BJP BRITISH JOURNAL OF PHARMACOLOGY
BPS BRITISH PHARMACOLOGICAL SOCIETY

BJP British Journal of Pharmacology

DOI:10.1111/j.1476-5381.2010.00963.x
www.brjpharmacol.org

Themed Section: Analytical Receptor Pharmacology in Drug Discovery

REVIEW

Use of the GTPγS ([^{35}S]GTPγS and Eu-GTPγS) binding assay for analysis of ligand potency and efficacy at G protein-coupled receptors

Philip G. Strange

School of Pharmacy, University of Reading, Whiteknights, Reading, UK

Correspondence
Philip G. Strange, School of Pharmacy, University of Reading, Hopkins Building, Whiteknights, Reading RG6 6UB, UK. E-mail: p.g.strange@rdg.ac.uk

Keywords
agonist; antagonist; inverse agonist; functional assay; GTPγS binding assay; efficacy; drug screening

Received
26 February 2010
Revised
10 May 2010
Accepted
1 July 2010

In this review I consider assays for G protein-coupled receptor (GPCR) activity based on the binding of labelled analogues of GTPγS ([^{35}S]GTPγS or Eu-GTPγS) to G proteins in tissues (GTPγS binding assays). Such assays provide convenient measures of GPCR activity close to the receptor in the signalling cascade. In order to set up a GTPγS binding assay, the requirements of the assay must be considered. These are tissue source, GTPγS analogue, G protein, GDP, Mg^{2+}/Na^{+} ions, saponin, incubation time. The assay, once optimized, can be used to generate concentration/response curves for GPCRs signalling via $G_{i/o}$ proteins (or to other G proteins with a modified assay) and actions of agonists, inverse agonists and antagonists may, in principle, be assessed. For agonists and inverse agonists, data for the maximal agonist effect, the concentration of ligand giving a half-maximal response and the Hill coefficient may be derived. For antagonists, data for the equilibrium dissociation constant can be obtained. The mechanistic basis of the assay is considered. Although the assay can be used to profile ligands, under the conditions it is used, it may not be measuring the same event that determines GPCR action in cells.

LINKED ARTICLES
This article is part of a themed section on Analytical Receptor Pharmacology in Drug Discovery. To view the other articles in this section visit http://dx.doi.org/10.1111/bph.2010.161.issue-6

Abbreviations

ARG, agonist/receptor/G protein complex; E_{max}, maximal agonist effect; EC_{50}, concentration of ligand giving a half maximal response; GPCR, G protein-coupled receptor; n_H, Hill coefficient; SPA, scintillation proximity assay

Given the importance of G protein-coupled receptors (GPCRs) as sites of action of current and potential future drugs, there is much interest in the development of simple flexible assays for the functional actions of drugs at these receptors. This is of particular relevance for development of agonists as drugs whether these are full or partial agonists. Also many currently used drugs are inverse agonists and it may be important to detect this activity in new drug candidates.

For GPCRs, their commonly accepted mechanism of action comprises formation of an agonist/receptor/G protein ternary complex (ARG) in which bound GDP is replaced by GTP leading to activation or deactivation of downstream signalling proteins such as adenylyl cyclase (Figure 1A). GTP binding is therefore a key step in the activation of the system and the first event following receptor activation. The realization that GTP binding is important for GPCR signalling lead to efforts to exploit this event as an assay for GPCR activation. The availability of labelled GTP analogues that are poorly hydrolysable ([^{35}S]GTPγS, Eu-GTPγS) allowed the development and application of this assay. Whereas in the case of GTP, the binding of this nucleotide to G proteins is followed by hydrolysis to GDP, for the poorly

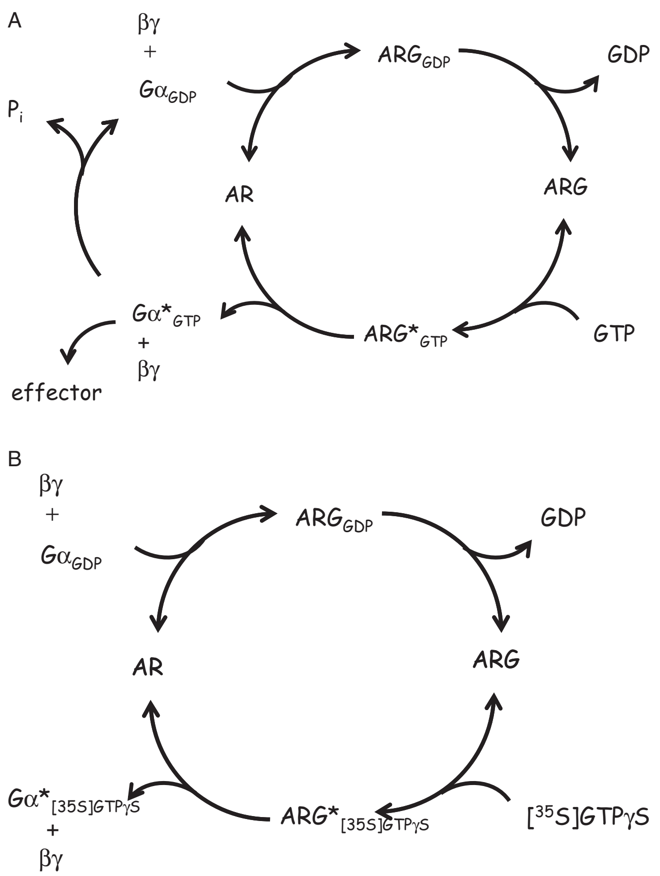

Figure 1

The G protein cycle. In panel A, the cycle is shown in the currently accepted form under cellular conditions ([GTP] ~50 μM). It should be noted that for G_i proteins the receptor/G protein (RG) complex may not dissociate into subunits (Bunemann *et al*., 2003; Frank *et al*., 2005). In panel B, the cycle is shown in the presence of a poorly hydrolysable analogue of GTP ($[^{35}S]$GTPγS), which increases the lifetime of the ARG.$[^{35}S]$GTPγS or Gα.$[^{35}S]$GTPγS species. The levels of GTPγS typically employed (~0.1 nM in the $[^{35}S]$GTPγS binding assay) are very low compared with GTP levels under cellular conditions.

hydrolysable GTP analogues the bound nucleotide is resistant to hydrolysis and the lifetime of the nucleotide-bound G protein is increased (Figure 1B). This allows the bound GTP analogue to be analysed.

The assay that has emerged from these considerations is superficially very simple and entails mixing a tissue sample, usually membranes containing the GPCR of interest, with GTPγS bearing a suitable label ($[^{35}S]$ or Eu), incubating the mixture and analysing the bound nucleotide. The assay then allows agonist stimulation of nucleotide binding to G proteins to be determined and this, in principle, allows assessment of GPCR activation close to the receptor with little amplification of signal. In practical terms the assay is attractive as it provides a measure of GPCR function but is performed in a manner similar to ligand binding assays thus avoiding the complexities inherent in other downstream assays.

Early use of the $[^{35}S]$GTPγS binding assay

Once it was recognized that a key aspect of G proteins was their ability to bind GTP, the binding of $[^{35}S]$GTPγS was used in the characterization of G proteins [see e.g. (Bokoch *et al*., 1984; Sternweis and Robishaw, 1984; Higashijima *et al*., 1987)]. Subsequently agonist-stimulated binding of $[^{35}S]$GTPγS to pure G proteins reconstituted with pure GPCRs was used to probe mechanisms of GPCR activation (Asano *et al*., 1984; Kurose *et al*., 1986). These assays use pure G proteins and pure GPCRs and so are not easily applicable to drug screening assays. The development of assays for agonist-stimulated $[^{35}S]$GTPγS binding to G proteins in membranes has, however, enabled these assays to become widely used in the context of drug screening.

One of the first descriptions of a membrane-based $[^{35}S]$GTPγS binding assay was for muscarinic acetylcholine receptors in cardiac membranes (Hilf *et al*., 1989). In this study, porcine atrial membranes were used and carbachol-stimulated $[^{35}S]$GTPγS binding was studied. Several important attributes of the $[^{35}S]$GTPγS binding assay were described in this early study that are now known to be applicable to the assay in general. It was shown therefore that the assay could be used to generate agonist concentration–response curves but that there was an absolute requirement for Mg^{2+} ions to see agonist stimulation. Also a second guanine nucleotide (preferably GDP) and addition of Na^+ ions were required for a favourable stimulated to basal ratio. In order to observe a robust agonist stimulation of $[^{35}S]$GTPγS binding over the basal level, it was necessary to purify the cardiac membranes. About 75% of the carbachol-stimulated $[^{35}S]$GTPγS binding was sensitive to *Pertussis* toxin treatment of the membranes, indicating an important role for $G_{i/o}$ proteins. The predominant muscarinic receptor in the atrium is the M_2 receptor coupled to G_i (Kitazawa *et al*., 2009), consistent with the *Pertussis* toxin sensitivity of a large part of the $[^{35}S]$GTPγS binding.

Current status of GTPγS binding assays

In the following discussion of the assay, I shall use the term GTPγS where I refer to generic aspects of the assay but this denotes either of the two poorly hydrolysable labelled forms of GTPγS ($[^{35}S]$GTPγS and Eu-GTPγS).

The GTPγS binding assay is typically run in the same way as a ligand binding assay where membranes expressing a receptor are mixed with labelled GTPγS under agonist stimulation and the bound GTPγS determined. The apparent simplicity of the assay belies some complexity and in this section I shall describe the current status of the GTPγS binding assay with respect to its practical use. I shall not give extensive technical details as these may be found in recent reviews (Labrecque *et al.*, 2009; Kara and Strange, 2010).

Tissue sources for GTPγS binding assays

GTPγS binding assays may be performed in various different tissues although the most popular tissue source, especially in industry, is membranes from recombinant cells, for example, CHO or HEK cells expressing a single cloned receptor. The expression level of the receptors is typically ~1 $pmol \cdot mg^{-1}$ protein or greater. Under these circumstances such a system can provide a robust response from the recombinant receptor usually with a good signal/background ratio (see below for examples). The signal/background ratio will, however, depend on the receptor concerned, its expression level and the assay conditions (see below) and can be variable in magnitude.

GTPγS binding responses have also been recorded using membranes derived from native tissues. This has been achieved for some receptors where there is a strong response from the receptor concerned and the basal response from the tissue is low. Examples of this include 5-HT_{1A} serotonin receptors in hippocampal membranes (Newman-Tancredi *et al.*, 2003; Martel *et al.*, 2007), CB_1 cannabinoid receptors in cerebellar membranes (Breivogel *et al.*, 1998) and CXCR3 chemokine receptors in activated T cells (Heise *et al.*, 2005). Sometimes a more favourable signal/basal ratio in GTPγS binding assays can be achieved in assays using brain membranes by suppressing effects of adenosine at A_1 receptors by addition of an inverse agonist, for example, DPCPX [see e.g. (Horswill *et al.*, 2007)].

Assays using membrane preparations suffer from the limitation that many components of the intracellular machinery may have been lost. Assays have therefore been developed using intact cell preparations. The assay has been used in an autoradiographic format to record [^{35}S]GTPγS responses in brain slices, for example, α_2 adrenoceptors (Newman-Tancredi *et al.*, 2000), 5-HT_{1A} serotonin receptors (Newman-Tancredi *et al.*, 2003), μ opioid receptors (Sim *et al.*, 1996). [^{35}S]GTPγS responses have also been recorded from whole cells (recombinant and native) expressing a GPCR after permeabilization with digitonin or saponin to allow the labelled guanine nucleotide to enter cells (Wieland *et al.*, 1995; Alt *et al.*, 2001; Breivogel *et al.*, 2004). Assays using whole cells have the obvious advantage that the intracellular machinery is intact and more valid comparisons of the activities of compounds may be made with other assays using whole cells such as neurotransmitter release assays [see e.g. (Breivogel *et al.*, 2004)]. In this latter study on rat cerebellar granule cells in culture the contribution of adenosine A_1 receptor activation to the basal level of [^{35}S]GTPγS binding was suppressed by addition of adenosine deaminase.

Which labelled guanine nucleotide?

The vast majority of GTPγS binding assays have been conducted using the radioactive version of the nucleotide, [^{35}S]GTPγS. This provides a very convenient assay with easy detection of signal by rapid filtration of membranes on glass fibre filters to separate bound and free nucleotide followed by liquid scintillation counting. Particularly in the industrial context, the assay has been adapted as a scintillation proximity assay (SPA) thus eliminating the need for a separation of bound and free nucleotide (DeLapp, 2004; Johnson *et al.*, 2008). SPA beads coated with wheat germ agglutinin (WGA) are used and the membranes bearing bound [^{35}S]GTPγS bind to the WGA-SPA beads enabling detection of the bound [^{35}S]GTPγS. This SPA-based assay has been adapted to detect stimulation of individual G proteins (DeLapp, 2004). This assay format requires SPA beads coated with anti-IgG antibodies and capture of G protein with bound [^{35}S]GTPγS using specific antibodies. The assay is then a single tube assay without separation when the antibody/G protein/[^{35}S]GTPγS complex associates with the anti-IgG-bound SPA bead. The assay has been used for recombinant and native systems (DeLapp *et al.*, 1999; Cussac *et al.*, 2002; DeLapp, 2004; Salah-Uddin *et al.*, 2008).

[^{35}S]GTPγS is typically used at a concentration of 100 pM in routine assays although we have found that a lower concentration (e.g. 50 pM) can sometimes improve signal/background levels for GPCRs giving only moderate signals. These low concentrations of nucleotide are entirely non-physiological (see below) but are necessary in order to measure the stimulation of GTPγS binding over the basal signal. In all the radioactive assays, the decay of the ^{35}S label needs to be taken into account but the labelled nucleotide is reasonably stable if stored appropriately.

A version of GTPγS labelled with the time-resolved fluorescent metal Europium (Eu-GTPγS) has been produced. The Eu label has been used to derive single tube assays for some GPCR-related activities, for example, in the time-resolved FRET-based assay for cAMP. In the case of the Eu-GTPγS binding assay, however, there is still a separation step, performed using 96 well filter plates, but the assay has the advantage of being non-radioactive. The Eu-GTPγS binding assay has not been widely used although some useful data have been reported for several GPCRs (Labrecque *et al.*, 2005, 2009; Leopoldo *et al.*, 2005; Koval *et al.*, 2010). The affinity of Eu-GTPγS for G proteins is about 10-fold lower than [^{35}S]GTPγS (Koval *et al.*, 2010) and correspondingly higher concentrations of the Eu-GTPγS are therefore used in assays (~5 nM).

A preliminary report has appeared recently describing a single tube GTPγS binding assay without separation based on quenching resonance energy transfer (Rozwandowicz-Jansen *et al.*, 2010). The assay uses Eu-GTPγS and takes advantage of the inaccessibility of the bound Eu-GTPγS to a fluorescence quencher. The fluorescence of the free Eu-GTPγS is, however, quenched. In the preliminary description, the assay has a lower signal to noise ratio than the other assays but concentration/response curves can be recorded with expected concentration of ligand giving a half maximal response (EC_{50}) values. With some development, the assay could be a very powerful new tool.

Although the labelled forms of GTPγS are poorly hydrolysable, the hydrolysis rate is not zero and this may complicate some mechanistic analyses. In order to model GPCR/G protein activation mechanisms it is important to include this hydrolysis rate so that correct conclusions can be drawn from mechanistic analyses (Brinkerhoff *et al.*, 2008).

Which G protein can be assayed?

GTPγS responses in the assay are typically confined to GPCRs coupled to G proteins of the $G_{i/o}$ subfamily. Responses for GPCRs coupled to G_s and G_q have been reported [see e.g. (Harrison and Traynor, 2003)] but are often very low. This seems to be due to the lower rate of exchange of guanine nucleotides at G_s and G_q together with relatively low levels of expression of these G proteins, leading to low levels of bound GTPγS. The problem here seems to be one of detection of signal over the basal level of GTPγS binding, contributed by GTPγS binding to heterotrimeric G proteins and to other guanine nucleotide binding proteins such as tubulin. In principle it might be possible to record signals for GPCRs coupled to G_s and G_q using longer incubation times but this has not been widely reported and in the author's lab this was not successful. Agonist-stimulated GTPγS binding signals have been recorded successfully for GPCRs linked to G_s or G_q by generating receptor/G protein fusions and using G protein-specific antibodies to isolate the bound GTPγS away from the membrane background (Milligan, 2003). In a related manner, antibody capture techniques have been used to examine $G_{q/11}$ signals (see below).

Although in a recombinant system there will be a single receptor responding to agonist, there may be more than one G protein responding to the receptor, depending on the composition of the host cell membrane. For example, in CHO cells the $G_{i/o}$ proteins are G_{i2} and G_{i3} and are found at levels of ~5 pmol·mg^{-1} and ~0.6 pmol·mg^{-1} respectively (Raymond *et al.*, 1993; Gettys *et al.*, 1994). It seems likely that GTPγS binding signals for a GPCR activating $G_{i/o}$ proteins will be to both of these G proteins. This could potentially lead to a complication in that if the agonist potency for stimulating GTPγS binding to the two G proteins were different this might lead to some flattening of the overall stimulation curve. Where this has been examined, however, differences in potency for agonists to stimulate G_{i2} and G_{i3} were slight for both dopamine D_2 and adenosine A_1 receptors (Wise *et al.*, 1999; Gazi *et al.*, 2003; Lane *et al.*, 2007) whereas for μ opioid receptors, agonists showed about fivefold higher potency for stimulation of G_{i3} over G_{i2} (Clark *et al.*, 2006).

The GTPγS binding signal due to a particular receptor/G protein pair may be examined using receptor/G protein fusions (Milligan, 2003) or using expression of defined receptor and G protein in insect cells (Cordeaux *et al.*, 2001; Gazi *et al.*, 2003; Nickolls and Strange, 2003). It has also been possible to examine GTPγS binding signals via individual G proteins in CHO cell membranes by using antibodies against G proteins to capture specifically the G_{i1-3} and $G_{q/11}$ signals linked to muscarinic acetylcholine receptors (using immunoprecipitation) (Akam *et al.*, 2001) or the G_{i3} and $G_{q/11}$ signals linked to 5-HT_{2c} serotonin receptors (using antibody capture and SPA, see above) (Cussac *et al.*, 2002). In the latter case, potency differences of up to eightfold are seen for some agonists when stimulating the different G proteins.

The requirement for GDP

The assay is usually run in the presence of a high concentration of GDP when agonists are examined. The GDP suppresses binding of GTPγS to non-heterotrimeric G protein targets so that the background GTPγS binding is lower. The GDP will also bind to heterotrimeric G proteins but addition of agonist will reduce the affinity of the G protein for the GDP so that GTPγS binding becomes apparent. It will be necessary to determine experimentally the appropriate concentration of GDP to maximize the agonist-stimulated GTPγS binding signal over the background signal. Concentrations in the 1–10 μM range are typically used in assays for agonists in membranes from cells expressing recombinant GPCRs but the concentration must be determined experimentally for each system. If the assay is run with a low concentration of GDP this will lead to increased basal levels of GTPγS binding and this may be useful in detecting inverse agonists (Roberts and Strange, 2005) (see below). For assays employing the autoradiographic format in tissue slices, higher concentrations of GDP are usually included [see e.g. (Sim *et al.*, 1996; Newman-Tancredi *et al.*, 2000)].

The concentration of GDP in an assay can also affect both the EC_{50} of an agonist and its relative efficacy. Higher concentrations of GDP lead to increases in EC_{50} (McLoughlin and Strange, 2000) and changes in relative efficacy. Reductions in relative efficacy of partial agonists have been reported with increased levels of GDP in some systems (Selley *et al.*, 1997; Pauwels *et al.*, 1998; Roberts *et al.*, 2004).

The concentration of GDP is therefore a very important factor in assay design and performance. It affects the ability to detect absolute and relative signals from agonists in the assay as well as their potency and should be carefully considered before embarking on use of the assay.

The requirement for Mg^{2+} and Na^{+} ions

Mg^{2+} ions are an absolute requirement for observing agonist stimulation of GTPγS binding. Effects of Mg^{2+} ions are optimal at 5–10 mM and assays are typically run using Mg^{2+} concentrations in this range (Harrison and Traynor, 2003).

The concentration of Na^{+} ions can influence the performance of the GTPγS binding assay. Na^{+} influences the strength of R/G coupling and typically a high concentration of Na^{+} is included in assays. This suppresses basal, agonist-independent, GTPγS binding thus increasing signal/background levels. The effect of Na^{+} ions is mediated through a conserved Asp residue on TM2 of GPCRs [see (Strange, 2008) for examples]. Typically, concentrations of Na^{+} ions in the 10–100 mM range are used in GTPγS binding assays with agonists, but the appropriate concentration of Na^{+} to use in an assay must be determined experimentally for each system.

The concentration of Na^{+} ions can alter the relative efficacy of partial agonists with higher concentrations reducing relative efficacy (Selley *et al.*, 2000). High concentrations of Na^{+} can also reduce agonist potency in some cases. A specific effect of the removal of Na^{+} ions on detection of partial agonism for aripiprazole has been reported for the D_2 dopamine receptor (see below) (Lin *et al.*, 2006).

The concentration of Na^{+} ions therefore is another key variable influencing assay design and performance in a similar manner to GDP.

Inclusion of saponin in GTPγS binding assays

In some cases, the detergent saponin has been included in the membrane-based assay leading to improved signal and signal/noise ratio (Cohen *et al.*, 1996; Heise *et al.*, 2005). It seems that the detergent aids accessibility of the labelled nucleotide to some G proteins. For the chemokine receptor CXCR4, addition of saponin to assays has been shown to increase the maximal stimulated Eu-GTPγS binding without affecting the EC_{50} of agonists (Labrecque *et al.*, 2005).

Incubation time

The GTPγS binding assay is frequently run as a single time point assay (typically 30–60 min) and is treated as a ligand binding assay (see above). As a result, certain assumptions are made about the assay, which may not be justified. In fact the assay is a kinetic assay that is not at equilibrium under normal conditions. This is seen clearly in experiments where the time course of [^{35}S]GTPγS binding is examined (Gardner *et al.*, 1996). The rate of [^{35}S]GTPγS binding is different for different agonists and accelerated over the basal rate but binding of [^{35}S]GTPγS continues well beyond the usual 30–60 min assay period. More detailed analysis of the time course of [^{35}S]GTPγS binding shows that this follows a pseudo first order reaction and at longer incubation times, [^{35}S]GTPγS binding reaches a plateau after about 180 min (Breivogel *et al.*, 1998; K. Quirk and P.G. Strange, unpublished). When the time course of [^{35}S]GTPγS binding is examined for different agonists or different concentrations of one agonist, the effect of agonist is on the maximal level of [^{35}S]GTPγS bound with little effect on the pseudo first order rate constant. This means that differences between agonists or different concentrations of one agonist are largely insensitive to the incubation time although this will affect the absolute signal recorded.

Although the single time point assay with a single low concentration of GTPγS is the usual assay format, some have used the assay in a saturation mode. In order to perform a saturation assay, the ‘cold ligand/hot ligand’ assay design is used. Thus a fixed concentration of [^{35}S]GTPγS is added in the presence of increasing concentrations of non-radioactive GTPγS and the total bound GTPγS is calculated by correcting the bound [^{35}S]GTPγS for the dilution factor. As the cold/hot ratio increases so the correction factor increases so that errors are multiplied accordingly and the saturation assay is quite difficult to perform accurately. It has been used to assess the numbers of G proteins in tissues (Gazi *et al.*, 2003) as well as to provide estimates of the number of G proteins activated by agonists (Selley *et al.*, 1997; Selley *et al.*, 1998).

When examining the effects of antagonists (see below) it will be necessary to consider assay design. If the antagonist is added together with the agonist, the two will not reach equilibrium immediately and it may be necessary to include a pre-incubation with the agonist/antagonist and membranes before adding GTPγS. The inclusion of a pre-incubation with agonist does not affect the performance of the assay (K. Quirk and P.G. Strange, unpublished).

High throughput screening using GTPγS binding assays

Although for the most part the assay is used in low throughput format, it can be adapted for use in high throughput screening. For the assay using [^{35}S]GTPγS a 1536 well assay has been described with WGA-SPA beads for detection (Johnson *et al.*, 2008). For the assay using Eu-GTPγS, changes to the assay format and use of automated liquid handling allowed a high throughput screening assay to be constructed (Labrecque *et al.*, 2005, 2009).

Use of the GTPγS binding assay to characterize ligands

In this section, I shall consider the use of the assay to characterize ligands in the most popular form of the assay, the membrane-based assay.

Agonists

The assay has been used to characterize agonist actions at many GPCRs, for example, adenosine A_1 (Lorenzen *et al.*, 1993; Cohen *et al.*, 1996), α_2 adrenergic (Tian *et al.*, 1994), cannabinoid CB_1 (Breivogel *et al.*, 1998), chemokine CXCR4 and CCR5 (Mueller *et al.*, 2002; Labrecque *et al.*, 2005), muscarinic acetylcholine (Lazareno and Birdsall, 1993; Lazareno *et al.*, 1993), dopamine D_2 (Gardner and Strange, 1998), 5-HT_{1A} serotonin receptors (McLoughlin and Strange, 2000), 5-HT_{1B} serotonin receptors (Pauwels *et al.*, 1997; Pauwels *et al.*, 1998), μ opioid (Selley *et al.*, 1997). For the most part these are GPCRs coupled to $G_{i/o}$ proteins as discussed above. The assay can be used to obtain concentration/response curves for agonists so that the EC_{50} and the maximal agonist effect (E_{max}) can be derived. These two parameters are discussed in more detail below but the E_{max} can be used to assess relative efficacy of agonists, allowing assessment of full and partial agonists.

Although the GTPγS binding assay is a very useful flexible assay for assessing agonist action, for some GPCRs the full agonist-stimulated level of GTPγS binding may be no more than twice the basal level (i.e. stimulation is about the same as the basal level). This means that the assay is not very sensitive for detection of low efficacy partial agonists. Given that partial agonists are of some interest in drug discovery, it would be useful to improve the sensitivity of the assay. Increased relative efficacy for partial agonists has been achieved by reducing the GDP or Na^+ ion concentrations in assays (Costa *et al.*, 1992; Selley *et al.*, 1997; Selley *et al.*, 2000; Roberts *et al.*, 2004). For the D_2 dopamine receptor a method has been developed whereby Na^+ ions in the assay are substituted with N-methyl D-glucamine (NMDG) (Lin *et al.*, 2006; Wood *et al.*, 2006). Substituting NMDG for Na^+ increases the basal level of [^{35}S]GTPγS binding and so the overall signal/noise ratio is reduced but under these conditions very low efficacy partial agonists such as aripiprazole give measurable signals, whereas such compounds are silent under standard conditions.

Antagonists

The GTPγS binding assay may be used to determine the effects of antagonists to inhibit the effects of agonists. This may be in a simple experiment where a range of antagonist concentrations is used to inhibit the effects of a single concentration of agonist or preferably using Schild analysis where agonist concentration/response curves are recorded using control conditions and in the presence of increasing concentrations of antagonist. The latter experimental design is preferable as it yields a value of the pA_2 for the antagonist as well as a Schild slope, which should be close to one for a competitive antagonist. Schild slopes different from unity may be seen if more complex mechanisms hold. Other methods have also been applied to analyse antagonist effects using [^{35}S]GTPγS binding assays (Lazareno and Birdsall, 1993). Care needs to be taken in assay design (see above) to allow agonist and antagonist to reach equilibrium and this may require a pre-incubation before addition of GTPγS. It may be necessary to test different pre-incubation times to be sure that equilibrium has been reached for ligands with slow binding kinetics (Haworth *et al.*, 2007).

The assay may be used to study the effects of compounds that act non-competitively/allosterically, that is, at sites different from the primary binding site of the receptor [see e.g. (Birdsall *et al.*, 1999)]. Where such mechanisms of antagonist effects are invoked, it will be necessary to ensure that assay artefacts (e.g. lack of equilibration) have been eliminated (Kenakin *et al.*, 2006).

Inverse agonists

It is now clear that many drugs that have been assumed to be antagonists do, in fact, exhibit inverse agonism, if assessed in a suitable assay (Kenakin, 2004). The GTPγS binding assay will detect inverse agonism if there is sufficient basal (agonist-independent) activity for the particular GPCR. Agonist-independent activity in the GTPγS binding assay seems to be very variable between GPCRs. For some GPCRs, for example, α_2 adrenoceptor (Tian *et al.*, 1994), 5-HT_{1A} serotonin receptor (McLoughlin and Strange, 2000), CCR5 chemokine receptor (Haworth *et al.*, 2007), cannabinoid CB_1 receptor (Bouaboula *et al.*, 1997), effects of inverse agonists can be readily detected using the $[^{35}S]$GTPγS binding assay. For these GPCRs, it seems that substantial agonist-independent activity is present. For other GPCRs, for example, D_2 dopamine receptor, basal activity seems quite low in this assay and inverse agonism is difficult to measure under standard conditions. Some improvement in inverse agonist detection can be achieved for such receptors by increasing the basal signal by working without GDP in the assay and substituting NMDG for Na^+ (Roberts and Strange, 2005). This provides a larger agonist-independent response for the inverse agonist to inhibit, thus improving detection of the inverse agonist signal. Under these conditions, the inverse agonist-inhibited signal may constitute only a small part of the basal signal so that although the inverse agonist signal can be detected, it may be subject to some error.

For the most part, manipulations of assay conditions do not affect the EC_{50} values of inverse agonists in GTPγS binding assays but there are examples of some compounds whose EC_{50} is sensitive to changes in GDP (McLoughlin and Strange, 2000).

Assay validation for agonists and inverse agonists

The $[^{35}S]$GTPγS binding assay can be used as a simple means to characterize the activities of compounds, but it is important to consider what the parameters derived from the assay represent; also how the measurements made relate to the *in vivo* activities of compounds and to the activities of compounds measured using other assays; also what event in the G protein cycle is being measured in the assay.

Parameters derived from the assay

In principle three parameters are accessible from concentration/response curves obtained using the $[^{35}S]$GTPγS binding assay [E_{max}, EC_{50}, Hill coefficient (n_H)]. The E_{max} is the maximal response seen at high concentrations of agonist or inverse agonist. Full agonists will give the same E_{max} whereas partial agonists will give sub-maximal responses. If E_{max} values for different compounds are assessed relative to a reference full agonist then, in principle, they can be used to provide relative efficacy values. Determination of E_{max} therefore for partial agonists allows rough scales of efficacy to be constructed but these scales fail for full agonists, which, by definition, give a full response in the assay but may nevertheless differ in terms of intrinsic efficacy (Strange, 2008). It may be possible to get round this limitation by manipulating assay conditions so that more agonists exhibit partial agonism. This could be achieved by changing the GDP concentration (see above) although this will also change the signal to basal ratio of the assay.

The EC_{50} is the concentration of an agonist or inverse agonist that achieves a half-maximal response. The EC_{50} is a function of the affinity of a drug for the receptor as well as the amplification between binding to receptor and response. The GTPγS binding event is close to the receptor and so there will not be great amplification (see below). Nevertheless, EC_{50} values for agonists in the assay are dependent on assay conditions, for example, concentration of Na^+/GDP (see above).

The n_H for a concentration/response curve denotes the shape of the curve and how the response depends on ligand concentration. n_H may be obtained by fitting data to the Hill equation. For a simple model where a drug binds to a single population of receptors, n_H close to one are expected. n_H differing from one are not always easy to interpret but suggest more complex mechanisms and some suggestions are given below.

In many of the published data, n_H are often not reported. Where they have been determined, the n_H of concentration/response curves are close to one in many cases. It is important to realize that, where concentration/response curves are obtained using log unit changes in concentration of ligand, it may be quite difficult to detect differences in n_H from unity unless these are substantial.

There are, however, clear cases in the literature where concentration/response curves have n_H that are less than one, for example, for muscarinic acetylcholine receptors expressed in CHO cells (Lazareno and Birdsall, 1993; Lazareno *et al.*, 1993). One possible mechanistic explanation for low n_H could be signalling via different G proteins and for the muscarinic receptor example, this was checked by eliminating $G_{i/o}$ effects with *Pertussis* toxin treatment. For M_2 and M_4 receptors, the acetylcholine-stimulated $[^{35}S]$GTPγS binding response was fully inhibited whereas for M_1 and M_3 receptors only

~60% inhibition occurred. For M_1 and M_3 receptors it seems likely that the low n_H correspond to effects at G_q and $G_{i/o}$ G proteins whereas for M_2 and M_4, in principle, effects at mixtures of G_{i2} and G_{i3} may be responsible although the latter explanation seems unlikely (see above).

Comparison with other assays

Here we wish to see if the GTPγS binding assay provides data that can be used to predict the properties of ligands in other assays including *in vivo* assays, so that it may be used with confidence in drug discovery. Ideally, this requires a comparison of the activities of a range of ligands in the GTPγS binding assay with their activities in other assays and there are very few published data sets allowing this comparison.

For antagonists, a detailed study has been performed for the muscarinic acetylcholine receptors and it was shown that the affinities of a range of antagonists were similar whether determined in [^{35}S]GTPγS binding assays using membranes of recombinant cells expressing the receptors or in ligand binding assays performed on animal tissues or on membranes of recombinant cells expressing receptors (Lazareno and Birdsall, 1993).

For agonists, such comparisons are complicated by differences in amplification in different assays. The GTPγS binding assay reflects events close to the receptor and so is not subject to strong amplification unlike other more downstream assays for GPCRs, for example, inhibition of adenylyl cyclase. In consequence, compounds that are full agonists in the adenylyl cyclase assay may appear as partial agonists in GTPγS binding assays and potencies of agonists may be less in GTPγS binding assays as compared with corresponding values in adenylyl cyclase assays [see e.g. (Payne *et al*., 2002)].

Crude comparisons may be made using E_{max} or rank orders of agonist potencies in different assays. For example, for the D_2 dopamine receptor, E_{max} data for five agonists obtained in [^{35}S]GTPγS binding assays agree reasonably with E_{max} data reported in electrophysiology assays (Strange, 2007). Also for the D_2 dopamine receptor, E_{max} data on a limited range of compounds agree for their activity in [^{35}S]GTPγS binding assays and for their effects on GIRK potassium channels (Heusler *et al*., 2007). For κ opioid receptors E_{max} data for a range of agonists obtained in [^{35}S]GTPγS binding and adenylyl cyclase assays agree (Bidlack and Jadrovski, 2000).

In order to use the parameters measured in the [^{35}S]GTPγS binding assay to predict responses in other assays it would be better to use parameters that are independent of amplification and some measures of agonist efficacy may allow this. For the D_2 dopamine receptor expressed in CHO cell membranes, a large data set exists for the effects of agonists on [^{35}S]GTPγS binding in membranes and to inhibit forskolin-stimulated adenylyl cyclase activity in whole cells (Payne *et al*., 2002). When K_A/EC_{50} or $E_{max}.K_A/EC_{50}$ values (Strange, 2008) (also see Appendix) are compared for the two assays, correlations are seen but there is some scatter (r^2 = 0.53, 0.59 respectively), but if the parameter E_{max}/EC_{50} is used, a much better correlation is observed (r^2 = 0.96). This parameter (E_{max}/EC_{50}), also known as the intrinsic relative activity, corresponds to the product of affinity and efficacy for an agonist (Ehlert *et al*., 1999) and may provide a useful practical tool for analysis of agonist action.

What molecular event does the assay measure?

It is important to analyse the GTPγS binding assay in terms of what species is being detected and how the assay relates to events in cells stimulated by agonists, as such knowledge will help validate the method as a suitable tool in drug discovery. According to current views of the G protein cycle, GTPγS binding should be to ARG yielding ARG.GTPγS, which then breaks down to Gα.GTPγS (Figure 1). We examined this possibility in [^{35}S]GTPγS dissociation binding assays and concluded that a large part of the bound nucleotide was in the form of ARG.[^{35}S]GTPγS with only a small proportion as Gα.[^{35}S]GTPγS (Quirk *et al*., 2007). One of the factors contributing to this apparent stability of ARG.[^{35}S]GTPγS seems to be the low concentration of nucleotide (~100 pM) used in these assays.

It is also important to determine which is the catalysed step in the G protein cycle that is being measured in the [^{35}S]GTPγS binding assay, and how this relates to the cellular situation given that in cells the GTP concentration is ~50 μM (Otero, 1990), whereas in the [^{35}S]GTPγS binding assay the [^{35}S]GTPγS concentration is ~100 pM. Figure 1 shows the G protein cycle under cellular conditions and under the conditions of the [^{35}S]GTPγS binding assay. In the latter case, owing to the low concentrations of [^{35}S]GTPγS used it is likely that the binding event is slow. We have verified that the [^{35}S]GTPγS binding event is indeed the rate-determining event in CHO cell membranes expressing D_2 dopamine receptors by conducting assays with varying concentrations of [^{35}S]GTPγS where the rate of binding is directly proportional to the concentration of [^{35}S]GTPγS (Roberts *et al*., 2004). Therefore, in the [^{35}S]GTPγS binding assay, the rate-determining step is the binding of [^{35}S]GTPγS to ARG. In the cell, the corresponding step, the binding of GTP to ARG is likely to be fast owing to the high nucleotide concentration and GDP release or ternary complex breakdown may be slow. This means that the [35S]GTPγS binding assay does not measure the rate-determining step in the G protein cycle under cellular conditions. Although the assay clearly does provide measures of agonist efficacy parameters, there could, in principle, be differences between the abilities of agonists to affect the different steps.

It will also be important to try to understand how the [35S]GTPγS binding step in the [35S]GTPγS binding assay is regulated and how do full and partial agonists differ. Does increased [35S]GTPγS binding occur because there is more ARG available in the presence of agonist or does the affinity of receptor/G protein complex for [35S]GTPγS increase in ARG? This has been addressed for opiate receptors where effects on both the maximum level of [35S]GTPγS binding and the affinity of [35S]GTPγS for ARG were observed (Selley *et al*., 1997; Selley *et al*., 1998).

Conclusion

The GTPγS binding assay provides a flexible assay for the analysis of functional effects of agonists, antagonists and inverse agonists at GPCRs. The assay is usually performed in the same rather simple format as is used for ligand binding assays although it is important to assess the conditions for performing assays so that optimal signals can be obtained. Although the majority of assays are still performed using [35S]GTPγS, the development of non-radioactive methods offers great promise for the future application of the assay.

Acknowledgements

I thank members of my lab for discussion on this topic and especially Ben Gardner who started this work and made me think. I thank the BBSRC and the Wellcome Trust who supported the work financially.

Conflict of interest

There is no conflict of interest.

References

Akam EC, Challiss RA, Nahorski SR (2001). G(q/11) and G(i/o) activation profiles in CHO cells expressing human muscarinic acetylcholine receptors: dependence on agonist as well as receptor-subtype. Br J Pharmacol 132: 950–958.

Alt A, McFadyen IJ, Fan CD, Woods JH, Traynor JR (2001). Stimulation of guanosine-5′-o-(3-[35S]thio)triphosphate binding in digitonin-permeabilized C6 rat glioma cells: evidence for an organized association of mu-opioid receptors and G protein. J Pharmacol Exp Ther 298: 116–121.

Asano T, Pedersen SE, Scott CW, Ross EM (1984). Reconstitution of catecholamine-stimulated binding of guanosine 5′-O-(3-thiotriphosphate) to the stimulatory GTP-binding protein of adenylate cyclase. Biochemistry 23: 5460–5467.

Bidlack JM, Jadrovski I (2000). Pharmacological profiles of kappa opioids. Correlation between [35S]GTP gamma S binding and cyclic AMP production. Ann NY Acad Sci 909: 264.

Birdsall NJ, Farries T, Gharagozloo P, Kobayashi S, Lazareno S, Sugimoto M (1999). Subtype-selective positive cooperative interactions between brucine analogs and acetylcholine at muscarinic receptors: functional studies. Mol Pharmacol 55: 778–786.

Bokoch GM, Katada T, Northup JK, Ui M, Gilman AG (1984). Purification and properties of the inhibitory guanine nucleotide-binding regulatory component of adenylate cyclase. J Biol Chem 259: 3560–3567.

Bouaboula M, Perrachon S, Milligan L, Canat X, Rinaldi-Carmona M, Portier M *et al*. (1997). A selective inverse agonist for central cannabinoid receptor inhibits mitogen-activated protein kinase activation stimulated by insulin or insulin-like growth factor 1. Evidence for a new model of receptor/ligand interactions. J Biol Chem 272: 22330–22339.

Breivogel CS, Selley DE, Childers SR (1998). Cannabinoid receptor agonist efficacy for stimulating [35S]GTPgammaS binding to rat cerebellar membranes correlates with agonist-induced decreases in GDP affinity. J Biol Chem 273: 16865–16873.

Breivogel CS, Walker JM, Huang SM, Roy MB, Childers SR (2004). Cannabinoid signaling in rat cerebellar granule cells: G-protein activation, inhibition of glutamate release and endogenous cannabinoids. Neuropharmacology 47: 81–91.

Brinkerhoff CJ, Traynor JR, Linderman JJ (2008). Collision coupling, crosstalk, and compartmentalization in G-protein coupled receptor systems: can a single model explain disparate results? J Theor Biol 255: 278–286.

Bunemann M, Frank M, Lohse MJ (2003). Gi protein activation in intact cells involves subunit rearrangement rather than dissociation. Proc Natl Acad Sci USA 100: 16077–16082.

Clark MJ, Furman CA, Gilson TD, Traynor JR (2006). Comparison of the relative efficacy and potency of mu-opioid agonists to activate Galpha(i/o) proteins containing a pertussis toxin-insensitive mutation. J Pharmacol Exp Ther 317: 858–864.

Cohen FR, Lazareno S, Birdsall NJ (1996). The effects of saponin on the binding and functional properties of the human adenosine A1 receptor. Br J Pharmacol 117: 1521–1529.

Cordeaux Y, Nickolls SA, Flood LA, Graber SG, Strange PG (2001). Agonist regulation of D(2) dopamine receptor/G protein interaction. Evidence for agonist selection of G protein subtype. J Biol Chem 276: 28667–28675.

Costa T, Ogino Y, Munson PJ, Onaran HO, Rodbard D (1992). Drug efficacy at guanine nucleotide-binding regulatory protein-linked receptors: thermodynamic interpretation of negative antagonism and of receptor activity in the absence of ligand. Mol Pharmacol 41: 549–560.

Cussac D, Newman-Tancredi A, Duqueyroix D, Pasteau V, Millan MJ (2002). Differential activation of Gq/11 and Gi(3) proteins at 5-hydroxytryptamine(2C) receptors revealed by antibody capture assays: influence of receptor reserve and relationship to agonist-directed trafficking. Mol Pharmacol 62: 578–589.

DeLapp NW (2004). The antibody-capture [(35)S]GTPgammaS scintillation proximity assay: a powerful emerging technique for analysis of GPCR pharmacology. Trends Pharmacol Sci 25: 400–401.

DeLapp NW, McKinzie JH, Sawyer BD, Vandergriff A, Falcone J, McClure D *et al*. (1999). Determination of [35S]guanosine-5′-O-(3-thio)triphosphate binding mediated by cholinergic muscarinic receptors in membranes from Chinese hamster ovary cells and rat striatum using an anti-G protein scintillation proximity assay. J Pharmacol Exp Ther 289: 946–955.

Ehlert FJ, Griffin MT, Sawyer GW, Bailon R (1999). A simple method for estimation of agonist activity at receptor subtypes: comparison of native and cloned M3 muscarinic receptors in guinea pig ileum and transfected cells. J Pharmacol Exp Ther 289: 981–992.

Frank M, Thumer L, Lohse MJ, Bunemann M (2005). G Protein activation without subunit dissociation depends on a G{alpha}(i)-specific region. J Biol Chem 280: 24584–24590.

Gardner B, Strange PG (1998). Agonist action at D2(long) dopamine receptors: ligand binding and functional assays. Br J Pharmacol 124: 978–984.

Gardner B, Hall DA, Strange PG (1996). Pharmacological analysis of dopamine stimulation of [35S]-GTP gamma S binding via human D2short and D2long dopamine receptors expressed in recombinant cells. Br J Pharmacol 118: 1544–1550.

Gazi L, Nickolls SA, Strange PG (2003). Functional coupling of the human dopamine D2 receptor with G alpha i1, G alpha i2, G alpha i3 and G alpha o G proteins: evidence for agonist regulation of G protein selectivity. Br J Pharmacol 138: 775–786.

Gettys TW, Sheriff-Carter K, Moomaw J, Taylor IL, Raymond JR (1994). Characterization and use of crude alpha-subunit preparations for quantitative immunoblotting of G proteins. Anal Biochem 220: 82–91.

Harrison C, Traynor JR (2003). The [35S]GTPgammaS binding assay: approaches and applications in pharmacology. Life Sci 74: 489–508.

Haworth B, Lin H, Fidock M, Dorr P, Strange PG (2007). Allosteric effects of antagonists on signalling by the chemokine receptor CCR5. Biochem Pharmacol 74: 891–897.

Heise CE, Pahuja A, Hudson SC, Mistry MS, Putnam AL, Gross MM *et al*. (2005). Pharmacological characterization of CXC chemokine receptor 3 ligands and a small molecule antagonist. J Pharmacol Exp Ther 313: 1263–1271.

Heusler P, Newman-Tancredi A, Castro-Fernandez A, Cussac D (2007). Differential agonist and inverse agonist profile of antipsychotics at D2L receptors coupled to GIRK potassium channels. Neuropharmacology 52: 1106–1113.

Higashijima T, Ferguson KM, Sternweis PC, Smigel MD, Gilman AG (1987). Effects of Mg2+ and the beta gamma-subunit complex on the interactions of guanine nucleotides with G proteins. J Biol Chem 262: 762–766.

Hilf G, Gierschik P, Jakobs KH (1989). Muscarinic acetylcholine receptor-stimulated binding of guanosine 5′-O-(3-thiotriphosphate) to guanine-nucleotide-binding proteins in cardiac membranes. Eur J Biochem 186: 725–731.

Horswill JG, Bali U, Shaaban S, Keily JF, Jeevaratnam P, Babbs AJ *et al*. (2007). PSNCBAM-1, a novel allosteric antagonist at cannabinoid CB1 receptors with hypophagic effects in rats. Br J Pharmacol 152: 805–814.

Johnson EN, Shi X, Cassaday J, Ferrer M, Strulovici B, Kunapuli P (2008). A 1,536-well [(35)S]GTPgammaS scintillation proximity binding assay for ultra-high-throughput screening of an orphan galphai-coupled GPCR. Assay Drug Dev Technol 6: 327–337.

Kara E, Strange PG (2010). Use of the [35S]GTPgS binding assay to determine ligand efficacy at G protein-coupled receptors. In: Poyner D, Wheatley M (eds). G Protein-Coupled Receptors: Essential Methods. Wiley-VCH: Weinheim, Germany, pp. 53–68.

Kenakin T (2004). Efficacy as a vector: the relative prevalence and paucity of inverse agonism. Mol Pharmacol 65: 2–11.

Kenakin T, Jenkinson S, Watson C (2006). Determining the potency and molecular mechanism of action of insurmountable antagonists. J Pharmacol Exp Ther 319: 710–723.

Kitazawa T, Asakawa K, Nakamura T, Teraoka H, Unno T, Komori SI *et al*. (2009). M3 muscarinic receptors mediate positive inotropic responses in mouse atria: a study with muscarinic receptor knockout mice. J Pharmacol Exp Ther 330: 487–493.

Koval A, Kopein D, Purvanov V, Katanaev VL (2010). Europium-labeled GTP as a general nonradioactive substitute for [(35)S]GTPgammaS in high-throughput G protein studies. Anal Biochem 397: 202–20.

Kurose H, Katada T, Haga T, Haga K, Ichiyama A, Ui M (1986). Functional interaction of purified muscarinic receptors with purified inhibitory guanine nucleotide regulatory proteins reconstituted in phospholipid vesicles. J Biol Chem 261: 6423–6428.

Labrecque J, Anastassov V, Lau G, Darkes M, Mosi R, Fricker SP (2005). The development of an europium-GTP assay to quantitate chemokine antagonist interactions for CXCR4 and CCR5. Assay Drug Dev Technol 3: 637–648.

Labrecque J, Wong RS, Fricker SP (2009). A time-resolved fluorescent lanthanide (Eu)-GTP binding assay for chemokine receptors as targets in drug discovery. Methods Mol Biol 552: 153–169.

Lane JR, Powney B, Wise A, Rees S, Milligan G (2007). Protean agonism at the dopamine D2 receptor: (S)-3-(3-hydroxyphenyl)-N-propylpiperidine is an agonist for activation of Go1 but an antagonist/inverse agonist for Gi1,Gi2, and Gi3. Mol Pharmacol 71: 1349–1359.

Lazareno S, Birdsall NJ (1993). Pharmacological characterization of acetylcholine-stimulated [35S]-GTP gamma S binding mediated by human muscarinic m1-m4 receptors: antagonist studies. Br J Pharmacol 109: 1120–1127.

Lazareno S, Farries T, Birdsall NJ (1993). Pharmacological characterization of guanine nucleotide exchange reactions in membranes from CHO cells stably transfected with human muscarinic receptors m1-m4. Life Sci 52: 449–456.

Leopoldo M, Lacivita E, Colabufo NA, Contino M, Berardi F, Perrone R (2005). First structure-activity relationship study on dopamine D3 receptor agents with N-[4-(4-arylpiperazin-1-yl)butyl]arylcarboxamide structure. J Med Chem 48: 7919–7922.

Lin H, Saisch SG, Strange PG (2006). Assays for enhanced activity of low efficacy partial agonists at the D(2) dopamine receptor. Br J Pharmacol 149: 291–299.

Lorenzen A, Fuss M, Vogt H, Schwabe U (1993). Measurement of guanine nucleotide-binding protein activation by A1 adenosine receptor agonists in bovine brain membranes: stimulation of guanosine-5′-O-(3-[35S]thio)triphosphate binding. Mol Pharmacol 44: 115–123.

Martel JC, Ormiere AM, Leduc N, Assie MB, Cussac D, Newman-Tancredi A (2007). Native rat hippocampal 5-HT1A receptors show constitutive activity. Mol Pharmacol 71: 638–643.

McLoughlin DJ, Strange PG (2000). Mechanisms of agonism and inverse agonism at serotonin 5-HT1A receptors. J Neurochem 74: 347–357.

Milligan G (2003). Principles: extending the utility of [35S]GTP gamma S binding assays. Trends Pharmacol Sci 24: 87–90.

Mueller A, Mahmoud NG, Goedecke MC, McKeating JA, Strange PG (2002). Pharmacological characterization of the chemokine receptor, CCR5. Br J Pharmacol 135: 1033–1043.

Newman-Tancredi A, Chaput C, Touzard M, Millan MJ (2000). [(35)S]-GTPgammaS autoradiography reveals alpha(2) adrenoceptor-mediated G-protein activation in amygdala and lateral septum. Neuropharmacology 39: 1111–1113.

Newman-Tancredi A, Rivet JM, Cussac D, Touzard M, Chaput C, Marini L *et al*. (2003). Comparison of hippocampal G protein activation by 5-HT(1A) receptor agonists and the atypical antipsychotics clozapine and S16924. Naunyn Schmiedebergs Arch Pharmacol 368: 188–199.

Nickolls SA, Strange PG (2003). Interaction of the D2short dopamine receptor with G proteins: analysis of receptor/G protein selectivity. Biochem Pharmacol 65: 1139–1150.

Otero AD (1990). Transphosphorylation and G protein activation. Biochem Pharmacol 39: 1399–1404.

Pauwels PJ, Tardif S, Palmier C, Wurch T, Colpaert FC (1997). How efficacious are 5-HT1B/D receptor ligands: an answer from GTP gamma S binding studies with stably transfected C6-glial cell lines. Neuropharmacology 36: 499–512.

Pauwels PJ, Wurch T, Palmier C, Colpaert FC (1998). Pharmacological analysis of G-protein activation mediated by guinea-pig recombinant 5-HT1B receptors in C6-glial cells: similarities with the human 5-HT1B receptor. Br J Pharmacol 123: 51–62.

Payne SL, Johansson AM, Strange PG (2002). Mechanisms of ligand binding and efficacy at the human D2(short) dopamine receptor. J Neurochem 82: 1106–1117.

Quirk K, Roberts DJ, Strange PG (2007). Mechanisms of G protein activation via the D2 dopamine receptor: evidence for persistent receptor/G protein interaction after agonist stimulation. Br J Pharmacol 151: 144–152.

Raymond JR, Olsen CL, Gettys TW (1993). Cell-specific physical and functional coupling of human 5-HT1A receptors to inhibitory G protein alpha-subunits and lack of coupling to Gs alpha. Biochemistry 32: 11064–11073.

Roberts DJ, Strange PG (2005). Mechanisms of inverse agonist action at D2 dopamine receptors. Br J Pharmacol 145: 34–42.

Roberts DJ, Lin H, Strange PG (2004). Mechanisms of agonist action at D2 dopamine receptors. Mol Pharmacol 66: 1573–1579.

Rozwandowicz-Jansen A, Laurila J, Martikkala E, Frang H, Hemmila I, Scheinin M *et al*. (2010). Homogeneous GTP binding assay employing QRET technology. J Biomol Screen 15: 261–267.

Salah-Uddin H, Thomas DR, Davies CH, Hagan JJ, Wood MD, Watson JM *et al*. (2008). Pharmacological assessment of m1 muscarinic acetylcholine receptor-gq/11 protein coupling in membranes prepared from postmortem human brain tissue. J Pharmacol Exp Ther 325: 869–874.

Selley DE, Sim LJ, Xiao R, Liu Q, Childers SR (1997). mu-Opioid receptor-stimulated guanosine-5′-O-(gamma-thio)-triphosphate binding in rat thalamus and cultured cell lines: signal transduction mechanisms underlying agonist efficacy. Mol Pharmacol 51: 87–96.

Selley DE, Liu Q, Childers SR (1998). Signal transduction correlates of mu opioid agonist intrinsic efficacy: receptor-stimulated [35S]GTP gamma S binding in mMOR-CHO cells and rat thalamus. J Pharmacol Exp Ther 285: 496–505.

Selley DE, Cao CC, Liu Q, Childers SR (2000). Effects of sodium on agonist efficacy for G-protein activation in mu-opioid receptor-transfected CHO cells and rat thalamus. Br J Pharmacol 130: 987–996.

Sim LJ, Selley DE, Dworkin SI, Childers SR (1996). Effects of chronic morphine administration on mu opioid receptor-stimulated [35S]GTPgammaS autoradiography in rat brain. J Neurosci 16: 2684–2692.

Sternweis PC, Robishaw JD (1984). Isolation of two proteins with high affinity for guanine nucleotides from membranes of bovine brain. J Biol Chem 259: 13806–13813.

Strange PG (2007). Mechanisms underlying agonist efficacy. Biochem Soc Trans 35 (Pt 4): 733–736.

Strange PG (2008). Agonist binding, agonist affinity and agonist efficacy at G protein-coupled receptors. Br J Pharmacol 153: 1353–1363.

Tian WN, Duzic E, Lanier SM, Deth RC (1994). Determinants of alpha 2-adrenergic receptor activation of G proteins: evidence for a precoupled receptor/G protein state. Mol Pharmacol 45: 524–531.

Wieland T, Liedel K, Kaldenberg-Stasch S, Meyer zu Heringdorf D, Schmidt M, Jakobs KH (1995). Analysis of receptor-G protein interactions in permeabilized cells. Naunyn Schmiedebergs Arch Pharmacol 351: 329–336.

Wise A, Sheehan M, Rees S, Lee M, Milligan G (1999). Comparative analysis of the efficacy of A1 adenosine receptor activation of Gi/o alpha G proteins following coexpression of receptor and G protein and expression of A1 adenosine receptor-Gi/o alpha fusion proteins. Biochemistry 38: 2272–2278.

Wood MD, Scott C, Clarke K, Westaway J, Davies CH, Reavill C *et al*. (2006). Aripiprazole and its human metabolite are partial agonists at the human dopamine D2 receptor, but the rodent metabolite displays antagonist properties. Eur J Pharmacol 546: 88–94.

Appendix

Glossary of pharmacological terms used

Full agonist: a compound able to produce the maximal stimulation of the functional response associated with a receptor at saturating concentrations.

Partial agonist: a compound able to produce only a sub-maximal stimulation of the functional response associated with a receptor at saturating concentrations.

Inverse agonist: a compound able to inhibit agonist-independent functional responses associated with a receptor.

Antagonist: a compound that can bind to receptors but is unable to alter the activity of the response system. It may, therefore, block the action of either agonists or inverse agonists.

AR: agonist/receptor complex.

RG: receptor/G protein complex.

ARG: agonist/receptor/G protein complex.

K_A: dissociation constant of agonist for binding to receptor.

E_{max}: maximal agonist-stimulated functional response in system.

EC_{50}: concentration of agonist that produces half its maximal functional response in system.

n_H: Hill coefficient for agonist concentration/response curve.