



Differential ligand efficacy at h5-HT_{1A} receptor-coupled G-protein subtypes: a commentary

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Received 16 April 2003; accepted 16 April 2003

Abstract

Activation of heterotrimeric G-protein involves the exchange by α subunits of GDP for GTP. The binding of the hydrolysis-resistant GTP analogue, guanosine-5'-O-(3-[³⁵S]thio)-triphosphate ([³⁵S]GTP γ S), provides a measure of agonist, antagonist and inverse agonist actions at G-protein-coupled receptors. However, classical [³⁵S]GTP γ S binding methods do not distinguish among the subtypes of G-proteins activated. This is a limitation in view of (i) promiscuity of receptor coupling to multiple families of G-proteins, and (ii) the differential influence of ligands on distinct signal transduction pathways. A recent development of [³⁵S]GTP γ S binding methodology employing antibody capture and scintillation proximity assays (SPA) permits the targeting of specific G α subtypes in both recombinant and native tissues. When applied to human serotonin_{1A} receptor (h5-HT_{1A}) expressed in Chinese hamster ovary (CHO) cells, this methodology revealed surprising patterns of G α_{i3} subunit activation. For example, low but not high concentrations of high-efficacy h5-HT_{1A} agonists direct receptor signalling to G α_{i3} . In contrast, partial agonists favour h5-HT_{1A} receptor signalling to G α_{i3} over a wide concentration range. Further, alterations in buffer sodium concentration reversed these actions of agonists: stimulation of G α_{i3} activation was observed at high sodium concentration, but inhibition was observed at low sodium concentration, suggestive of

Abbreviations: [³⁵S]GTP γ S, Guanosine-5'-O-(3-[³⁵S]thio)-triphosphate; 5-CT, 5-carboxyamidotryptamine; CHO, Chinese hamster ovary; ERK, extracellular signal-regulated kinase; GPCR, G-protein coupled receptor; h5-HT_{1A}, human serotonin_{1A} receptor; HEK, human embryonic kidney cells; S14506, 1-[2-(4-fluorobenzoylamino)ethyl]-4-(7-methoxynaphthyl)piperazine; S18127, *N*-[1-(2,3-dihydro[1,4] dioxin-5-yl)piperid-4-yl] indan-2-yl-amine; SB224,289, 1'-methyl-5-[2'-methyl-4'-(5-methyl-1,2,4-oxadiazol-3-yl)biphenyl-4-yl]-2,3,6,7-tetrahydro-spiro[furo[2,3-f]indole-3,4'-piperidine]; SPA, Scintillation proximity assay; WAY100,635, *N*-{2-[4-(2-methoxyphenyl)-1-piperazinyl]ethyl}-*N*-(2-pyridinyl)-cyclohexane-carboxamide.

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protean efficacy, i.e. a switch from positive to negative efficacy dependent on receptor tone. These results indicate that complex changes in both magnitude and direction of response to receptor ligands can occur for specific G-protein subtypes. Interestingly, the inverse agonist, spiperone, inhibited constitutive h5-HT_{1A} receptor-mediated G α_{i3} activation under all conditions tested, consistent with the hypothesis that it selectively stabilises distinct receptor conformation(s). Further, whereas classical [³⁵S]GTP γ S binding assays in CHO cell membranes failed to demonstrate negative efficacy for the neuroleptic, haloperidol, this compound exhibited robust inverse agonism for the activation of G α_{i3} subunits. These data suggest that haloperidol may selectively inhibit constitutive activation of this G-protein subtype and, thus, exhibit inverse agonist-mediated trafficking of receptor signalling. © 2003 Elsevier Science B.V. All rights reserved.

Keywords: G-proteins; G α_{i3} ; 5-HT_{1A} receptors; Antibody capture; Chinese hamster ovary; Protean agonism; Inverse agonism; Trafficking

1. Introduction

The superfamily of heptahelical cell surface receptors which couple to heterotrimeric intracellular guanine nucleotide binding proteins (G-proteins) has been intensively investigated, both at the ligand-binding and at the G-protein activation levels. Indeed, under the influence of agonists, G-protein-coupled receptors (GPCRs) promote nucleotide exchange by G-protein α subunits, which release GDP and bind GTP. This exchange triggers subunit dissociation and the transmission of signalling to a variety of intracellular second messenger systems. Hydrolysis of GTP by the G α subunit and re-association with G $\beta\gamma$ subunits re-establishes the initial basal state. The GDP/GTP exchange step is therefore of key interest in the study of ligand efficacy and can be investigated by use of the hydrolysis-resistant GTP analogue, guanosine-5'-O-(3-[³⁵S]thio)-triphosphate ([³⁵S]GTP γ S). In the case of 5-HT₁ receptors, [³⁵S]GTP γ S binding has yielded extensive information concerning ligand efficacy (positive and negative) at human serotonin_{1A} (h5-HT_{1A}) [1–3], h5-HT_{1B} [4–6] and h5-HT_{1D} [6,7] receptors. However, the majority of these studies have employed membrane preparations expressing mixed populations of G-proteins, and the resultant [³⁵S]GTP γ S binding is therefore composed of individual responses of all the G-protein subtypes activated or inhibited by the receptor [8]. Total [³⁵S]GTP γ S binding measures, which lack resolution at the level of specific G-protein subtypes, may, therefore, require re-evaluation in light of differential coupling of receptors to specific G-protein subtypes as well as ligand-dependent activation of specific transduction pathways.

2. Differential coupling to G-protein subtypes: h5-HT_{1A} receptors

The stimulation or inhibition of [³⁵S]GTP γ S binding by agonists and inverse agonists, respectively, has been studied at recombinant human 5-HT_{1A} receptors expressed in various cell lines, including HeLa, Chinese hamster ovary (CHO) and C6 glial cells [1,9–11]. However, these cell lines are known to express differing subpopulations of G-proteins: HeLa cells express predominantly G α_{i3} subunits, whereas CHO cells express

predominantly $G\alpha_{i2}$ subunits [12,13]. These cell-specific differences are pharmacologically relevant because 5-HT_{1A} receptors favour coupling to some $G\alpha$ subunits more than others. Thus, h5-HT_{1A} receptors couple to G-proteins with the following order of preference: $G\alpha_{i3} > G\alpha_{i2} \geq G\alpha_{i1} \geq G_o > G_z$ [14–17]; see Raymond et al. [13] for review.¹ Differences in coupling to G-protein subtypes may influence ligand profiles at 5-HT_{1A} receptors. For example, the selective 5-HT_{1A} receptor ligand, *N*-{2-[4-(2-methoxyphenyl)-1-piperazinyl]ethyl}-*N*-(2-pyridinyl)-cyclohexane-carboxamide (WAY100,635), behaved as a neutral antagonist in standard G-protein activation (³⁵S]GTP γ S binding) assays in CHO cells [9]. In comparison, the same ligand exhibited sodium-sensitive inverse agonist activity in a study employing HeLa cells [21], suggesting that the differing populations of G-proteins expressed in these cell types were influencing the observed efficacy of the ligand.

Similar considerations apply to physiological tissues. Indeed, in a coimmunoprecipitation study, rat brain 5-HT_{1A} autoreceptors in raphe nuclei coupled preferentially to $G\alpha_{i3}$ subunits, whereas post-synaptic 5-HT_{1A} receptors in hippocampus coupled preferentially to G_o [22]. In addition, although the density of 5-HT_{1A} receptors in these brain regions is similar, agonist-induced [³⁵S]GTP γ S labelling was markedly lower in pre-synaptic than in post-synaptic regions in some studies [23,24, but see 25]. Further, no coupling of 5-HT_{1A} receptors to adenylyl cyclase inhibition was detected in rat raphe, whereas a robust response is observed in the hippocampus [26]. It may be speculated that differential coupling to intracellular G-proteins may explain some of these contrasting responses of pre- and post-synaptic 5-HT_{1A} receptors.

3. Ligand-directed trafficking of receptor signalling: application to 5-HT_{1A} receptors?

The concept that agonists may selectively direct receptor signalling towards specific intracellular pathways has been attracting increasing interest [27–29]. In the case of h5-HT_{1A} receptors, accumulating evidence indicates that agonist-dependent differential coupling is likely to occur. In ligand binding experiments, for example, the naphthylpiperazine derivative, 1-[2-(4-fluorobenzoylamino)ethyl]-4-(7-methoxynaphthyl)piperazine (S14506), and the anti-psychotic, clozapine, contrasted with other agonists by exhibiting much smaller decreases in binding affinity when receptor-G-protein dissociation was induced by GTP [30–32]. These data suggest that S14506 and clozapine selectively bind to receptors which are preferentially coupled to a tightly associated G-protein subtype.

At the G-protein level, Gettys et al. [33] provided an early indication that serotonergic ligands differentially induce h5-HT_{1A} receptor-mediated $G\alpha$ subunit activation. Using a CHO cell line, these authors labelled activated G-proteins with a photoreactive GTP analogue (4-azidoanilido-[α -³²P]GTP) and immunoprecipitated specific $G\alpha$ subtypes. The

¹ The issue of 5-HT_{1A} coupling to Gs is unclear. Malmberg and Strange [18] reported low-potency agonist stimulation of signalling via Gs in HEK cells, whereas Raymond et al. [19] and Barr et al. [20] failed to detect coupling to Gs in CHO, HeLa or Sf9 cells.

data indicated that, whereas some agonists (5-HT and 8-OH-DPAT) more potently activated $G\alpha_{13}$ than $G\alpha_{i2}$, rauwolscine activated both subunits with similar potency.

Further complexity in coupling may be identified in downstream responses. Thus, whereas the studies cited above indicate preferential coupling of h5-HT_{1A} receptors to $G\alpha_{13}$, a study of extracellular signal-regulated kinase (ERK) activation in Sf9 cells showed that, following inactivation of endogenous Gi/o G-proteins with pertussis toxin, $G\alpha_{i1}$ and $G\alpha_{i2}$ were the most effective $G\alpha$ subunits in rescuing h5-HT_{1A} receptor-mediated ERK phosphorylation [34]. Thus, activation of $G\alpha_{i1}$ and $G\alpha_{i2}$ may favour ERK phosphorylation, whereas activation of $G\alpha_{13}$ may direct intracellular signalling to other transduction cascades. This may provide a basis for the distinctive behaviour of clozapine mentioned above [32]. Indeed, clozapine only modestly stimulates ERK phosphorylation, compared with other 5-HT_{1A} receptor agonists [35]. Taken together, these studies strongly suggest that different agonists favour coupling of h5-HT_{1A} receptors to distinct G-protein populations. It should be noted that evidence of inverse agonist-directed trafficking of receptor signalling has not been available, but drugs that selectively attenuate constitutive activation of specific transduction pathways may exist (see below).

4. Investigating agonist and inverse agonist influence on specific G-proteins

Several efforts have been made to characterise the pharmacological profiles of ligands at 5-HT_{1A} receptors by examining coupling to specific G-protein subtypes. Firstly, specific G-proteins have been co-expressed or reconstituted with 5-HT_{1A} receptors in bacterial, insect and mammalian cells [14–17,36]. Secondly, h5-HT_{1A} receptor-G-protein fusion constructs have been designed to constrain the coupling of the receptor to specific G-protein subtypes in human embryonic kidney (HEK293) cells [37–39]. Thirdly, in the presence of co-expressed Regulator of G-protein Signalling (RGS) proteins, such constructs display enhanced agonist and inverse agonist signals [39], at least concerning GTPase activity measures. While these strategies have yielded much useful information, they are not adapted to detect regulatory mechanisms that depend on the availability of multiple G-proteins. In addition, such techniques, which require the use of recombinant systems expressing mutant receptor constructs, are unsuitable for investigation of receptor coupling in native tissues.

Recently, De Lapp et al. [40] reported a novel approach for determining G-protein subtype activation by muscarinic receptors using a rapid methodology which lends itself to the investigation of numerous ligands and experimental conditions. The experimental principle is based on rapid immunoprecipitation and [³⁵S]GTP γ S binding, whereby activated G-proteins (i.e. [³⁵S]GTP γ S-bound) are captured by specific antibodies. These are detected using scintillant-impregnated polymer beads coated with a secondary antibody (see Fig. 1). While no data were reported concerning the action of inverse agonists, De Lapp et al. [40] demonstrated that this technique allows detection of activation of differing G-protein families ($G\alpha_{q11}$ and $G\alpha_{i1,2,3}$) in membranes prepared from both recombinant CHO cells and native rat striatum. The applicability of this methodology to serotonergic receptors was demonstrated when the same approach was applied to h5-HT_{2C} receptors expressed in CHO cells [41]. h5-HT_{2C} receptors exhibited preferential activation of $G\alpha_{q11}$ subunits but,

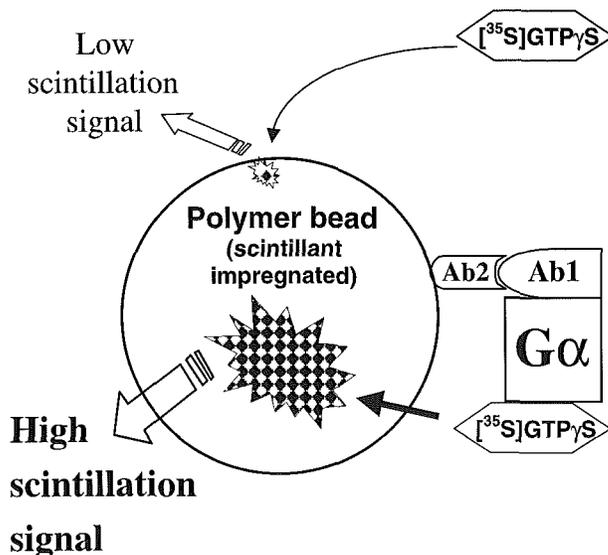


Fig. 1. Detection of G-protein $G\alpha$ subunit activation employing antibody capture and scintillation proximity assay (SPA) technology. Schematic representation of the antibody capture/SPA procedure [40]. A [^{35}S]GTP γ S-bound $G\alpha$ subunit is captured by a specific antibody (Ab.1). Ab.1 is itself recognised by the secondary antibody (Ab.2), which is covalently attached to the surface of a scintillant-impregnated polymer bead. Thus, the [^{35}S]GTP γ S- $G\alpha$ -Ab.1 complex is brought into proximity with the bead. Emission of β particle by the $G\alpha$ -bound [^{35}S]GTP γ S molecule produces scintillation. Emission of β particles by [^{35}S]GTP γ S molecules which are not bound to captured $G\alpha$ subunits is responsible for background non-specific scintillation signal.

at higher receptor expression levels, efficient coupling to $G\alpha_{i3}$ was also demonstrated. The authors employed the edited (VSV) isoform of h5-HT $_{2C}$ receptors which displays low constitutive activity [41,42]. Accordingly, no inverse agonists were identified.

In contrast, at h5-HT $_{1B}$ receptors expressed in CHO cells, robust constitutive activity was detected for the activation of $G\alpha_{i3}$ subunits [43]. This constitutive activation could be attenuated by the known inverse agonists, methiothepin and 1'-methyl-5-[2'-methyl-4'-(5-methyl-1,2,4-oxadiazol-3-yl)biphenyl-4-yl]-2,3,6,7-tetrahydro-spiro[furo[2,3-f]indole-3,4'-piperidine] (SB224,289) [4,6,44], whereas the agonists, 5-HT and almiditan, robustly stimulated $G\alpha_{i3}$ activation. Both agonist and inverse agonist actions were concentration-dependently reversed by a low-efficacy, selective 5-HT $_{1B/1D}$ ligand, *N*-[1-(2,3-dihydro[1,4] dioxin-5-yl)piperid-4-yl] indan-2-yl-amine (S18127). Taken together, these data support the validity of the antibody capture/scintillation proximity assay (SPA) procedure for investigating $G\alpha_{i3}$ subunit activation.

5. $G\alpha_{i3}$ activation at h5-HT $_{1A}$ receptors: conformational selection, protean agonism and inverse agonism

At h5-HT $_{1A}$ receptors, the antibody-capture/SPA procedure revealed complex interactions in receptor-G-protein coupling [45]. Indeed, 5-HT and other high-efficacy agonists,

such as 5-carboxyamidotryptamine (5-CT), S14506 and (+)8-OH-DPAT, yielded bell-shaped $[^{35}\text{S}]\text{GTP}\gamma\text{S}$ binding isotherms with peaks at nanomolar concentrations (Fig. 2A). Hence, the direction of the response to agonists (stimulation or inhibition) was dependent on the ligand concentration range examined and suggested that a conformational change occurs by which h5-HT_{1A} receptors ‘switched’ signalling to other G-protein subtypes. This hypothesis is supported by the fact that, in classical $[^{35}\text{S}]\text{GTP}\gamma\text{S}$ binding assays which do not distinguish G-protein subtypes, 5-HT-induced stimulation is biphasic, consistent with sequential activation of at least two G-protein subtypes (Fig. 3). The identity of the second G-protein is probably G α_{12} , in view of its high level of expression in CHO cells, and its known interaction with h5-HT_{1A} receptors [33,46].

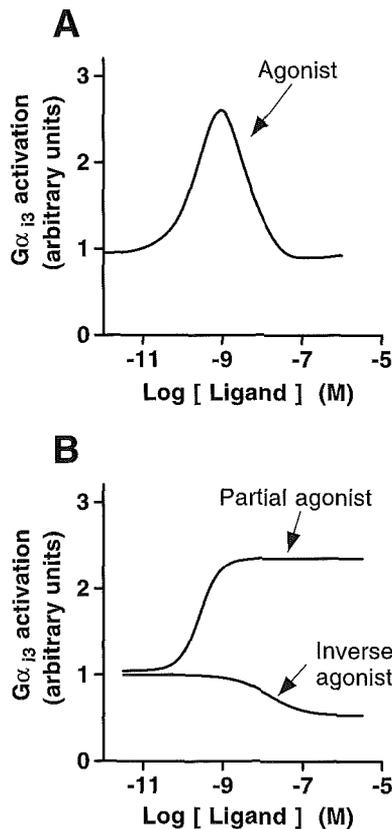


Fig. 2. Schematic representation of h5-HT_{1A} receptor-mediated G α_{13} subunit activation in CHO cells. Application of antibody-capture/SPA methodology to h5-HT_{1A} receptors resulted in three different patterns of response to serotonergic ligands [45]. Drugs that are known as high-efficacy agonists in classical $[^{35}\text{S}]\text{GTP}\gamma\text{S}$ binding experiments yielded bell-shaped isotherms for G α_{13} activation (Panel A). In contrast, partial agonists yielded sigmoidal stimulation isotherms and inverse agonists yielded sigmoidal inhibition isotherms (Panel B). Similar data have been reported for adenosine A₁ receptors expressed in CHO cells, although the precise G-protein subtypes were not identified [53,54].

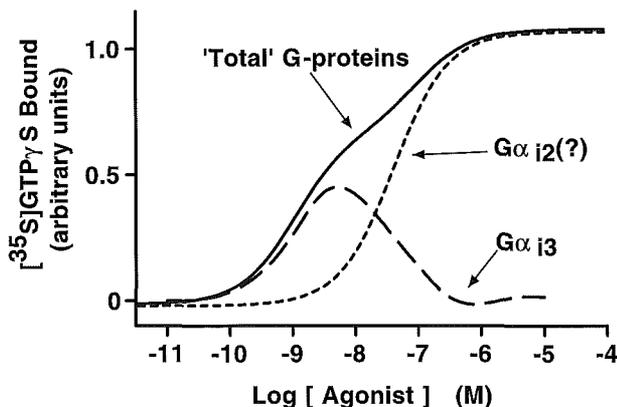


Fig. 3. Sequential activation of distinct G-protein subtypes by agonists acting at a G-protein-coupled receptor. Standard [^{35}S]GTP γ S-binding experiments often detect total G-protein activation, without distinguishing the contributions of individual G-protein subtypes. In the case of h5-HT $_{1A}$ receptors, the solid line represents the biphasic, [^{35}S]GTP γ S binding curve observed with 5-HT employing classical methodology [45]. This composite isotherm may be constituted of a bell-shaped agonist concentration–response isotherm for G α_{13} (dashes) and a sigmoidal concentration–response isotherm (dots) for another G α subunit. The latter may be G α_{12} , which is highly expressed in CHO cells [12].

Several comments should be made. Firstly, previous observations of bell-shaped drug concentration–response isotherms in *in vitro* models have classically been attributed to the presence of cross-reacting receptors [47,48] or promiscuous coupling to two or more signal transduction systems (for example to both stimulation and inhibition of adenylyl cyclase activity) [18,49]. In contrast, in the case of G α_{13} activation by h5-HT $_{1A}$ receptors, no interfering receptors were present in the recombinant system, as demonstrated by the blockade of agonist and inverse agonist actions by the selective 5-HT $_{1A}$ ligand, WAY100,635, and by the absence of response in membranes from non-transfected cell membranes. Further, only a single G-protein subtype was investigated, and the proximity of the G-protein activation response to receptor–ligand binding excludes the possibility of intervening promiscuous regulatory steps.

Secondly, unlike 5-HT, (–)pindolol displayed sigmoidal stimulation isotherms for G α_{13} activation, suggesting that it is not capable of inducing the conformational switch observed with higher efficacy agonists. Thus, when acting at h5-HT $_{1A}$ receptors, (–)pindolol maintains h5-HT $_{1A}$ receptor signalling to G α_{13} over a wide concentration range. Pindolol has been previously reported to behave as a partial agonist in classical [^{35}S]GTP γ S binding assays [50], but its maximal stimulation of G α_{13} activation was comparable to that of 5-HT. (–)Pindolol may therefore display higher efficacy at G α_{13} compared with other G-protein subtypes.

Thirdly, ligand actions on G α_{13} activation were highly susceptible to modulation by sodium ions. Indeed, when the sodium concentration was lowered (increasing basal G α_{13} activation), the actions of (–)pindolol were reversed from activation of G α_{13} to inhibition. Conversely, WAY100,635, which exhibits sodium-dependent inverse agonist properties in HeLa cells [21], stimulated G α_{13} activation in CHO cell membranes (in the presence of

sodium), indicating that it possesses mild agonist properties under these conditions. Sodium ions also profoundly influenced $G\alpha_{i3}$ activation by 5-HT: only stimulatory actions were observed at high sodium concentration, whereas only inhibitory ‘pseudo-inverse agonist’ actions were seen at low sodium concentration. Thus, for this G-protein subtype, the actions of the endogenous agonist, 5-HT, can be altered not only in magnitude but also in direction. More generally, ligands which display variable direction of response, depending on the level of constitutive activity (or ‘tone’), are known as ‘protean agonists’ [28,51] and the results discussed above for h5-HT_{1A} receptors suggest that $G\alpha_{i3}$ subunit activation may be particularly sensitive to such ligands.

Fourth, the inverse agonists, spiperone and methiothepin, sigmoidally inhibited [³⁵S]GTP γ S binding. Their isotherms were not mirror images of those of 5-HT: no stimulatory phase was observed, producing U-shaped curves. Further, spiperone and methiothepin did not exhibit protean behaviour, that is, even when sodium concentration was modified, they invariably exhibited inhibition of basal activity. These data suggest that spiperone and methiothepin selectively stabilise/induce receptor conformation(s) which are distinct from those recognised by agonists or partial agonists. Further, these observations indicate that, to distinguish true inverse agonism from protean agonism, assays must be carried out under a variety of conditions (e.g. different sodium ion concentrations) favouring or suppressing constitutive activity. It is interesting that spiperone exhibited greater negative efficacy than methiothepin in CHO cell membranes [2,9,10] but opposite effects were observed in HeLa cells [1]. Further, in HeLa cells, the negative efficacy of spiperone, but not methiothepin, was significantly increased by removal of sodium from the incubation buffer [1], providing additional support for differential influence of these ligands on receptor-G-protein coupling [52].

The anti-psychotic agent, haloperidol, exhibited inverse agonist activity for $G\alpha_{i3}$ subunit activation, whereas classical [³⁵S]GTP γ S binding assays in CHO cells, and GTPase assays at h5-HT_{1A}- $G\alpha_{i1}$ and h5-HT_{1A}- $G\alpha_{o1}$ fusion proteins failed to detect this activity [2,39]. These results suggest that haloperidol may preferentially exert its h5-HT_{1A} receptor inverse agonist properties via $G\alpha_{i3}$ subunits, i.e. haloperidol may provide an example of inverse agonist-directed trafficking of receptor signalling. This is consistent with the finding that it robustly inhibits [³⁵S]GTP γ S binding in HeLa cells, which express predominantly $G\alpha_{i3}$ subunits [1,13]. Another anti-psychotic agent, risperidone, weakly stimulated [³⁵S]GTP γ S binding in filtration assays in CHO cells [43] but inhibited it in HeLa cells in the absence of sodium [1]. Taken together, it is tempting to speculate that such differences in magnitude and direction of G-protein activation may be due to coupling of h5-HT_{1A} receptors to distinct G-protein populations in these cellular expression systems and raise the possibility of identifying inverse agonists which selectively inhibit signalling via specific G-proteins subtypes.

6. General comments

Recent discoveries in receptor pharmacology, including promiscuous coupling of receptors to multiple G-protein subtypes and ligand-directed trafficking of receptor signalling, have highlighted the need to refine descriptions of drug efficacy for G-protein

activation. In this context, the antibody-capture/SPA methodology provides a useful means of elucidating diverse receptor interactions with specific G-protein subtypes. The complex actions of agonists and inverse agonists on h5-HT_{1A} receptor-mediated G α_{i3} activation discovered using this methodology may be paralleled by similar effects at other GPCRs. Indeed, in a preliminary study, Browning et al. [53,54] reported bell-shaped [³⁵S]GTP γ S binding isotherms at adenosine A₁ receptors expressed in CHO cells. The precise G-protein subtypes activated were not identified, but the presence of bell-shaped isotherms was dependent on high receptor expression levels and, as in the case of h5-HT_{1A} receptors, the use of high efficacy agonists, whereas an inverse agonist exhibited sigmoidal inhibition isotherms [53,54]. These data suggest that changes in receptor conformation producing sequential activation of different G-protein populations may occur for a variety of GPCRs. Although the mechanistic basis is unclear, the possibility of receptor desensitisation, dimerisation and allosteric interactions has been evoked [43,53,54].

In conclusion, further biochemical and pharmacological studies are necessary to investigate the nature of protean and inverse agonism at h5-HT_{1A} receptors coupled to diverse G-protein subtypes. Further, it would be interesting to apply antibody capture/SPA methodology to native tissues in order to determine whether the complex phenomena observed for 5-HT_{1A} receptors in CHO cell membranes are paralleled in brain regions associated with control of serotonergic transmission.

Acknowledgements

I thank Cristina Cosi and Mark Kleven for useful comments on the manuscript and Gabrielle Guerin for assistance in its preparation.

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Discussion 9

M. Lohse

In one of your first slides, when you introduced the antibody to the Gα₁₃ subunit, it appeared to me that it was the high affinity component that was Gα₁₃ specific. Then you showed the bell-shaped curves and you lost GTPγS binding at high concentrations of 5HT. And you also said this was Gα₁₃.

A. Newman-Tancredi

No, the high potency was the Gα₁₃. In this slide you can see a Gα₁₃ component which seems to be resistant to the dissociation of the GTPγS. But at high concentrations of 5HT we have our very rapid dissociations of GTPγS.

M. Lohse

If it is coupling to another subunit from which GTPγS can rapidly dissociate by whatever mechanism, do you have any idea what this subunit could be?

A. Newman-Tancredi

We're suggesting that it's Gα₁₂. Because we know Gα₁₂ is highly expressed in the CHO cells. We've also carried out very preliminary experiments using this antibody capture strategy directed at Gα₁₂.

M. Lohse

This bell-shaped yellow curve you showed, is that an ordinary GTPγS binding assay, or is that an assay to which you added GTPγS?

A. Newman-Tancredi

This is just a summary and schematic illustration of what we think is happening. What's measured is that other one, where we have this bell shape here following differential dissociation from different Gα subunits. It looks like the Gα₁₃ is resistant to the GTPγS dissociation. But you only see it up until a certain concentration.

M. Lohse

Why would the receptor Gα₁₃ complex, which has high affinity, dissociate at high agonist concentrations, where then the receptor then captures Gα₁₂? Why would this high affinity complex not remain stable, as in ordinary biphasic binding experiment?

A. Newman-Tancredi

I think you have put your finger on the point. Why does it come down? At the moment I don't have an answer for that. Nigel Birdsall from the Medical Research Centre

in London has reported bell-shaped isotherms, like that, for adenosine A₁ receptors. He did standard GTP γ S binding experiments without targeting individual G proteins. Results were dependent on the efficacy of the ligands that he tested, so he only saw this for high efficacy agonists, but not for partial agonists or even for inverse agonists. He also showed that was dependent on receptor expression level. There is a paper that he is now going through, which should be appearing in a few months, where he shows this at α_{2a} -adrenoceptors. All these studies were carried out on CHO cells. Is there something in CHO cell membranes which can associate to a receptor under certain conditions and change a confirmation so it will push the signalling to another G protein? I know there are certain RGS proteins present in CHOs-RGS3, I believe-so is there something present in CHO cells, specifically, which could be directing this? Maybe someone can comment on that.

R. Bond

I'm still not convinced that this Occam's razor explanation for this is what someone would call strength of signal. The 5HT is so efficient at producing R* that you saturate one G protein and move on to the other. And the low efficacy agonists can't do that, they're not capable. And the second thing that I wanted to ask you was about the role of 5HT, which is obviously unique, that it can be a hormone and a neurotransmitter, and it has a nanomolar and micromolar affinity.

A. Newman-Tancredi

Let me just pick up on one of those two points. As for the strength of signal point, I find it hard to use that in this situation, because the signal is switching off. We're not seeing activation of one G protein at low concentrations and then additive activation of another G protein on top of that. We're seeing activation of one G protein, which then stops, and then activation of another G protein. Now if it's a strength of signal response, I wouldn't expect to see that, I would expect to see something else which keeps adding on, as you push the concentration up, or as you push the receptor expression up. So that doesn't seem to fit, in my mind.

R. Bond

But I would assume that the receptor could bind to G α subunits. I mean, you'd have to get rid of one to get the other one.

We have seen data on whether the inverse agonist is faithful, and it doesn't flip around, and agonists can do both. You said something like "the agonist can do both, and the inverse agonist always stays as an inverse agonist". But from the receptor point of view, the constitutively active receptor is faithful to one G protein, whereas the ligand-activated receptor is promiscuous. Is that right?

A. Newman-Tancredi

Imagine two extremes from a full agonist to a full inverse agonist. In between you'd have partial drugs, which would switch around and go one way or the other, depending on receptor tone. What surprised us and even shocked us, that a full agonist such as 5HT looked like an inverse agonist under those low-sodium conditions. That made me ask the question "Is there a drug which cannot switch around?" If 5HT does, is there a drug which can't? Is there such a thing as a true, absolute inverse agonist, which will always remain inverse? Should we just drop the definition of "full agonist" and "full inverse agonist" and just talk about protean drugs?

R. Bond

I use them but, when I lecture to the graduate students, I say everything to me is a ligand. You get binding and after that all bets are off. I've seen data with isoprenaline as an inverse agonist in old TG4 mice from Sir James Black's group. I believe that we will still continue to say that epinephrine is an agonist, adrenaline is an agonist and ICI-118551 is an inverse agonist, but I like the ambiguity.

L. Prezeau

GABA is the endogenous agonist on GABA-B receptors, but when we mutate the receptor in a special way, we have constitutively active receptors, and then GABA is an inverse agonist. We don't know exactly the mechanism for that but it's really puzzling.

M. Brann

If you look at this problem from a thermodynamic perspective, you look at the modelling of the R and R*, and the kind of data that predicts about the idea of agonists, antagonists and inverse agonists, the differential affinity between states, those models can predict the affinity of ligands. It's not a state-dependent thing. Agonists should be agonists, antagonists should be antagonists, and inverse agonists should be inverse agonists, and it's all predicted by a relative affinity difference for the states in question. Our experience actually, through random mutagenesis, very large number of compounds coupling to very large numbers of G proteins with large numbers of receptors, reveals that first, second and third approximation, it works. The majority of the exceptions to this rule are things that we've appreciated in pharmacology for years: receptor reserve fakes you out, you think of partial agonists as a full agonist. One of the things that fakes people out is that they don't realise how huge the effects of things like receptor reserve can be. I've seen receptor reserve shift the EC₅₀ of agonists one thousand-fold. You can convert things that look like completely pure full agonists to antagonists with just receptor reserve. Just invoking the old things we've known about for 50 years, you can explain most of the phenomenology that we talked about in terms of differential pharmacology. An inverse agonist is an inverse, you look at in every system that you want to look at it in, and a partial agonist can be almost anything depending on what the receptor reserve is. Although there are exceptions, those rules actually usually do work.

H. Giles

I think the only way you can classify ligands is on a relative basis. And I think that if you start using "relative", then you can say that one compound is more of an inverse agonist than the other one. But from my experience, a single ligand doesn't behave the same when you start crossing systems. Then you only need to look at the clinical trials that have gone on with compounds which were supposedly antagonists and they turn out to be agonists once you get into man. And I'm sure we'll start seeing the same things with inverse agonists. So relativity is, for me, the way to quantify.

R. Bond

I do agree with Mark Brann on the fact that fundamentals of receptor theory are often ignored and the concept of receptor reserve. But a lot of those principles are forgotten, and we add complexity that we don't need. But there are still a substantial number of drugs that convert from inverse agonism-from negative efficacy to positive efficacy, and that can't be done in a two-state model.

W. Clarke

One of the things that I agree with Mark Brann, is that with a number of ligands drugs that we've looked at on our system, studying multiple signal transduction pathways coupled to a single receptor, you can see that they have the same, or similar *qualitative* properties, they're inverse agonists for example. But they may differ in their quantitative relative efficacy, which really cannot be explained with a two-state model. Differences in relative efficacy have to be explained by a more complicated model than a two-state model.

Beyond that I think that where we're going to see that the largest differences in ligand efficacy is when we're studying receptor signalling to disparate signalling systems. Most of us here have been talking about G proteins, but we should to call these GPCRs seven transmembrane spanning receptors. They can couple to a variety of other signalling cascades, and I suspect that if you think about receptor confirmations that may be promoted or stabilised by different ligands, that the likelihood of the signalling molecules detecting those different confirmations will be greater if were looking at a G protein-mediated response versus a non-G protein-mediated response, as opposed to two G protein-mediated responses that interact with a similar region of the receptor. Within G protein-mediated responses we may not see much difference in qualitative nature of ligands. But between responses mediated by disparate signalling mechanism, that's where the qualitative differences may show up. And then in vivo, all bets are off. The biggest black box that I see in pharmacology today is the dissociation between what we do at the cellular level-in dissecting R^* , and $GTP\gamma S$ binding and then second messenger effects-to what happens in a living breathing human being. Until we figure that out, some of these questions are going to be very difficult to work out.

A. IJzerman

In fact, to me, "receptor reserve" is a nice term. But what we try to do here is to find some molecular mechanisms behind nice terms, like what is inverse agonism? I've never felt very comfortable with the words "receptor reserve" to explain what we see in many experimental studies. One should like to know what it in fact means. Is there something between R and G coupling efficiency that's going on? Is it really about receptor numbers? Even with a term such as "receptor reserve" that shows up in every textbook, eventually one should like to know what is going on at the cellular level.

M. Brann

As definition of "receptor reserve", we all know the pharmacological predictions of having a very well-coupled system that you only need a tiny proportion of the receptors to give you a maximum response, or a ligand that has a tiny proportion of the maximum that can be effective. You can't see the difference of it, and one is much more robust, because it's so sensitive. I mean, that's one of the most fundamental things that we all learn in pharmacology, and there's an awful lot of phenomena that can be explained by that. And it's actually amazing, in the modern molecular pharmacology literature, how many papers are written, obviously, by people who never learned those very basic principles.

W. Clarke

Yes, there are a number of cases where, certainly people have referred to trafficking of receptor stimulus which can be explained by differential receptor reserve toward different signalling pathways. That's an important thing that needs to be evaluated, and it's often

mistaken. But there are still cases where there are differential relative efficacies of ligands, in fact reversal of efficacy/potency order occur, that can't be explained by a receptor reserve issue.

T. Schwartz

If you go to IM channels and GABA, there's some interesting things previously commented on that a ligand will stabilise a conformation with a sort of highest affinity for certain conformation of the receptor. Then it's really a matter of what that leads to. If you take an IM channel, there are very good examples where a compound open or close the channel, or will hold it in the closed conformation. But then if you make a mutation, that might be the open case. What that leads to really depends on the interaction of the whole cellular system, and that will change, which means whatever you're looking at will change. Then it is true that a ligand is a ligand, and it does something to the receptor. But it depends on all the connecting things later on, that the system, whether that is agonism on one G protein, or change to another one. The ligand does something, that is very simple, but what that leads to varies tremendously, it depends on the co-players, where in the cell it is, what it has, and how much it gets influenced by other mediators.

M. Brann

I don't dismiss or doubt the data that's been spoken about, where inverse agonists become agonists, and I don't have any explanation for that in the R* simple models. I think this is something where a lot of the very recent molecular pharmacology literature may be misleading people, and my point of view is that for a first approximation, the simple explanations, those old, robust pharmacological interpretations, usually do work. The concept of inverse agonist is a useful concept, and we shouldn't teach our students to think that every single time it's going to be a state-dependent thing, because at every single time it's a state dependent thing, forget about drug discovery, forget about pharmacology, pharmacology should give up.

M. Lohse

I'd like to come back to this issue of the two-state model. About two years ago we had a conference in Barcelona, and many of us attended it, and during that conference I think it was agreed that the two-state model doesn't exist, or it's just an approximation. We do know that the two-state model is a big simplification which works for many things, but if you look at rhodopsin, as the simplest possible receptor, we know there are at least eight different states, and they adopt it in a sequence. So, you know, binding of a ligand to a receptor may induce one conformation at one time point, and then another conformation at another time point. Now we are dealing with three-dimensional switches, so they will adopt all sorts of conformations, some of which are active towards some signalling pathways, others may be active towards other signalling pathways, and they may change over time. And we know the two-state model is easy and successful to explain the switch but wrong.

H. Giles

We have to oversimplify in order to provide frameworks for thought. I think that generalities can often fit within that framework. But when you come down to details, we all know that actually the simple models don't work. For example, in this general framework the two-state model just provides some basis for thought and for the understanding that there are partial, full or inverse agonists. But when we're trying to

understand detailed scientific data we have to remember that those are not big overarching general principles.

P. Strange

The two-state model is an operational model which describes pharmacological observations, but it doesn't describe any mechanisms. It doesn't mention G proteins. Although the paper is very nice and very well written, I think it's been enormously misleading, actually.

W. Clarke

One other thing I'd like to point out about the more multi-state models is that even though there may be multiple active conformations, that their activity is dependent upon the cell system. And a conformation that may be active towards one signalling pathway could indeed be inactive towards another. Additionally, a receptor that stabilises one or more of those other active conformations could therefore act as an inverse agonist by shifting the population for R* and R**. If it stabilises R** it could reduce the quantity of R*. And thereby act as an inverse agonist towards one pathway, and act as an agonist towards another.

R. Bond

I'd still say that adrenaline is an agonist and stimulates. I still say that β -blockers will usually decrease heart rate, and I still believed that inverse agonism was important to us. But when you have drugs succeeding and failing, and it becomes that one's an inverse agonist, the other one's an antagonist, or the other one is less of an inverse agonist, or whatever, I think some of us have to keep in mind that every time we teach or we edit, we never tell the truth. Because if you gave every exception, you would never be able to tell a story and we need to tell stories. I think we use concepts as agonist, antagonist and inverse agonist, but there are exceptions which may be very relevant in drug discovery.