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Different mechanisms of negative efficacy. Distinguishing inverse agonists from negative antagonists

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Abstract

The subject of negative efficacy at seven transmembrane receptors has attracted increasing interest during the past years. We briefly review how that started and the reasons why this phenomenon is commonly interpreted as an indication that some ligands can inhibit the constitutive association between receptors and G proteins. However, as shown for a cannabinoid receptor antagonist, negative efficacy may also be the outcome of ligands that stimulate receptors to lock G protein in nonactive form. We suggest that inhibition of spontaneous receptor activity and stimulation of receptor-mediated G protein sequestration underlie different mechanisms of negative efficacy and justify the distinction between negative antagonists and inverse agonists, respectively. Using a simplified model to describe ligand-induced shifts in guanine nucleotide binding, we propose that these diverse mechanisms of efficacy may correspond to distinct patterns in the way agonists affect the binding properties of the G protein.

Keywords: Inverse agonism; Negative antagonism; G protein coupled receptors; Drug efficacy; GDP binding

1. Introduction

The terms negative or inverse efficacy refer to the property of some ligands to elicit a receptor response opposite to that of natural or synthetic agonists.

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In its broadest sense, negative efficacy may result from a variety of different mechanisms, especially if we consider the heptahelical family of G protein-coupled receptors (7TM or GPCR) and the host of molecules that regulate their activity. Potential candidates for inverse agonistic activity are not only ligands that bind to the receptor at, or outside of the agonist-binding region, but also compounds that interact with the G protein subunits at their regulators recognition sites or with molecules involved in desensitization.

From the perspective of drug discovery, this wider range of potential GPCR-related targets has not been extensively explored so far, and there is no doubt that it may disclose new potentially useful medicines in the future [1,2]. However, in discussing molecular mechanisms, it is useful to restrict the subject according to the primary target of interaction. In this paper, therefore, we only consider the subset of inverse agonists that interact with the agonist-binding pocket of the receptor. One may call such ligands *competitive antagonists with negative efficacy* or *isosteric inverse agonists*, although, as proposed later in this chapter, such apparently equivalent terms might better be adopted to distinguish different mechanisms of negative efficacy.

2. History, in pills

The idea that some competitive antagonists can have negative efficacy at 7TM receptors was first proposed on theoretical grounds by Wreggett and De Lean [3] and experimentally demonstrated later for a peptide antagonist of the delta opioid receptor [4]. But the term inverse agonist is actually older, as it was originally introduced [5] to describe the effects of β-carbolines on the benzodiazepine-binding site of GABA-gated channels [6]. That site, however, is located on a subunit distinct from that interacting with the natural agonist [7].

Although agonist-independent GPCR receptor signaling and negative effects of antagonists were even observed in "physiological" experimental systems [8,9], the issue remained little more than scientific extravaganza for some time. To raise widespread interest on negative efficacy was the observation that discrete mutations of receptor sequence can generate or greatly enhance the level of ligand-independent receptor activity [10,11]. The finding that receptor-activating mutations can occur spontaneously and may be a cause of inherited diseases [12,13] suggested at least one first potential therapeutical application for antagonists with negative efficacy. Therefore, antagonists with negative efficacy have been searched and found for many types of GPCRs (see reviews Refs. [14–18]).

3. Models and mechanisms

The concept most often evoked to explain negative efficacy is based on the "linkage theory" of Wyman [19] or the equivalent "free-energy coupling" principle of Weber [20], both of which are generally used to rationalize allosteric reactivity in proteins. Accordingly, we can regard the receptor, or the G protein, (or also, obviously, both), as interacting proteins existing in two or more functionally distinguishable and interconvertible states.

Ligands would then act by stabilizing such states to produce activity [21], or to reverse it if the intrinsic equilibrium of the system permits some extent of spontaneous activation to occur and be detectable in the experimental system [22]. This concept does not identify a precise mechanism, of course, but it does allow to draw a number of possible reaction schemes or "models" of receptor activation, which, in turn, may be useful to test hypotheses about mechanisms. Thus, starting from the original "ternary complex model" [3,23], several others were proposed [11,24–29], which differ in the number of receptor states or paths that are explicitly considered and, consequently, in the number of parameters that are needed to balance the thermodynamic-free energy changes.

Despite the differences, all such models describe efficacy primarily as a property of the ligand–receptor complex, which reflects the ability of each different ligand to facilitate or hinder the interaction of the receptor with the G protein. The transducer side of the activation process is a default, i.e. G protein response to receptor activation (or inactivation) is considered to vary only quantitatively and not qualitatively with changes of ligand efficacy [29,30].

This limitation is not intrinsic in the models, as they may be reformulated to explicitly address qualitative differences in the G protein response. It rather reflects two orders of experimental restraints.

One is the result of earlier studies on the kinetics of GTPase turnover and α-subunit activation in purified Gαβγ trimers [31–34], which points to GDP-release as the sole limiting step that receptors control to trigger G protein subunit dissociation and activation. Reconstitution experiments with purified receptors and G proteins have largely confirmed such notion [35–37], although only in few cases, ligands of different efficacies were compared [38,39]. In a study, where the activations by full agonist and partial agonist-occupied receptor were confronted, different efficacies appear to result from differences in receptor-Gα association, not in turnover of GTP hydrolysis [40]. This again points to a scenario where agonists only direct receptor-Gαβγ association, not a conformational perturbation that occurs in the G protein.

A second factor is the difficulty to measure the effect of ligand-occupied receptor on G nucleotide binding parameters, even in cells overexpressing recombinant receptors. To overcome the problem, reconstitution protocols, eventually aided by chaotropic extraction/denaturation of endogenous α-subunits, were proposed [38,39], but this requires the technically demanding purification of Gα and Gβγ subunits on a routine basis.

Without reconstitution, the effect of receptor activation on nucleotide-binding isotherms in cell membranes is quite small, being masked by the background-binding activity of all GTP-binding proteins of the cell. This makes it impossible to measure how diverse agonists alter the apparent affinity of GDP or other properties of the Gα subunit nucleotide recognition site. What is usually measured in these experiments, instead, is an agonist-induced change in GTPγS binding at a fixed concentration of GDP, which only allows to explore variations in ligand concentrations, not in G nucleotide binding affinities.

Moreover, the inclusion of nucleotide binding reactions in the models of receptor G protein interactions, although feasible, surely adds enormous complexity in terms of number of parameters and allosteric factors to the extent that it may render the system virtually untreatable. For example, a model describing all the interactions among the five relevant species, receptor, Gα, Gβγ, ligands and G nucleotides was described [25], but it

required a computer-driven automated exploration of the parameter space to be analysed. Although the number of possible configurations consistent with experiments was reduced down to a few hundreds from a seven-digit figure, the predictions of that model remain far too blurred to be testable.

4. Inverse agonist vs. negative antagonist

Perhaps, for all such reasons, very little progress beyond what had been firstly proposed, was made in term of mechanisms, despite the growing number of studies on inverse agonism.

There is, however, one outstanding exception. Bouaboula et al. [41] reported neat experimental evidence that an antagonist at CB_1 cannabinoid receptors (SR141716A) can suppress constitutive receptor activity through a quite different mechanism. Rather than reducing the spontaneous association of the receptor with the G protein, this ligand appears to share with agonists the ability to stabilize that interaction. Unlike agonist-bound receptor, however, the SR141716A-receptor complex seems capable to block the Gα subunit into an inactive form.

Although the cubic ternary complex model (CTC) [26] can be used to explicitly account for a type of ligand that while facilitating receptor-G protein interaction results in an unproductive ternary complex species, it is clear that the implications of that finding may go much farther than the plain suggestion of a way to interpret previous models. In fact, in pending the identification of the exact mechanism, this finding may even challenge the prevailing idea that the Gα subunit can be regarded as a simple on–off switch, where the receptor is the catalyst of GDP release and its ligands only regulate, in positive or negative way, its catalytic efficiency [42].

It is not entirely clear yet how SR141716A inhibits Gα activity. It has been shown that CB_1 receptors occupied by this ligand can reduce the stimulation of GTPγS binding induced by a mastoparan analogue. If mastoparan in membranes directly stimulates Gα subunits at the receptor contact site, as it does in purified proteins [43,44], then SR141716A may simply promote the receptor to sequester Gαi in its basal state. However, the ligand also inhibits IGF-1 and insulin-mediated stimulation of MAP kinase, which requires PI-3 kinase and Gβγ subunits. While the mechanism of growth factor receptor-promoted βγ release from α subunits still needs clarification, it is not probably mediated by the GPCR interaction surface of Gα. This suggests that the SR141716A-occupied receptor might exert a more "active" inhibitory role on Gα, such as, for instance, enhancing Gβγ affinity, thus preventing its release by other stimuli. If so, the compound must change through the receptor the basal properties of the Gαβγ heterotrimer.

Also important is the fact that although SR141716A stabilizes receptor → Gα association, its receptor binding affinity is enhanced by guanine nucleotide. This behavior, according to current models, should instead be expected for ligands that stabilize the uncoupled form of the receptor. Which means that the nucleotide's effect on ligand binding may not result from dissociation of receptors from α-subunits, but from the direct transfer of conformational perturbations between the binding sites of the two preassembled proteins.

Regardless of the exact mechanism, such results designate at least two different modes of negative antagonism. Ligands acting like SR141716A share one trait in common with agonists, the ability to stabilize receptor → Gα association, and another common to ligands with negative efficacy, the ability to reduce spontaneous receptor activity. For such reason, we should reserve the term *inverse agonist* for this type of ligands, and ought to employ that of *negative antagonist* for ligands which reduce receptor-Gα interaction, thus residing in the opposite edge of the spectrum of efficacy compared to agonists.

5. Alternative views of ligand efficacy at GPCR

Are all "negative ligands" inverse agonists like SR141716A or does either kind exist? And if so, can both occur at the same class of receptor-G protein pair? And further, should we not imagine also diverse means to generate full agonism?

All such questions add new texture to the problem of efficacy. In principle, the experimental strategy used by the Sanofi group [41] could be applied to all known negative ligands to discriminate inverse agonists from negative antagonists, eventually. Which is far easier to say than make, however, as not many experimental models allow detecting how diverse signalling paths can compete for and saturate a single class of intermediating G protein.

Even if further experimental enlightenment is needed, it is perhaps time to think of alternative ways to model ligand efficacy at GPCRs.

There are two particular features that, if experimentally established, could not be accounted for by current models of ligand efficacy. (a) One is the possibility that a ligand, upon binding to the receptor, can alter G protein properties beyond its basal or maximally active state. (b) Another is that guanine nucleotides by binding to the Gα subunit may change the affinity of the ligand for the receptor, regardless of their effects on receptor-Gα association and dissociation.

One possible way to explain such phenomenology is that the Gαβγ trimer can "read-out" the conformational changes that ligands induce on the receptor, and react proportionally. This prospects a new scenario, where there is full transfer of conformational perturbations between the partners of the receptor-Gαβγ complex, so that the whole repertoire of their intrinsic and extrinsic interactions can be affected. Efficacy then may depend on the "flexible" structural properties of every ligand-receptor–G protein assembly, rather than on the ability of ligand-receptor complexes to exert differential catalytic activation on a variable pool of competing G proteins.

Pushed to the farthest, this notion lets us think of GPCRs as preassembled R-G multimeric entities, pretty much in the same way as we usually consider ionotropic receptors the result of precoupled multiple subunits (at this class of receptors, incidentally, the issue of ligand-induced changes of binding affinity among subunits has never been an obsession for building models of agonism).

In such precoupled R-G units, agonist binding may not exclusively affect the stability of the complex, but also influence concurrently and differentially several G protein properties, such as transition into active form, binding affinities for GTP and GDP,

strength of Gα–Gβγ association. If so, ligand efficacy would then result from the balance of multifactorial perturbations transmitted across the electrostatically binding surfaces of receptor and Gαβγ, rather than from changes in the probability of their interaction. Thus, instead of regarding GPCRs as membrane enzymes that have evolved to become catalysts of the ubiquitous GTPase superfamily, we might consider heterotrimeric G proteins as GTPases that have specialised to become subunits of the 7TM receptor superfamily.

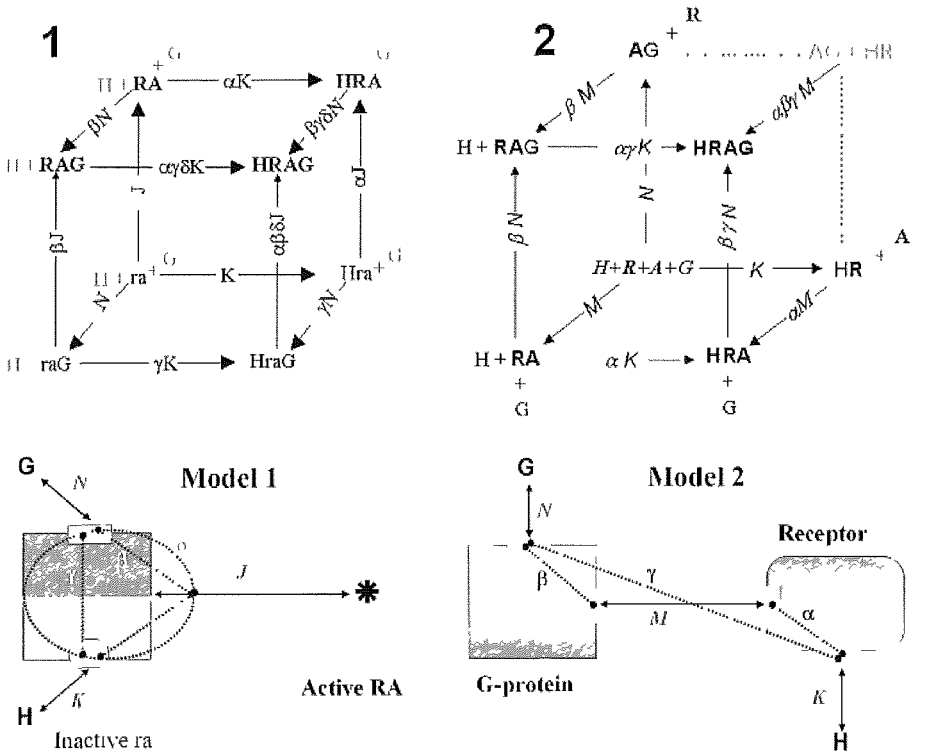
6. Ligand efficacy seen from the perspective of the guanine nucleotide binding site

The ternary complex model was originally build to predict the effect of G protein on the binding of ligands to the receptor [23]. This legacy, shared by all its offspring, is the main reason why current models of efficacy ignore the nucleotide binding site of the G protein. However, efficacy can also be described in the other direction, from ligand effects on guanine nucleotide binding affinity.

Here, we present two equivalent, albeit different, models designed to express ligand efficacy on nucleotide binding in the simplest possible way. To maintain ease, we did not include Gβγ as a separate reactant in either model, which does not mean to ignore its role. The implicit assumption here is that both Gα and Gβγ are in contact with the receptor [45–47], and Gβγ dissociation (provided that it does occur [48]), can be implicitly assumed to concur with GDP release. Obviously, the explicit addition of βγ [25] is a next necessary step, once the ability or inability of the simplified models to explain experimental data will be fully explored.

In one case (Model 1, Fig. 1, left), we treat receptor and Gαβγ as a stable assembly of proteins (ra), with two separate binding sites for ligand and G nucleotide, respectively. The complex is thought to shuttle between two functional states according to a single stability constant, J=[RA]/[ra] (in which are embedded the intermediate substates Ra and rA and their corresponding stabilities). In the other case (Model 2, Fig. 1, right), we draw the reactions among four binding partners, ligand (H), receptor (R), Gαβγ (A) and nucleotide (G), where the interaction between R and A is governed by a macroscopic bimolecular binding constant, M=[RA]/([R][A]) (which would comprise substates resulting from the multiple interactions of Gβγ and Gα within themselves and with several domains of the cytosolic loops of the receptors [49]). Results of both models are equivalent, particularly when J (in model 1) or M[R] ≈ M[A] (in model 2) are ≥ unity. However, while model 2 allows the study of the possible competition between different kinds of receptors interacting with one G protein, or viceversa, model 1 dissects activation (interconversion) and nucleotide binding as distinct steps.

We named the allosteric constants of the two models to bear similar meanings. Thus, α and β represent the effects of receptor ligands (H) and nucleotides (G), respectively, on either the isomerization process of model 1, or the R → A interaction of model 2. In both, γ gauges the allosteric interaction between the H- and G-binding sites. The 2nd order constant δ, which is only necessary in model 1, can be set to unity for simplicity, and ignored at the moment.



Allosteric constants

constant	Model 1	Model 2
α	Effect of H binding on RA interconversion	Effect of H binding on the stabilization of R → A
β	Effect of G binding on RA interconversion	Effect of G binding on the stabilization of R → A
γ	Effect of H binding on the binding of G	Effect of H binding to R on the binding of G to A
δ	2nd order constant	---

Fig. 1. Reactions schemes describing the effects of receptor ligands on Guanine nucleotide binding. Equilibrium binding of a receptor ligand (H) and guanine nucleotide (G) at their respective sites are depicted as arrows with superimposed binding or stability constants. In 1, the receptor and the Gαβγ heterotrimer are treated as single protein complex interconverting between two functional states ra and RA. In 2, the receptor (R) and the G protein (A) interact according to a bimolecular association constant (see text for more details). The cartoons underneath each model illustrate the linkage relations associated with the allosteric factors, named similarly in the two models. Their meaning is given in the table at the bottom.

In the presence of two diverse G-nucleotides (say, GTP-and GDP-like) which, respectively, may promote ($\beta>1$) and demote ($\beta<1$) the state transition (or the $R \rightarrow A$ interaction in model 2), the addition of ligand will differentially affect their relative binding depending on the factor α and γ. Both factors measure ligand's efficacy since they influence R/A reactivity (regardless of whether that is functional interconversion or interaction). However, while α describes effects of ligands on nucleotides binding, which results indirectly from ligand-mediated changes of R/A reactivity, γ does the opposite. Thus, in both models, γ is the explicit statement that the ligand-bound receptor can affect the nucleotide-binding site in a qualitatively different manner than the empty receptor does.

To illustrate the role of these two components of ligand efficacy, we have imagined two distinct scenarios and use model 2 to generate G nucleotide binding data (but quite similar results can be produced with model 1).

In both scenarios, the simulated system consists of two different guanine nucleotides, G_1 and G_2, in the absence or presence of saturating concentrations of receptor ligands of varying efficacies. The simulated experimental observable is the fractional binding of G_1 (assumed to be radiolabeled), under increasing concentrations of G_2. The factors β_1 and β_2 are set ≥ 1 and $\ll 1$ for G_1 and G_2, respectively. Thus, the system alludes to experiments where unlabeled GDP is in competition for the binding of radiolabeled GTPγS [50–52].

In the first scenario (Fig. 2a), ligands efficacy is assumed to vary only for the α value, being γ constant and set to 1 for either nucleotide. In this situation, the increase of ligand efficacy shows primarily as a shift of the apparent affinity of nucleotide G_2 (i.e. GDP, $\beta \ll 1$), while the ligand-induced increase in binding of G_1 (alias, GTPγS) is magnified at relatively high concentrations of G_2 (as generally observed in such sort of experiments [50,51]). In contrast, the effect of a negative antagonist ($\alpha<1$) is best evident at low GDP concentrations, as also experimentally reported [51]. Note, however, that if we simulate the same binding data (Fig. 2b) in the presence of a second and preponderant Gα subunit which does not interact with the receptor (i.e. in the presence of the background nucleotide binding activity of a transfected cell), rather than a shift in affinity, the displacement curves exhibit the emergence of a low affinity component, best evident only for top values of ligand efficacy [50,51].

In the second scenario, all ligands have equal α, set as $\gg 1$, which means that they all promote $R \rightarrow A$ association in the same way. Different efficacies are assumed to result only from diverse γ_1 and γ_2 values, with respect to the two nucleotides. In this situation, there is no strict correlation between ligand-induced shift in GDP (G_2) apparent affinity and enhancement of G_1 binding (Fig. 2c). For example, the relative abilities of a full agonist ($\gamma_2=0.001$, $\gamma_1=3$) and a partial agonist ($\gamma_2=0.5$, but $\gamma_1=6$) to stimulate GTPγS (G_1) enhancement of binding may become equal or even reversed depending on the concentration of GDP. A strong inverse agonist can be simulated as a ligand having $\gamma_2=1$ and $\gamma_1=0.01$. This ligand stabilizes $R \rightarrow A$ interaction ($\alpha>1$), but induces the GDP-bound form of the G protein to a greater extent than it does the empty receptor. Note that the peculiar properties of such system are still apparent even in the presence of extensive background binding activity in the cell (Fig. 2d).

It turns out that these two theoretically simulated distinct scenarios are very similar to guanine nucleotide binding responses experimentally measured with receptor ligands for δ

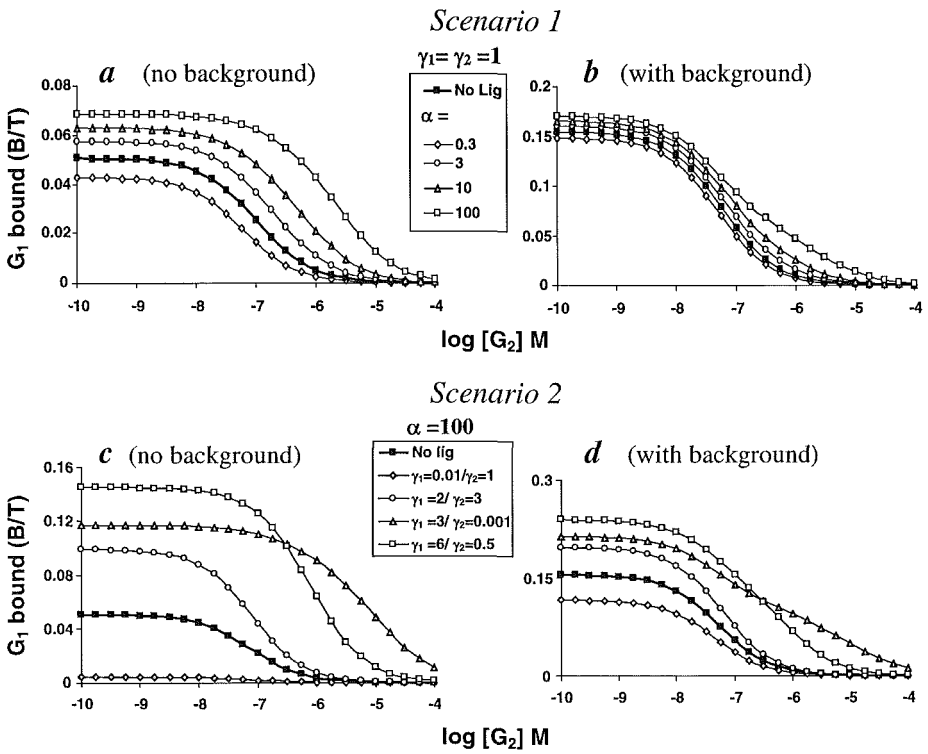


Fig. 2. Two possible scenarios of the relationship between ligand efficacy and shifts in GDP competition curves. Simulations were generated using model 2. In both scenarios, the system consists of one ligand (H), one receptor (R) one or two (right panels) G proteins (A) and two guanine nucleotides (G_1 and G_2), with G_1 present at fixed concentration (0.2 nM) and G_2, varying as indicated in abscissa. Free species were computed by an iterative procedure as described [22,25]. Curves representing the fractional binding (bound over total) of G_1 as a function of G_2 concentration were computed either in the absence (No ligand) or presence of receptor ligands (at concentrations three orders of magnitudes larger than their K_d). The following parameter values are equal in both scenarios: $M=0.03\ pM^{-1}$, $N=2$ and $0.03\ nM^{-1}$ for G_1 and G_2, respectively, $[R_{tot}]=[A_{tot}]=40$ pM. The factors β_1 and β_2 for G_1 and G_2 are 3 and 0.01, respectively, while δ values are all equal to unity. In both scenarios, right panels (labeled "with background") represent a system identical to that in the left, except for the presence at 4-fold larger concentration of a second G protein (A_2) with no affinity for the receptor ($M=0$). In scenario 1, ligands are assumed to vary only in their α factors (as indicated), while in scenario 2, all α are set = 100 and ligands differ in the values of γ_1 and γ_2.

opioid and β_2 adrenergic receptors, respectively, expressed as fusion proteins with their cognate $G\alpha_o$ and $G\alpha_s$ subunits.

7. Conclusions

Although the reaction schemes presented above are a very crude simplification of the interactions occurring between GPCR receptors and G proteins, they can produce realistic simulations of the effects that ligands of diverse efficacy can exert on GTPγS binding in the

presence of GDP. The visibility of such effects depends on the use of experimental strategies that can reduce the nucleotide binding background of the membrane, such as reconstitution assays [38,39] or transfection of receptor fused to Gα subunits [53]. Despite this limitation, the study of ligand-induced shifts on nucleotide binding can provide interesting information about efficacy and the different modes of agonism and inverse agonism.

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Discussion 1

R. Adan

Why did you use GDP for displacement studies and not GTPγ[S] itself? Aren't you concerned that the GDP itself may influence the interaction between the receptor and the G protein?

T. Costa

We have here a system with a ligand at very high concentration and what we want to see is how that ligand can influence GDP binding. I thought at the beginning that there were no effects on GTPγ[S] binding itself and all the effects would reflect changes in GDP binding. You have to think that the G protein is always filled up with GDP, and the effect on GTPγ[S] is a reflection of changes in GDP binding. But you are right in the sense that at least for the β-adrenergic system, we have definite data that receptor is affecting GTPγ[S] binding directly, independently of whether the G protein is filled up with GDP or not. We chose GDP as the ligand because it should show an effect symmetrical to that of the agonist.

P. Strange

Perhaps I haven't understood the experiments, but you are basically doing kinetic experiments there, but modelling in equilibrium terms aren't you? And, what concentration of GTPγ[S] are you using?

T. Costa

No, I am doing equilibrium experiments modelled in equilibrium terms. Both, of course, might be irrelevant from a kinetic point of view. But I think that there is no real antagonism between kinetics and equilibrium because kinetics is what really happens, and equilibrium tells you why that happens. If you are looking for the driving forces, you need to know the equilibrium, which is behind the kinetics.

We are using a trace concentration of GDPγ[S] about 0.1 nM.

M. Lohse

Perhaps we can see some differences in the modelling depending on the context. In our hands, the GTPγ[S] did not dissociate, so if we try to do kinetic experiments and if we let the experiments run for long times, we always got the same maximum binding. If you let this experiment incubate over a weekend, do you get always the same maximum binding?

T. Costa

We can have more or less dissociation in some of these systems. There is a discrepancy when we use purified components and membranes. In purified component, GTPγ[S] is virtually impossible to dissociate. And you never know if the G protein or the GTPγ[S] are getting inactivated, thus it is very hard to perform a very long type of experiment. I noticed that there is a big difference between membrane work and purified α-subunit work.

G. Milligan

In our own work it makes a big difference to GDP affinity depending on which amino acid you choose to substitute the pertussis toxin sensitive toxin cysteine for. Have you tried to use a range of those, to see if this is one of the differentials between the opiate receptor work and the results you were seeing with the β-receptor?

T. Costa

Not yet. We have done mutation studies of the β-adrenergic receptors, so far, but no studies on the G protein site. We are using isoleucin for the opioid receptor fusion protein as the standard way to block the system. There is a small difference between the isoleucin system and the regular system, even if in that case you have some contribution from the endogenous system.

G. Milligan

If we look at the work of Seifert, he would argue that it makes a big difference whether you are using the long or the short isoform of $G_{S\gamma}$. Again, have you managed to compare these directly?

T. Costa

No, we haven't done any work on the G protein side.