there probably is no such thing as a neutral ligand, that it's our ability to discriminate. But I also don't think that, whether it's an agonist or an inverse agonist, is a quality of the ligand. It's certainly dependent on the system which you measure. I can well believe that alprenolol may be an inverse agonist in some systems, and an antagonist in others. In heart failure you have a unique situation, but it does argue whether inverse agonism as it matters. The $\beta_1$-receptors are down-regulated and uncoupled, and the $\beta_2$-receptors are not down-regulated, they're uncoupled. You have a relative shift in the proportion, where in the heart failure patient, maybe half the population is $\beta_2$, and if you don't discriminate between $\beta_1$ and $\beta_2$-receptors, there probably is a correlation between inverse agonism and therapeutic efficacy. I'll just say: ligand efficacy of β-receptors in the heart, just a general statement of that nature.

C. Maack

I agree with you on that point. One possible way to estimate the inverse agonist activity of a β-blocker in heart failure patients is if you take a look at their heart rate during the nighttime. This is the time when sympathetic activation is much lower than during the daytime. Thus, a partial agonist (e.g. xamoterol) displays its partial agonist activity especially by increasing heart rate at night, while during the day, this partial agonist still reduces heart rate probably since its intrinsic efficacy is lower than that of endogenous catecholamines. But inverse agonists like metoprolol or carvedilol, which do have

beneficial effects in heart failure, decrease heart rate during both day- and nighttime. Interestingly, bucindolol, a β-blocker that did not reduce mortality in heart failure patients, did neither increase nor decrease heart rate at night. Accordingly, in vitro experiments indicate that this β-blocker is either a weak partial agonist or weak inverse agonist, depending on the tissue investigated. Thus, I believe there may be a correlation between the effects of a β-blocker on heart rate and the effects on survival that may correlate with the degree of inverse agonist activity. This would mean that inverse agonism is desirable and partial agonism adverse in the treatment of heart failure.

P. Kitabgi

We have an example of a neutral ligand for the neurotensin receptors subtype 2. What is very weird in this case is that the neutral antagonist is the only known natural ligand of the receptor, that is neurotensin itself. When we express the human neurotensin subtype 2 receptor in cells, it has constitutive activity. Neurotensin behaves as a neutral antagonist because it can reverse both the effect of inverse agonist and of agonist. Of course, I don't know the physiological significance or relevance of these findings.

G. Milligan

There are receptors coming through in the literature now that have started to just diverge away from the native receptor. Maybe the one that's going to come through very soon now is the class of receptors for small aliphatic acids, like propionic acid, where there are closely related receptors that appear to be nonfunctional. So you don't think the situation with the neurotensin 2 is it's actually almost becoming a pseudogene through divergence over time?

P. Kitabgi

I don't think so if you mean that it would be a receptor without functional significance. This receptor is found in all the different species that I've looked at. Actually we have good indication that the well-known analgesic effect of centrally administered neurotensin is mediated through this receptor.

T. Schwartz

The virally encoded chemokines receptors have very high constitutive activity. For example the ORF74 receptor that binds a series of difference CXC-chemokines. They form three groups where some of them are beautiful agonists and others are inverse agonists, and then you have the last group that are actually neutral ligands. All the CXC-chemokines bind round 1 nM affinity, whereas the classical inflammatory chemokines are neutral ligands in the way they don't do anything, and they don't act as antagonists, which is really interesting.

G. Milligan

But presumably that reflects quite a distinct binding site.

T. Schwartz

Which is going to be really hard, because these are pretty big proteins and they have interactions. It probably reflects the fact that the receptor can be found in different sub-populations, and they bind to a different sub-population, they might not reflect the same one.

G. Milligan

I don't think anyone's really going to talk about this topic at the meeting, but we are well aware of examples of polymorphic variance in receptors leading to differences in

agonist efficacy or potency. Do we know of any examples from the work around the groups about that being true for inverse ligands, as well?

R. Leurs

There is a paper in the $H_2$ field. Although first of all I have to make a comment that the polymorphic variant studies is under fire if this is really occurring in the population or not. In this case, a Japanese group is going after the phenotype of these receptors, and they seem to lose constitutive activity. Some of you might know that $H_2$ receptor in its wild-type form is highly constitutively active, and with the inverse agonism you have up-regulation. And in this case what they show is that you lose constitutive activity and you lose the up-regulation with the ligands again. In that paper they would like to study, in fact, gastric acid secretion in the population. However, in their Japanese population, they couldn't find the polymorphic variant, so that was the end of the story.

G. Milligan

I think too often when we go to some public databases, the supposed polymorphisms that are in there are generally sequencing errors rather than true polymorphisms. Fortunately, some of the rather better databases that are available in companies are a little more trustworthy, but you can spend a lot of time messing around with supposed polymorphisms just by reading the literature to find that actually they come from high-level sequencing that maybe hasn't been terribly well controlled.

H. Giles

In my experience, I have never come across a neutral antagonist yet, across any of those GPCR systems. If you look hard enough, you can make them behave either as low efficacy partial agonists or you can see some inverse agonists properties, and I've not seen any exceptions to that yet.

G. Milligan

It just struck me conceptually that if something is going to bind to a receptor, it must do something to it as well as just bind. I would just be surprised if you couldn't set up an assay that you could determine efficacy in one direction or another.

P. Kitabgi

I'm coming back to this neurotensin receptor 2. We have evidence that neurotensin, in the systems we've been studying, is a neutral ligand. We can't see any positive or negative efficacy of the peptides. Now, if this were true in vivo, how could it work? Now, you can hypothesise that there exists, in vivo, a natural agonist for this receptor, and even a natural inverse agonist. And that you can get a sort of complex regulation of the systems by the interplay between these three different ligands—the agonist, the inverse agonist and the neutral ligand. And in this case the neutral ligand will show its activity by interacting either with an agonist or an inverse agonist. Of course, this is only speculation, but it gives room for neutral agonists in the real world.

G. Milligan

I wouldn't disagree with that, and obviously taking on Thue Schwartz's point, where clearly some of the chemokines can compete effectively at the same receptor and show agonism and inverse agonism, and maybe indeed, neutral that might well be true.

W. Clarke

I think that it's very important to recognise that receptors couple to more than one signalling cascade inside cells, and that whether or not one calls a ligand an agonist, an

inverse agonist, partial agonist or neutral will depend on the response measured—even at the same receptor in the same cell—the ligand's characteristics can be different. I think we also have to be aware of the time parameter, that even in the same cell with different effector mechanisms studied under different conditions or after its previous exposure to a ligand, we can see differences in the quality of what a ligand does, whether it's an agonist or an inverse agonist.

G. Milligan

Maybe one of the points is that clearly a number of GPCRs now are well known to send signals that don't require activation of the G protein. And it may well be that different end points there would give us quite different pharmacologies. We may see some molecules actually inhibiting one pathway but acting as an agonist at a second.

R. Adan

If we make membranes from tissues, from wild-type animals, and we're measuring GTPγS binding, what effect are we measuring? Is this always measuring constitutive receptor activity? Is it really showing us the existence of endogenous relevance for constitutive activity or not?

G. Milligan

I certainly don't think it is, if all you do is measure GTPγS binding. Because there are clearly many other proteins that are not traditional G proteins that will bind GTPγS. I think if you then pull out the G protein and show indeed that it was loaded, then I think that will show you constitutive activity.

R. Adan

But in the membrane preparation you can imagine that you don't have these small GTP binding proteins.

G. Milligan

I think there's still many GTP binding proteins in what many of us would be happy to describe as our plasma membrane preparation.