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COMMENTARY

G-protein Coupled Receptors

CONFORMATIONS AND STATES

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ABSTRACT. Activation of G-protein coupled receptors by agonists is thought to involve the stabilisation of a ternary complex of agonist/receptor/G-protein, leading to effector activation, but this mechanism may be an oversimplification, as follows: (a) Agonist binding to the free receptor (uncoupled from G-proteins) is not a neutral event, but includes a component of the activation process and may be described in terms of the stabilisation of a partly activated form of the receptor (R*) that is able to couple to the G-protein. Stabilisation of R*, therefore, may contribute to agonist efficacy. Also, determinations of agonist affinity even in the absence of G-protein coupling do not necessarily describe the affinities of agonists for the ground state of the receptor. (b) R* is a partly activated intermediate between the ground state of the receptor (R) and the activated form coupled to G-protein (R*G). There is some indication that different agonists may stabilise different conformational states of the receptor, i.e. different R* species. (c) Agonists also stabilise the activated, coupled form of the receptor (AR*G), and for some agonists acting at a single receptor, the activated states may be similar, although there is evidence for other agonists that different activated states with different activities may be stabilised. (d) Two or more efficacy-generating steps are involved in the activation of G-protein coupled receptors by agonists: the stabilisation of R*, the stabilisation of R*G, and possibly the modulation of the activity of the activated state (AR*G). (e) The experimentally observed excess of G-proteins over receptors in membranes is inconsistent with data obtained from ligand-binding assays on these receptors. Receptors and G-proteins, therefore, may exist in some form of higher order array with cooperative interactions. BIOCHEM PHARMACOL **58**;7:1081–1088, 1999. © 1999 Elsevier Science Inc.

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The act of agonist binding to its receptor leads ultimately to a response in the system linked to the receptor. From the point of view of the function of the system, there are two properties of the agonist that are important: its affinity for the receptor and its ability to induce a response, often referred to as efficacy. Efficacy is exhibited in terms of the maximal effects of agonists and the degree of amplification from binding to response, i.e. the extent to which agonist-mediated effects occur at concentrations lower than those needed for agonist binding. There has been some debate recently about mechanisms of GPCR† activation [1, 2], and in this commentary I wish to consider some aspects of these mechanisms.

THE ACTIVATION PROCESS

For GPCRs it is widely believed that the critical event in agonist action is the stabilisation of the ternary complex of agonist/receptor/G-protein [3], and the better this ternary complex is stabilised, the more efficacious is the agonist. The formation of this activated state must involve a series of interactions between the agonist and amino acid side chains of the receptor [4]. Notionally we can divide this activation process up into different stages. First the agonist interacts with the free receptor to form an agonist/receptor complex. This then interacts with the G-protein, leading to the formation of the ternary complex, the activated state. If the formation of this activated state determines the efficacy of signalling, then the difference in free energy between the agonist/receptor complex in the ground and activated states determines efficacy. The additional interactions between agonist and receptor in the activation process lead to this free energy difference. To understand efficacy, it is necessary to determine this free energy difference. This has been approached by determining agonist affinities for the free receptor and for the receptor coupled to G-protein [5–7], but there may be some problems with this approach, as I shall show below.

AGONIST BINDING TO THE FREE RECEPTOR

Agonist binding often is performed *in vitro* under conditions where interaction of receptor and G-protein is suppressed,

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† *Abbreviations*: GPCR, G-protein coupled receptor; and GTPγS, guanosine-5′-*O*-(3-thio)triphosphate.

e.g. in the presence of GTP. Under these conditions, it is assumed frequently that the affinity determined is the ground state affinity for the receptor. The binding of the agonist to the free receptor, however, is likely to use some of the amino acid side chains that interact with the agonist in the activated state. Hence, it seems intuitively likely that when the agonist binds to the free receptor, it stabilises the receptor in a conformation that partly resembles the activated state of the receptor. This stems from the fact that the interactions in the two states must bear some resemblance to one another. Therefore, the act of agonist binding even to the free receptor is not neutral but moves the conformation of the receptor toward that of the activated state. Therefore, even this simple act of binding includes some of the free energy of the activation process, and agonist affinities measured under these conditions contain both a binding component and an efficacy component, as discussed by several authors [8, 9]. This can be expressed mathematically by proposing that the receptor exists in a ground state (R) and a partly activated conformation (R*). The equilibrium lies well toward R in the absence of agonist, but it is R* that has a higher affinity for agonist, and so the presence of the agonist stabilises R* (see Appendix 1).

These ideas are very similar to those proposed in the extended ternary complex model [3], which was based on the results of mutations in the β_2-adrenergic receptor that lead to increases in the affinity of the free receptor for agonist and constitutive activation of the signalling pathway. It was proposed that the free receptor (R), must therefore exist in a second conformation (R*) that could couple to the G-protein; free receptor could not (Appendix 1). The mutations of the receptor were leading to an increased tendency for the receptor to exist in the R* state. This second receptor conformation, inferred from the use of mutants, probably is related to the conformation that I have argued is stabilised by the agonist when it binds to the free native receptor. The two are not, however, necessarily identical. If the two are related, then we can make some inferences about the properties of the states. The existence of this second receptor conformation has significant implications for understanding efficacy, as I shall show.

Some calculations of possible affinities based on the R/R* equilibrium are shown in Appendix 2. Two important conclusions emerge. First, differential binding of agonist (A) to R and R* can contribute to efficacy; that is, the ability of the agonist to stabilise R* can be one efficacy-determining step for the agonist. Second, in a ligand-binding assay where coupling to G-proteins is suppressed, if there is significant accumulation of AR*, then the apparent affinity will not be the affinity for the ground state of the receptor. Measurements of agonist affinity have been made in the absence and presence of GTP to estimate the stabilisation of the activated state by agonists [5–7], but if there is significant stabilisation of AR* in the presence of GTP, then the overall stabilisation from ground state to activated state will be underestimated. This may contribute to some of the problems encountered in some of these studies, discussed below.

An important question is, therefore, does R* accumulate to any significant extent under normal cellular conditions? R* is an unstable conformation of the native receptor, but if mutations are made [3] in certain parts of the receptor, then its formation can be more favourable. Interaction with a G-protein also can stabilise R*, as seen from the agonist-independent activation observed for some receptors [3], but is it present for the native receptor and standard agonists in the absence of G-protein coupling? We cannot directly answer this question, but as I have argued, the act of agonist binding to receptor is not a neutral event, and unless the receptor conformation is very fixed, there must be a change in conformation upon agonist binding. Given that the potential interactions between agonist and receptor are limited, then agonist binding must move the receptor in the direction of the activated state. What is required now is to make biophysical measurements of receptor conformation to analyse these changes, and one study has begun this process for the β_2-adrenergic receptor [10] in the absence of G-proteins. Here, the conformation was monitored with a fluorescent reporter group, and it was possible to show a change in fluorescence upon addition of agonists, which appeared to be related to their efficacy. This change has the correct properties for the R/R* conformational change, and since it can be observed, this is some evidence that AR* does indeed accumulate for the native receptor. Further evidence for this conformational change comes from studies of the β_2-adrenergic receptor, where the extent of phosphorylation of the agonist-occupied receptor by β-adrenergic receptor kinase (in the absence of G-protein coupling) was correlated with the efficacy of the agonist [11]. These observations suggest that there may be an agonist-induced conformational change in the receptor that is related to the efficacy of the agonist. Also for the β_2-adrenergic receptor, studies of the kinetics of ligand binding suggested that there was a conformational change in the receptor upon agonist binding in the absence of G-protein coupling [12]. Activation of rhodopsin by light leads to the formation of a new conformational form, metarhodopsin(II), which has a high affinity for the G-protein transducin, so that metarhodopsin(II) is the equivalent of R* in this system [13].

In summary, the simple consideration of agonist binding requires the postulation of an isomerisation of the receptor to a partly activated state, which I have termed R*. This isomerisation is one of the efficacy-determining steps for receptors, and it may be missed or underestimated using ligand-binding assays.

Relation of AR to the Activated Species*

In the extended ternary complex model [3], the R* species is an obligatory intermediate between the ground state and the activated state. As I have argued above, the R* state may be inferred as being a partly activated state between R and the activated state. So where exactly does R* fit

between R and the activated state? To address this question, we have to consider the nature of the activated state. It was assumed that the activated state is the ternary complex of agonist/receptor/G-protein, but now it is clear that G-proteins can be activated by receptors in the absence of agonist to form a binary complex, so-called constitutive activation [3]. In the ternary complex (AR*G) the receptor must be in an activated conformation derived from R*, but this is unlikely to be the same as R*, as there is G-protein bound as well. The receptor conformation in the binary complex (R*G) must be similar to that in AR*G but may not be identical, as agonist is not present (see Ref. 13). Given that there is an increase in agonist affinity in forming AR*G, then AR* must lie between R and AR*G. It is difficult at present to quantitate this, but it may be accessible using suitably chosen receptor mutants. For example, the affinities of agonists for constitutively active mutant receptors should approximate their affinities for R* as outlined in Appendix 2. Conversely, the affinities of mutants where R* cannot form will approximate their affinities for the R state. For the monoamine receptors, mutants in the conserved aspartic acid residue in TM2 may provide such a disabled receptor. For a number of monoamine receptors, mutation of this Asp yields a receptor that is unable to couple to G-protein, and in several cases the mutant receptor exhibits a decreased agonist affinity independent of G-protein coupling [14–19]. Allosteric regulation of the receptor by H^+ and Na^+ also is prevented, so that these mutants may be examples of receptors that cannot form a key intermediate in signalling, i.e. they are unable to undergo the R/R* transition, or at least this is severely impaired.

There is also the related question of whether there is a single R* species. As I have proposed above, a convenient way to express these ideas is to assume a single R* species and suggest that different agonists have different affinities for R and R* and thus stabilise R* to different extents, and this difference contributes to their efficacy. In this view, the receptor exists in two major conformations with different affinities for agonists. An alternative way of viewing this would be that each agonist stabilises a different R* state, and this contributes to efficacy. At present there is no way of distinguishing these two possibilities, but there are certain compounds that do not appear to fit the simple theory of a single R* conformation. For the D_2-like dopamine receptors and the 5-HT_{1A} serotonin receptor, the ergopeptines have markedly different properties compared with other agonists. They are agonists at these receptors, and yet they are insensitive to the effects of GTP in ligand-binding assays [20, 21], unlike other agonists of similar intrinsic activity. It is tempting to suggest that these compounds achieve agonism by strongly stabilising the R* state that is common to other agonists, but they have the same affinity for R* and R*G. As outlined in Appendix 1, however, this mechanism still would give rise to guanine nucleotide-sensitive binding. An alternative explanation for these observations is that the ergopeptines stabilise the receptor in a different conformation (R^{*1}), in the absence of G-protein coupling, that is very similar to the conformation in the coupled state, i.e. AR^{*1} for these compounds is very similar to AR*G. Because the conformation of AR^{*1} is similar to that in AR*G, there will be little or no gain in energy in forming AR*G. Hence, the affinities of these compounds for the G-protein coupled and uncoupled states are similar. These compounds have a large peptide side chain, and its interaction with the receptor may induce a different conformation close to that in the activated state, so that the majority of the binding energy of the ligand is realised in the binding of the ligand to the free receptor. These observations show that not all agonists induce the same AR* state at these receptors. Further evidence for this contention comes from the work of Schimerlik and colleagues [22, 23], who have shown that at the muscarinic acetylcholine receptor, agonists of different efficacies induce states of the receptor that have different affinities for the G-protein.

Leff and colleagues [1, 24, 25] have extensively developed models of receptor action based on a two-state (and three-state) formulation. In their earlier models [1, 24], the receptor exists in R and R* states, the latter stabilised by agonists, but no explicit account is taken of G-protein coupling except to subsume it in the "active" conformation, R*. These models assume that there is one efficacy-determining step for agonists, the stabilisation of R* over R. This account of agonist action, therefore, ignores the complexity that I have summarised above, and the R and R* states discussed in the present article are not equivalent to those of Leff and colleagues [1, 24]. More recently, they have included receptor/G-protein interaction in their models [25], using a formulation whereby the agonist has different affinities for R and R* but identical affinities for R* and R*G. As summarised in Appendix 1, such a model can generate agonism. In addition, such a model is unlikely to be applicable to all situations, since it would not allow for GTP-sensitive agonist binding in mutants where R* formation is favoured, and such sensitivity has been described [3] (Appendix 1).

THE ACTIVATED STATE

It is thought that the activated state of a GPCR is the ternary complex of agonist/receptor/G-protein (AR*G) and that in this complex GDP/GTP exchange occurs; once GTP is bound, the complex disintegrates, yielding α and $\beta\gamma$ subunits of the G-protein, which can alter effector activity. The guanine nucleotide exchange event is thought to be the rate-limiting step in the activation process in the absence of the catalytic effect of the receptor [26].

There is, then, the question as to whether there is a single activated species stabilised to different extents by different agonists or whether there are different activated species for different agonists. There have been differing opinions on this point [24, 25, 27–29], but there is little direct evidence one way or the other. As I have argued

before [2], the maximal rates of guanine nucleotide exchange, measured in [^{35}S]GTPγS binding assays, provide some information. At the D_2 dopamine receptor, maximal rates of [^{35}S]GTPγS binding are similar for several agonists [30, 31], suggesting the same or a very similar active conformation. Similar data have been reported for the somatostatin$_5$ receptor [32].

Information that could be interpreted in terms of differences in the active conformation comes from work on stimulation of [^{35}S]GTPγS binding via opiate receptors [33]. In this study, [^{35}S]GTPγS binding was determined after a particular time in the presence of a range of concentrations of nonradioactive GTPγS. The data were subjected to Scatchard analysis, which showed that different opiate agonists lead to different apparent levels of [^{35}S]GTPγS binding, and binding occurs with different apparent affinities [33]. Superficially, this might indicate different activated conformations. The assay, however, is not at equilibrium, and if different agonists mediated different rates of [^{35}S]GTPγS binding as has been shown for other receptors, then this could give rise to the behaviour seen. This would then not constitute evidence for different activated states.

If different activated states existed for different agonists, then it might be expected that these states would exhibit different properties, such as different guanine nucleotide exchange rates or rates of breakdown. Different rates of ternary complex breakdown have indeed been inferred for the β_2-adrenergic receptor [34], and different guanine nucleotide exchange rates have been shown for the A_1 adenosine receptor [35]. Some indirect evidence for this also has been found when agonists were assessed in ligand-binding and functional ([^{35}S]GTPγS binding and adenylyl cyclase) studies at the D_2 dopamine receptor. Certain agonists were shown to stabilise formation of the ternary complex well but did not exhibit full agonism [30, 31]. This suggests that the activity of the ternary complex (guanine nucleotide exchange or ternary complex breakdown) [36] is low for these agonists. Several studies, therefore, suggest different activated conformations, but in these studies one must exclude the possibility that the differences seen are not due to activation of different G-proteins by different agonists. Indeed, some evidence has been presented to show that combinations of one receptor with different G-proteins exhibit different pharmacological profiles [27], so that in principle different agonists could activate different pools of G-proteins.

THE NUMBER OF EFFICACY-GENERATING STEPS

From the discussion above, it is clear that there is more than one step that influences the efficacy of agonists in the pathway from the free receptor to the active complex. First, there is the conversion of the free receptor to the partly activated state (R*) by the agonist to form AR*; this is followed by the conversion of AR* to AR*G. Therefore, two steps provide the minimum mechanism for efficacy generation, as summarised in Refs. 3 and 9, but should the ternary complexes generated by different agonists differ in their functional activity [36], then this will provide a further efficacy-generating step.

Several studies have attempted to provide measures of agonist efficacy from ligand-binding data, as this would be of great use for drug design. Generally this has entailed determining a ratio of agonist affinities for the low and high affinity states seen in agonist binding or from separate determinations of agonist affinity determined in competition versus a radiolabelled antagonist and agonist (K_l/K_h ratio). Correlations have been reported between measures of agonist efficacy and the K_l/K_h ratio for several receptors [6, 7, 37, 38], but other studies have failed to see a correlation [30, 31, 39]. Where correlations have been seen, this may mean that determinations of K_l provide a good estimate of the ground state affinity of the agonist for the receptor, so that K_l/K_h is a good estimate of the overall stabilisation of the ternary complex from the ground state. Where correlations are not seen, this may be due to problems in defining the affinity of the agonist for the ground state of the receptor so that the importance of the AR/AR* step in stabilisation of the ternary complex is underestimated, or it may reflect differences in ternary complex activity.

ORGANISATION OF RECEPTORS AND G-PROTEINS

The relative levels of receptors and G-proteins will affect the signalling properties of the receptor system, and there has been some discussion on this topic. For several receptors including the β_2-adrenergic receptor, determinations of total receptor and G-protein have been made, and these suggest a preponderance of G-protein [40]. These data do not fit with ligand-binding data on a number of receptors, where agonists have been shown to exhibit complex binding curves that may be modelled in terms of higher and lower affinity states [30, 31, 41]. This kind of behaviour is most consistent with an excess of receptor over G-protein if a ternary model holds [40, 41]. If, indeed, there is functionally a preponderance of receptor, this implies some kind of compartmentalisation or restricted access for the receptors. Alternatively, the models of receptor/G-protein interaction may be incomplete, and receptors and G-proteins may interact in some form of higher order array [40–42].

The use of receptor/G-protein fusions has shed some light on this matter. The β_2-adrenergic receptor has been fused with the α-subunit of G_s and expressed under conditions where coupling to endogenous G-proteins is unlikely [43]. The agonist-binding properties of the fusion are very similar to those of the β_2-receptor expressed in an unfused form in cells containing G-proteins. In both cases, agonist binding curves are seen that may be resolved into higher and lower affinity states. The fusion guarantees that the receptor/G-protein stoichiometry is 1:1, and it has been

shown that if a ternary model describes receptor/G-protein interaction, then deviations of binding curves from a single site model are slight for a 1:1 R/G stoichiometry (see Ref. 41 and Appendix 1). Therefore, the origin of the complex binding curves seen for the fusion and for unfused receptor/G-protein may result from some other physical arrangement, as suggested above.

I thank Sam Hoare for valuable comments on the manuscript and Paul Leff and Clare Scaramellini for the generous gift of the extended ternary complex model in Excel

NOTE ADDED IN PROOF

After the submission of this paper, an article appeared that considers some of the same material (Colquhoun D, Binding, gating affinity and efficacy: The interpretation of structure-activity relationships for agonists and of the effects of mutating receptors. *Br J Pharmacol* **124:** 924–947, 1998).

APPENDIX 1. THE EXTENDED TERNARY COMPLEX MODEL

$$R \rightleftharpoons R^* \rightleftharpoons R^*G \rightarrow \text{response}$$

For the scheme shown, the receptor exists in R and R* states, and the R* state is a partly activated state of the receptor that can couple to the G-protein to form R*G, the activated species. An agonist stabilises the activated state by binding with higher affinity to R* than to R and with higher affinity to R*G than to R*, as outlined in the text. This model can be used to simulate data from agonist binding experiments, as follows.

Agonist binding is taken to be [AR] + [AR*] + [AR*G], and Fig. 1 shows this quantity for different values of the equilibrium constant governing R*/R*G, equivalent to having different concentrations of guanine nucleotide in assays. For ease of presentation, I have taken the total concentrations of R and G to be the same at 100 pM. The equilibrium constant governing R*/R has been taken to be 0.1, and values for the equilibrium association constants for the binding of A to R, R*, and R*G have been taken as $3.33\ 10^6$, $3.33\ 10^7$, and $2.5\ 10^8\ M^{-1}$, respectively. The data of Fig. 1 show agonist binding for situations where R*G is stable, e.g. in the absence of added guanine nucleotides, and where it is very unstable, e.g. in the presence of GTP, and a "GTP shift" of 25-fold is seen.

It is also possible for a compound to be an agonist by stabilising the R* state over the R state but having equal affinities for R* and R*G. Figure 2 shows simulations of agonist binding using the same parameters as above, but with the association constants for agonist binding to R* and R*G being the same at $2.5\ 10^8\ M^{-1}$. The "GTP shift" is lower than that above, but still significant at 6-fold.

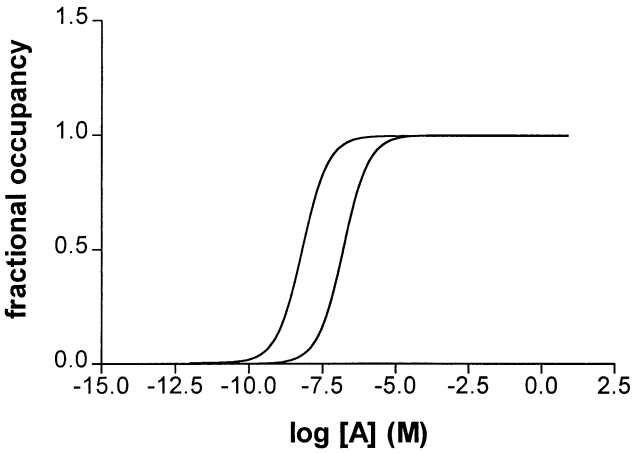


FIG. 1. Agonist binding simulated as in the text for a model where the agonist stabilises both R* and R*G. Binding curves for low and high stabilisation of R*G are shown (values of the equilibrium constant for R*/R*G formation of 10^{-8} and 10^{-12} M^{-1}, respectively). Data were simulated in Excel and analysed using Prizm.

These simulations show that, even if a compound does not discriminate between R* and R*G, it can be an agonist if it discriminates between R* and R, and the resultant agonist binding will be sensitive to GTP.

This latter case is important for considering the ergopeptines, whose binding to D_2 dopamine and 5-HT_{1A} serotonin receptors is GTP-insensitive and therefore might be considered to bind as in Fig. 2, i.e. with no discrimination between R*G and R*. These simulations show, however, that such a case would still generate GTP sensitivity. This conclusion depends on the R*/G to R*G transition being favourable in the absence of agonist and guanine nucleotide, so that when AR* is formed it will couple to G. Evidence that this must be so for some receptors comes from the agonist-independent activation seen for some

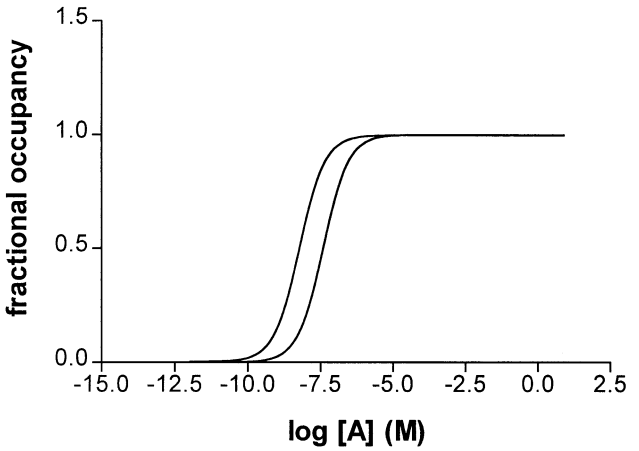
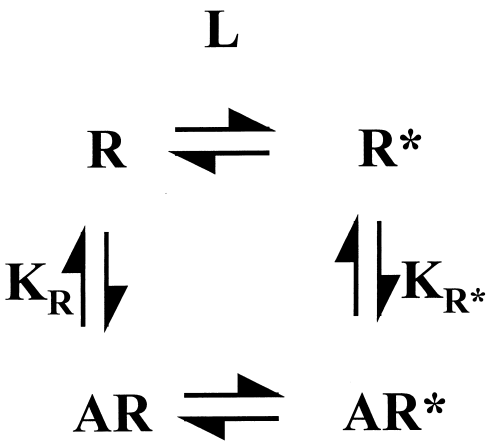


FIG. 2. Agonist binding simulated as in the text for a model where the agonist stabilises R* but has equal affinity for R* and R*G. Binding curves for high and low stabilisation of R*G are shown (values for the R*/R*G equilibrium constant of 10^{-12} and 10^{-8} M^{-1}, respectively).

receptors, including the D_2 and 5-HT_{1A} receptors [44, 45]. Therefore, for the ergopeptines a different receptor conformation may be produced (AR^{*1}) that couples weakly to G-proteins. AR^{*1} is not stabilised greatly by coupling to G-proteins, so that ligand binding appears to be guanine nucleotide-insensitive, and this also could account for the partial agonism typically seen with these agonists.

Others have suggested that agonists at GPCRs may function primarily via stabilisation of R* over R [25, 46]. If, however, this were the mode of function of a receptor and a mutant of the receptor were made in which R* formation was favoured, i.e. $R^*/R > 1$, then this mutant receptor would not exhibit GTP-sensitive agonist binding. Such mutant receptors, however, do exhibit GTP-sensitive agonist binding [3], so this mechanism cannot be a general one.

APPENDIX 2. R AND R* STATES OF RECEPTOR IN THE ABSENCE OF G-PROTEIN COUPLING



For the scheme shown, the receptor exists in R and R* states, and coupling to G-proteins is suppressed, for example by addition of GTP. The present discussion, therefore, relates to ligand-binding assays in the presence of GTP and not to any functional assays. L is the equilibrium constant governing the relative stability of R and R*, and K_R and K_R^* are, respectively, the equilibrium association constants for agonist binding to R and R*. L is the R/R* equilibrium constant and is given by R*/R. The dissociation constant for agonist binding (K_d) is given by

$$K_d = \frac{1 + L}{K_R + LK_R^*}$$

If L is very low ($\ll 1$) and lower than K_R/K_R^*, then $K_d = 1/K_R$, and the receptors behave as if the R* state does not exist as far as ligand binding is concerned. In this case, determinations of the affinity of the agonist by ligand binding are good estimates of the affinity of the ground state of the receptor. If L is very high ($\gg 1$) and higher than K_R/K_R^*, then $K_d = 1/K_R^*$, and the receptors behave as if they are all in the R* state. For intermediate values of L, K_d will depend upon L, K_R, and K_R^*, and a single affinity will be seen, but this will not be a good estimate of the ground state affinity. It is instructive to consider some examples here in order to understand the consequences of this equilibrium.

If R* formation is relatively unfavourable ($L = 0.01$), and R binds A rather weakly ($K_R = 10^3\ M^{-1}$), and R* binds A well ($K_R^* = 10^6\ M^{-1}$), then K_d is 91 μM. At saturation the proportion of the total receptor that is R* is 91%. The apparent K_d of the receptor for A is 91 μM, and this corresponds to the favourable binding of A to an unfavoured species. If we now consider the corresponding case where R* formation is favourable ($L = 100$) but the affinities for the R and R* states are the same, then K_d = 1 μM. Therefore, a shift in the stability of R* has increased apparent agonist affinity by a factor of about 100-fold.

This comparison corresponds to the effects of the mutants described in Ref. 3 for the β_2-adrenergic receptor. R* formation was unfavourable in the native receptor, but favourable in the mutants. In that study the maximum effect of the mutation was an increase in affinity of about 30-fold. The effect of the mutation was, however, different for different agonists and seemed to be related to agonist efficacy, less efficacious agonists exhibiting a smaller shift in potency. This can be accounted for by proposing that different agonists have different K_R/K_R^* ratios, so that the effect on the apparent affinity following the change in L caused by the mutation will be quantitatively different for the different agonists. If the shift in affinity caused by a change in L is related to agonist efficacy, then the K_R/K_R^* ratio also must be related to efficacy.

If we consider a determination of agonist affinity in a ligand-binding assay (with coupling to G-proteins suppressed), then in the case described above for the native receptor, the apparent affinity observed will be 91 μM. This is neither a reflection of the affinity of A for the free receptor ($K_R = 10^3\ M^{-1}$) nor a direct reflection of the affinity of A for R* ($K_R^* = 10^6\ M^{-1}$). It is largely a reflection of the favourable binding of A to an unfavoured species (R*). Importantly, however, ligand-binding assays under these conditions do not determine the true ground state affinity of the receptor. If efficacy depends on the free energy difference between the ground state and the activated state of the receptor, then it will be underestimated by affinity determinations for the ground state using assays under these conditions. One of the efficacy-determining steps for the agonist will not have been assessed properly. For a receptor or an agonist where AR* does not form significantly under normal conditions, e.g. $L \ll 1$, these problems are absent.

There are now a number of studies for different receptors reporting mutations of the kind discussed earlier, which give increased agonist affinity in the absence of G-protein coupling and constitutive activation of signalling systems. The mutations give effects on agonist affinity of between 10- and 100-fold [3, 47–49]. Effects of this magnitude are consistent with significant affinity differences of A for R

and R* (i.e. $K_R/K_R^* < 1$) and values of $L < 1$ for the native receptor.

A value for L can be estimated for the β_2-adrenergic receptor based on values for the dissociation constants of isoprenaline for the constitutively active mutant receptor [3], where $L > 1$ and $K_d \sim 1/K_{R^*}$, and for mutants where the conserved aspartic acid in TM2 has been mutated [14, 15], $L < 1$, and R* formation is impaired greatly so that $K_d \sim 1/K_R$. These data may be used to calculate a value of 0.037 for L for the native receptor and values of $6.7 \cdot 10^5$ M^{-1} and $1.52 \cdot 10^8$ M^{-1} for the association constants for the R and R* states respectively.

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