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# Binding, gating, affinity and efficacy

# Binding, gating, affinity and efficacy: The interpretation of structure-activity relationships for agonists and of the effects of mutating receptors

**David Colquhoun**

University College London, Gower Street, London WC1 6BT

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## The nature of the problem

The nature of the problem is illustrated by the curves in Figure 1. A mutation in a receptor is seen to produce 100 fold increase in the $EC_{50}$ for an agonist (Figure 1a). A ligand binding experiment with the same agonist, on the same mutant receptor (Figure 1b) shows that the measured affinity for the binding of the agonist has also been reduced by about 100 fold. Obviously the mutation has affected the agonist-binding site, and the mutated amino acid is likely to be part of that site? No! It is not in the least obvious. The example in Figure 1 was calculated on the basis that the affinity for the binding step of the reaction was totally unaffected by the mutation (the equilibrium constant for this step was 100 $\mu$M for both wild type and mutant). The only difference between wild type and mutant receptor in this example is the ability of the receptor, once the agonist has bound, to change conformation to its active state. There is no reason at all why the amino acids that affect the ability to change conformation should be anywhere near the agonist binding site.

- Binding experiments do not measure affinity (in any sense that is useful for learning about the binding site), for any ligand that causes a conformation change.
- The term 'apparent affinity' is often used to describe $EC_{50}$ for the response but it is meaningless (unless you define what you mean by 'apparent').

Making this distinction between effects on binding and effects on conformation change is arguably the fundamental problem of modern molecular studies of receptors. It is not an easy distinction to make, but unless it can be solved, the interpretation of structure-function studies is quite likely to be nonsense.

It is not just a theoretical problem; this is how ion channels actually behave. Nevertheless, the very existence of the problem has not always been recognized. For example, statements like the following are not at all uncommon*.

(a) '*Simplistically, the efficacy of a full agonist can be set equal to 1, that of an antagonist to 0, and that of a partial agonist to a value between 0 and 1*' (Ross, 1996, in Goodman & Gilman, 9th Edition). This statement obscures the point that is crucial, both for the interpretation of structure-activity relations and of mutant studies, that efficacy has no upper limit in principle, and that when it is large, changes in it are indistinguishable from changes in affinity.

*I can only apologize to the authors of the particular papers that are cited in order to emphasize this point. They were an entirely arbitrary choice from a large number of possibilities.

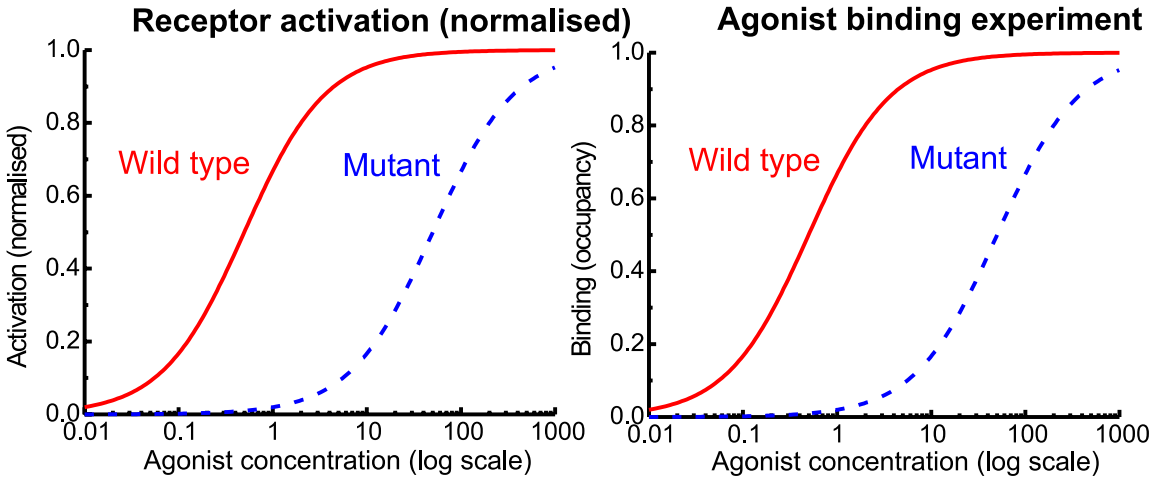



**Figure 1** Concentration-response curves (left) and agonist-binding curves (right). Calculated from the del Castillo-Katz (Scheme 1). The binding reaction has an equilibrium constant of $K_A = 100$ $\mu$M for both wild type and mutant receptors, so the mutation does not affect the binding site at all. The equilibrium constant for isomerisation to the open state (the gating reaction) is 200 for the wild type (high efficacy), but only 1 for the mutant. The mutation has affected only the ability of the protein to change its conformation; the binding site is unaffected.

(b) '*This change in the sensitivity of the Y190F mutant could be due either to a change in the binding affinity of the receptor or to a change in the energetics of opening of the channel after binding. To distinguish between these possibilities, we assessed the ACh binding affinity of the mutants by measuring the ability of ACh to compete for* [$^{125}$*I*]-α-bungarotoxin binding' (Tomaselli *et al*., 1991). Figure 1 shows clearly that such measurements do not make the required distinction.

(c) '*Of particular interest in this regard is the ability to measure independently both ligand receptor affinity (by ligand binding techniques) and biological activity (*$ED_{50}$ *and* $E_{max}$*). These two independent processes provide information about . . .*' (Hollenberg, 1991). The whole point of the problem is, of course, that these measures are far from being independent. If binding affects activation (transduction, gating), then activation must affect binding.

(d) '*Measurement of full-agonist-affinity can be made by a procedure developed by Furchgott*' Kenakin (1997a, p272). This would be true only if activation had no effect on binding, which is not true for any currently proposed mechanism of agonist action (Colquhoun, 1987).

The problem of interpreting the effect of mutations has been discussed before (Colquhoun & Farrant, 1993). It now seems timely to consider what can be done about it. Although difficult, it is not impossible, and, at least for ion channels, there are sensible things that can be done.

Before going on to the modern problem, it is appropriate to recognise that this is not a new problem at all. Essentially the same difficulty occurs in the interpretation of the structure-activity relationships of a series of different agonists on the same receptor, and in this context it has been around since the 1950s.

# Some history

## The classical era

Stephenson (1956) had pointed out the very important fact that the action of an agonist could not be described by an affinity constant alone. In addition some measure of the ability of the agonist to activate the receptor (e.g. to open an ion channel) was essential too (he termed the latter, the efficacy of the agonist). This postulate was, very rightly, enormously influential. Two quantities (at least!) were needed to describe the action of an agonist, affinity (for the initial binding reaction), and efficacy (to measure the ability to activate once bound). No sense could be made of structure-activity relationships for agonists unless these two quantities could be disentangled, because the effects of a change in agonist structure may be (and often is) quite different for each of them. Unfortunately these separate quantities, affinity and efficacy (for which, in an ion channel context we may read binding and gating), have proved very hard to measure. Various methods have been proposed to measure them, but because of an error in Stephenson's argument these methods are not valid. Stephenson's original error has been propagated to the progeny of his paper. In particular, it is present in Furchgott's (1966) method for measuring the affinity and efficacy of a full agonist (which is unfortunate because it is essentially the only method that has been proposed for use when no mechanism is known). It is also present in the 'operational model' of Black & Leff (1983), which is identical with Stephenson's argument except for the addition of the additional assumption of a particular (hyperbolic or Hill) stimulus-response relationship. The same error has propagated to many other papers e.g. the method of Venter (1997), and the discussion of Clarke & Bond (1998). Likewise, attempts to 'validate' Furchgott's method by showing that the 'affinity' it produces agree with those by direct binding measurements (e.g. Morey *et al*., 1998) are futile because agreement is expected (Figure 1 and Colquhoun, 1987), but the value produced by both methods is not affinity in the sense intended by Stephenson, or in the sense that is useful for making inferences about the binding site (e.g. see eq. 1, below). These methods cannot, therefore, be expected to work, and it is the purpose of this discussion to consider what can be done about it.

The nature of Stephenson's error is enlightening. There are various ways in which it can be stated, but the essential point is that his theory contained a parameter, $p$, described as the receptor occupancy, which he supposed to be related to concentration in a simple Langmuirean manner, and to depend only on affinity. In fact, in any physically realistic mechanism, the receptor occupancy must depend on all the reaction steps, not on only the affinity for the initial binding reaction. This is a consequence of the basic physical principle of reciprocity (if A affects B then B must affect A; see Edsall & Wyman, 1958; Wyman & Gill, 1990). This reciprocity is built into every current proposal for the mechanisms of both ion channels and G protein-coupled receptors, but it is absent from Stephenson and his progeny. That is why they are wrong. Stephenson's paper is ambiguous about whether the term 'occupancy' is intended to represent what you would measure in a ligand binding experiment, though that is actually what he had in mind (R. P. Stephenson, personal communication). The problem lies in the fact that, for any ligand that produces a conformation change, the total amount of binding (as measured in a ligand binding experiment) depends not only on the affinity of the initial binding, but also on the extent to which the conformation change takes place once ligand has become bound. The result of a binding experiment depends on both affinity and efficacy (or, in ion channel language, on both binding and gating).

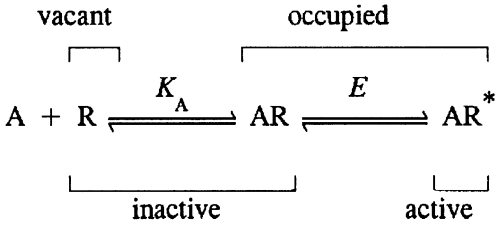
## The interaction between binding and conformation change: a conceptual leap forward

Stephenson's approach was essentially rooted in the classical era. The approach to receptor problems hardly changed between 1909, when A. V. Hill first derived the Langmuir equation, and the early 1950s. An enormous advance was made when it was realized that some proteins could undergo global conformation changes, and that the ligand binding properties of the two conformations might be very different. The first clear statement of that idea was, as far as I am aware, in a seminal paper by Wyman & Allen (1951). Enough was known of the structure of haemoglobin at that time for it to be realised that the molecule existed in two distinct conformations. Wyman and Allen suggested that the properties of haemoglobin (and perhaps of enzymes too) could be explained very economically if the change in affinity during binding of oxygen were actually based on a concerted change in structure from one conformation to the other (i.e. all four subunits flip together). Furthermore it was known that the structure of oxyhaemoglobin was very similar to that of carboxyhaemoglobin, so this hypothesis also provided an elegant explanation for the identity of the Bohr effect for both oxygen and carbon monoxide. The ion-channel equivalent of this prescient statement would be to say that the properties of an open ion

channel should be independent of the agonist that caused it to open (the only difference being the length of time the channel is open for), a prediction that has generally been found to be true. Indeed the direct observation of a single channel 'snapping' from one conductance state to another has provided the most direct evidence available in any field that a functional receptor protein exists, to a good approximation, in two (or a few) distinct conformations.

### *The simplest case: del Castillo-Katz mechanism*

The idea of a conformation change also appeared, during the same decade, when del Castillo & Katz (1957) wrote the binding step and the conformation change as two separate steps, as an attempt to explain partial agonism. Their paper was a year after Stephenson's, and it took a very different approach. Stephenson had tried to formulate the transduction mechanism as a black box, and to provide a very general treatment. This had worked very well for the analysis of competitive antagonists, in the way suggested by Schild a bit earlier. For antagonists the use of null methods allowed valid estimates of antagonist equilibrium binding constants in a way that was remarkably independent of any knowledge of the transduction mechanism (e.g. Colquhoun, 1973). For a long time it was hoped that a similar trick would work for agonists too (e.g. Furchgott, 1966; Black & Leff, 1983), but sadly it is not so simple. Katz, on the other hand, postulated a simple and explicit transduction mechanism (for the endplate nicotinic receptor-channel). Their mechanism postulated that binding of the agonist (A) to a receptor (R) resulted in a complex (AR) which was still inactive (channel shut), which could then undergo a conformation change to the active (open) state. The del Castillo-Katz mechanism was



Scheme 1

The agonist, A, binds to a vacant receptor, R, to form a complex AR (the channel is still shut), and this can then flip conformation to the open state, AR*. The equilibrium dissociation constant for the binding step is denoted $K_A$, and the equilibrium constant for the shut-open isomerisation is denoted $E$ (this is defined as opening rate constant/shutting rate constant, so the larger the value, the greater the fraction of occupied channels that are open at equilibrium). If we want to know about the binding site then we must be able to determine $K_A$. This is not so easy because this mechanism predicts that both response and agonist binding will follow simple hyperbolic (Langmuirean) curves, as in Figure 1, and that both of these curves will have exactly the same $EC_{50}$. This $EC_{50}$ will be given by

$$EC_{50} = \frac{K_A}{1+E}. \qquad (1)$$

This depends on both binding ($K_A$) and on conformation change ($E$), so knowledge of its value does not tell us anything directly about the binding site. The problem is to separate these two quantities. The $EC_{50}$ in (1) is what is often known as $K_d$ when talking about binding, or 'apparent affinity' when talking about responses. For the example in Figure 1, the $EC_{50}$ is 0.498 $\mu$M for wild type and 50 $\mu$M for mutant, whether binding or response is measured.

The actual equilibrium (or rate) constants for individual reaction steps (like $K_A$ and $E$) are known as microscopic constants, and they are what tell us about what is going on. In contrast, equation (1) defines a macroscopic constant; it describes what we see, but does not tell us what is going on underneath.

The maximum fraction of receptors in the active state (at very high agonist concentration) is

$$p_{AR*}(\infty) = \frac{E}{1+E}. \qquad (2)$$

which increases from 0 to 1 as $E$ increases. If this maximum can be measured then $E$ can be estimated separately, but (a) it can be measured (on an absolute scale) only for a few ion channels, and (b) $E$ can be found only if it is not too large. It is a crucial property of this simple mechanism (and all the others discussed here) that changes in binding ($K_A$) and in conformation change ($E$) become indistinguishable for very efficacious agonists, when $E$ is large the maximum response, from (2), will always be near 1. This is made clear by plotting concentration response curves for receptors with different conformation changes (different values of $E$), but all with the same binding (value of $K_A$). These are shown in Figure 2a for values of $E$ from 0.1 to 10,000. When $E$ is less than 10 or so, we see obvious partial agonism (as expected from (2)), but for all $E$ values from 10 or so up to 10,000, the maximum responses are essentially indistinguishable; the curve just shifts progressively to the left as though binding affinity had changed. But binding affinity, in the sense that tells you about the binding site ($K_A$) has not changed. The total binding (as measured in a binding experiment) does change of course, as expected from (1), and this is plotted in Figure 2b, but this too clearly does not tell you about the binding site.

The curves in Figure 2a are very similar to the theoretical and experimental curves shown in Stephenson (1956) (though he calculated them in a different way). Now, as then, they show that any attempt to measure the efficacy of an agonist on a scale from 0 to 1 (as maximum response as fraction of that for a 'full agonist) is unhelpful and misleading, if the aim is to discover something about the structure-activity relationships of agonists, or about the effects of a mutation in a receptor. Of course, if real receptors always had rather low efficacies then this objection would not be serious, but that is not the case. For the muscle nicotinic receptor, $E$ is at least 30–100 for acetylcholine (see Table 1). In the case of a protein that is better characterized than most receptors, $E$ has been estimated as 3000 for haemoglobin (see below). The problem is not pedantic, it is real.

### *Two states and constitutive activity*

Once the idea of a global conformation change had taken root, it was natural, indeed it was a thermodynamic necessity, to consider how much of the receptor was in its active conformation in the absence of agonist. Wyman's postulate converged with Katz's approach when Monod *et al.* (1965) proposed their well-known mechanism for cooperative enzymes.

In the case of a single subunit, this amounts merely to addition of one extra state to the del Castillo-Katz mechanism, the unliganded active state (R*) which will produce 'constitutive activity', as shown in Scheme 2. Here $E_0$ is the conformational equilibrium constant in the absence of agonist, and is therefore a measure of constitutive activity, whereas $E_1$

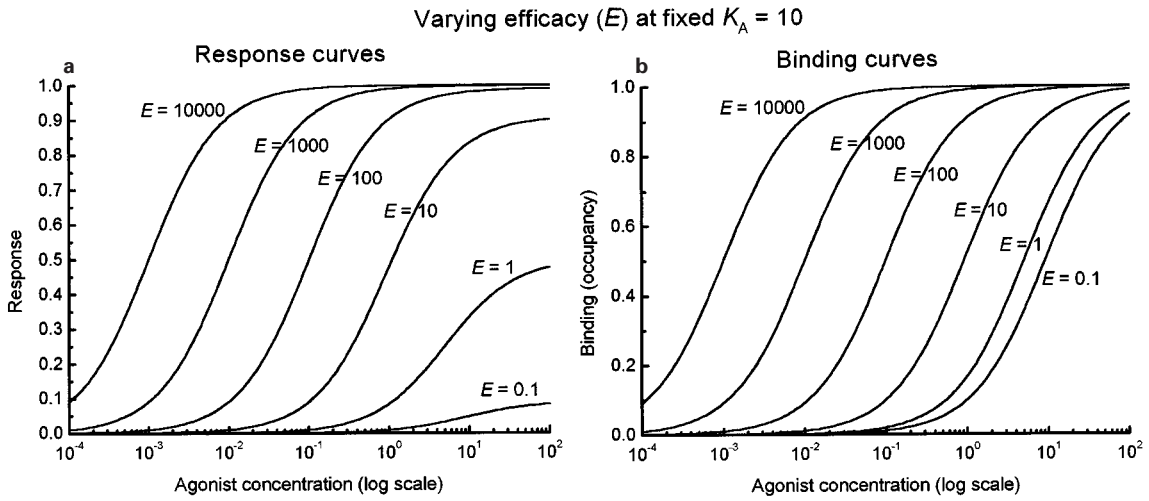
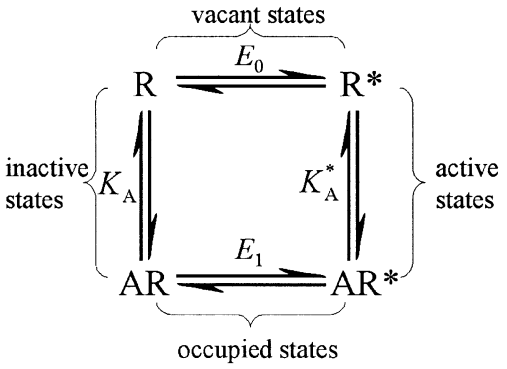



**Figure 2** Illustration of the effect of changing the ability to change conformation (the value of E in the del Castillo-Katz scheme) for a series of agonists (or of the receptors) that all have the same affinity for the binding reaction. (a) shows the fraction of active receptors, (b) shows the corresponding agonist binding curves.

is (like E above) a measure of efficacy. As before, both response and binding are hyperbolic at equilibrium, and again both have the same $EC_{50}$. And as before this $EC_{50}$ depends on all of the equilibrium constants. If we want to know about the binding site we have to find a way to estimate $K_A$.




Scheme 2

*Haemoglobin as a receptor*

This sort of mechanism (extended to four subunits, by analogy with Scheme 5, below) has been applied to haemoglobin (e.g. Edelstein, 1975), though it is only an approximate description. This makes an interesting analogy with a drug receptor. The deoxy (or T) state of haemoglobin corresponds to the resting receptor (denoted R here). Addition of oxygen (the 'agonist') causes, in a proportion of molecules, a concerted conformation change of the entire molecule to the oxy- conformation (known in the haemoglobin literature as the R state, corresponding to the active state, R*, here). In the absence of 'agonist', only about 1 in 9,000 molecules are active ($E_0 = 1.1 \times 10^{-4}$, very little constitutive activity, in receptor terms). The 'agonist' binds more tightly to the 'active form' by a factor of $M = K_A/K_A^* = 71$. Thus (see eq. 3) the conformational equilibrium constant for the mono-liganded molecules is $E_1 = E_0M^1 = 7.8 \times 10^{-3}$ (still little effect), for bi-liganded molecules it is $E_2 = E_0M^2 = 0.55$ (about 36% change conformation), for molecules with three ligands bound it is $E_3 = E_0M^3 = 39$ (about 98% change conformation), and for the fully-liganded molecule it is $E_4 = E_0M^4 = 2795$, i.e. almost all molecules are in the 'active state' (the oxy-conformation) at high 'agonist' concentration. In these terms, oxygen is a very high efficacy agonist. The fact that, for such agonists, it is difficult to distinguish a change in efficacy from a change in affinity has caused problems for the interpretation of experiments on haemoglobin, just as it has for receptors.

## What does 'allosteric' mean?

In the context of receptors, the description allosteric is now widely used. It is, perhaps, not helpful for clarity of thought that different authors often use it to mean somewhat different things.

At one extreme, the term 'allosteric antagonist' can often be translated as 'we have got an antagonist and we are not sure what it does, but it appears not to be competitive'. This means much the same as 'non-competitive', a word which pharmacologists had always supposed to mean action at a different site, though with no postulate as to how the effect was mediated. In fact 'non-competitive' usually meant (and still does) nothing more than 'not competitive', and therefore says nothing about mechanisms.

At the other extreme, Monod *et al.* (1965) gave a sharply delimited definition. Their definitions were as follows (slightly paraphrased for brevity).

'(1) Allosteric proteins are oligomers the protomers of which are associated in such a way that they all occupy equivalent positions.
(2) There is one site on each protomer, for each ligand that can combine with it.
(3) The conformation of each protomer is constrained by its association with other protomers.
(4) Two (at least) [conformational] states are accessible to allosteric oligomers.
(5) As a result, the affinity of one (or several) of the sites towards the corresponding ligand is altered when a transition occurs from one to the other state.
(6) When the protein goes from one state to another state, its molecular symmetry is conserved.'

The term allosteric ($\alpha\lambda\lambda o\sigma$ = other, different, $\sigma\tau\varepsilon\rho\varepsilon o\sigma$ = solid) was introduced by Monod & Jacob (1961) who said, in a discussion of end-product inhibition, ''From the point of view

of mechanisms, the most remarkable feature of the (inhibition of the synthesis of a tryptophan precursor by tryptophan) is that the inhibitor is not a steric analogue of the substrate. We propose therefore to designate this mechanism as 'allosteric inhibition' ''. At this stage, the word allosteric meant little other than what pharmacologists would have referred to as non-competitive antagonism. Soon afterwards Monod *et al.* (1963) said, concerning such non-competitive regulation of enzyme activity, 'The effect of these regulatory agents appears to result exclusively from a conformational alteration (allosteric transition) induced in the protein when it binds the agent'. This shifted the emphasis towards the central role of conformation changes, as postulated by Wyman & Allan (1951), and discussed above. This emphasis culminated in the influential paper by Monod *et al.* (1965) (see also Changeux, 1993, for an account of the background).

Probably the nearest thing there is to a consensus at the moment is that allosteric refers to any mechanism in which a protein can exist in two (or more) distinct conformations, which differ in their affinity for a ligand. This usage has been endorsed by Wyman (Wyman & Gill, 1990). And an allosteric regulator is anything that binds better to one conformation than the other (i.e. almost everything). Although the definition of Monod *et al*., (1965) explicitly limits the term to oligomeric molecules that show cooperativity, it is now common to use the term for mechanisms like that in Scheme 2, which do not fall into this category.

## What is efficacy in terms of mechanism?

It is obvious that the binding equilibrium, $K_A$, in schemes 1 and 2 represents affinity, in the sense that tells us about the binding site. Similarly, the equilibrium constant for conformation change in the fully occupied receptor reflects efficacy (e.g. $E$ in scheme 1, $E_1$ in scheme 2 and $E_2$ in Schemes 3–5). It is argued below that the roots of efficacy, and hence of partial and inverse agonism, lie in the receptor itself, rather than later events (i.e. in constants like $E$, or their analogues for G protein-coupled receptors). However, these receptor properties are never the only things that influence the maximum response. For example, in the case of an ion channel, the size of the response per occupied receptor will depend on the single channel conductance too (and on many other things if depolarization or some later response is being measured). Similarly, for G protein-coupled receptors, the nature of the coupling to G protein (and the concentration of G protein) also affect the overall efficacy (see below), as well as the coupling to the eventual response. Of course these things contribute to the relative efficacy of two agonists (on the same receptor) only insofar as they are dependent on the nature of the agonist, and often they are not. On the other hand, when comparing wild type and mutant receptors, it is not uncommon for things like single channel conductance to change (though luckily that, at least, is easily measured).

## What is a pure binding effect?

Mechanisms, like that in Scheme 2, which postulate that the receptor protein can exist in two different conformations (inactive, R, and active, R*) are often known as two-state mechanisms. Because they are cyclic, the principle of microscopic reversibility dictates that there are only three independent equilibrium constants in Scheme 2, rather than four. The fourth can be found from the other three, e.g. from

$$E_1 = E_0 \frac{K_A}{K_A^*}. \quad (3)$$

This says that the ability of the agonist-receptor to change conformation (as measured by $E_1$) depends simply on the relative affinities of the agonist for the inactive and active conformations. It shows that the 'efficacy', $E_1$, is inextricably linked to the binding affinities, so at first sight it is not obvious what we mean when we ask whether we are seeing a 'pure binding effect', or a 'pure gating (activation) effect'? The answer is simple if we bear in mind that the aim is to see how we can find evidence that the binding reaction is what has changed, rather then the ability to change conformation.

First consider the case where one agonist is tested on two receptors, say a wild type and a mutant receptor.

- A 'pure binding effect' would mean that both affinities, $K_A$ and $K_A$*, were changed by the same factor when the mutation was introduced, and both $E_0$ and $E_1$ were unchanged. This would be good evidence for an effect on the binding site.
- At the other extreme, a 'pure gating effect' would mean a change in the agonist-independent constant $E_0$, i.e. a change in the level of constitutive activity (though it is quite possible that even the larger value would give too little constitutive activity to be detected in an experiment). For a pure gating (activation) effect, both affinities, $K_A$ and $K_A$*, would be unchanged by the mutation, so the 'gating constant', $E_1$ would be changed in direct proportion to the change in $E_0$.

Next consider the case where we test two agonists on the same receptor. Is the difference between their potencies ($EC_{50}$s) a result of different binding, or different ability to activate once bound? In some ways this is a bit trickier. The tendency to activate in the absence of agonist is measured by the equilibrium constant $E_0$, so $E_0$ is the same for both agonists. Thus if the 'efficacy', $E_1$, changes this means that one, or both, affinities must change too. So is this a 'gating (activation) effect', or is it a 'binding effect'?

- For a pure binding effect, both affinities, $K_A$ and $K_A$*, would have to differ by the same factor for each agonist, so $E_1$ would be the same for both of them. This would be good evidence for a change in the binding site itself.
- For a pure gating (activation) effect the initial binding to the shut state, $K_A$ would have to be the same for both agonists, but $E_1$ would be different for each agonist, and hence binding to the open state, $K_A$*, must also be different. The change in binding to the active state means that the active state produced by each agonist must, to some extent, be different. In that case how can we call the mechanism 'two-state'? We know from ion channel studies (see below) that the active (open) state differs from one agonist to another, because different agonists hold the channel open (on average) for different lengths of time. However it is also found that (almost always) the conductance of the open channel does not depend on which agonist is used. It thus seems that the global conformation of the active (open) state is much the same for all agonists, but that some agonists can stabilize the open state better than others.

This sort of question has given rise to much discussion in the context of G protein-coupled receptors, which are dealt with later.

There is, of course, another possibility; the reaction rates could change without changing the equilibrium constants. For example, denote the opening rate constant for an ion channel as $\beta_1$ and the shutting rate constant as $\alpha_1$, so $E_1 = \beta_1/\alpha_1$. A mutant receptor in which both $\beta_1$ and $\alpha_1$ were

halved would have the same 'efficacy', $E_1$, but would nevertheless show a change in gating (the mean open time would be doubled but openings would be rarer). An experimental example of this phenomenon is shown in Table 1.

## What *is* the binding site?

Most of the discussion here supposes that the binding site is a well-defined set of amino acids that interact with the bound agonist. But, since the agonist binds more tightly to the active conformation of the receptor, the binding site obviously changes when the receptor changes conformation. Many proteins undergo quite large conformation changes (e.g. hexokinase, ribose binding protein), so it is quite possible that in the active conformation some part of the molecule hinges down onto the agonist and causes it to be trapped. If this is the case, it is likely that more amino acids will interact with the ligand in the active conformation (and it is even possible that interactions that are present in the inactive conformation would be lost). Clearly, even in those cases in which it is possible to estimate $K_A$, the value we get will tell us only about binding to the inactive conformation, and that is certainly something we want to know about, because that is the first event in producing a response, the *sine qua non* for all that follows (at least for receptors with low constitutive activity). In the context of the two-state view, it is clear from the discussion above that binding to the active conformation is part of the 'efficacy'. From the practical point of view, that is the case too, in the sense that binding to the active state will depend on the ability to change conformation, and is likely to be affected by mutations which affect that ability.

On the other hand, it is easy to envisage a different possibility. Imagine that, when the receptor is in its active conformation, several amino acids (that were not close to the ligand in the inactive conformation) now clamp down onto the ligand and form part of its binding site. A mutation in one of these amino acids could have no effect at all on the ability of the receptor to change conformation (e.g. $E_0$ unchanged in Scheme 2), but might nevertheless reduce the extent to which the ligand was bound (increase $K_A$*). This would decrease the 'efficacy' ($E_1$), and if that were reduced sufficiently could result in partial agonism.

## Are the terms affinity and efficacy still useful?

It is the general thesis of this paper that it is futile to think that firm conclusions can be drawn about structure-activity relationships of agonists, or the effect of mutations on receptors, without some knowledge of mechanisms. To that extent, our aim is simply to identify a mechanism that describes physical reality (to a sufficient approximation), and to identify the rate and equilibrium constants for the transitions between the states in which the receptor can exist. Then, for example, changes in $K_A$ will tell us something about the structure of the binding site in the inactive conformation, and changes in $K_A$* will tell us something about the structure of the binding site in the active conformation. The old terms affinity and efficacy are entirely redundant from this point of view. On the other hand, they do serve well to draw attention to a general problem, without getting bogged down in the details of particular mechanisms, and to that extent I still find them useful.

## Is our knowledge of mechanisms sufficient to allow physically meaningful conclusions?

The answer is that we don't know. It seems likely that our knowledge of G protein-coupled receptor mechanisms is still inadequate (see below). In the case of ion channels the situation is better. It seems very likely, for example, that physically meaningful conclusions can be drawn about channel blockers. The binding-gating question is harder, but it seems quite likely that, in a few cases at least, this may also be analysed in a physically realistic way. Nevertheless, there are, even for ion channels, many potential complications. A couple of these are as follows.

Almost all work has assumed, implicitly or explicitly, that the effect of changing the receptor structure (e.g. making a mutation), or changing the agonist structure, has the effect of changing the balance between existing states of the receptor. In other words, Schemes like (1) to (5) (and extensions of them that include desensitization) are still good descriptions of the physical mechanism. All that happens is that the structure change alters the rate (and hence equilibrium) constants, thus changing the balance between the various states. There is actually next to no hard evidence about whether or not this is true. It is not hard to imagine that this is an oversimplification, but until such time as there is convincing evidence to the contrary, this approach can be justified by application of Occam's razor.

Another, related, possibility is that the agonist binding itself induces a conformation change, which precedes, and is separate from, the global conformation change that is called 'activation' here. Some such conformation change is inevitable, but it is usually assumed to be small and local (see Shortle, 1992). It is also implicitly assumed to be induced, so inability to cause this change could not itself produce partial agonism. It is a simple matter to incorporate such a conformation change in any mechanism, but once again there is no hard evidence that there is need for such a step, so Occam's razor prevails again.

It is certainly a danger of the more sophisticated analyses that are mentioned below that the mechanisms on which they are based may be oversimplified to the point that the aim of measuring physically meaningful quantities may be foiled. In the words of the late William Paton, 'God does not shave with Occam's razor'. Or, as I would prefer to put it, you cannot expect a random process (such as evolution) to produce a simple and elegant system, but only a system which, however baroque, allows procreation.

# Experiments on ion channels

In the case of ion channels, single-channel recording allows us to 'see' the active state of the receptor very directly. If we could see with equal clarity when a molecule became bound to the receptor, the problems would go entirely, but that cannot be done. Nevertheless, it is no surprise that it is only for ion channels that anything at all rigorous has been achieved so far.

## What can be inferred from mutation studies?

The use of mutated receptors has undoubtedly led to enormous advances, but some sorts of experiments are much easier to interpret than others. It is no accident that the earliest studies concentrated on looking at the properties of a channel while it

was open (i.e. at ion permeation), rather than tackling the much harder problems involved in binding and gating. Such studies rapidly and clearly established that the M2 region of the nicotinic channel (rather than the initially postulated amphiphilic region) formed part of the channel lining, and the effects of charges on permeation (Imoto *et al.*, 1986, 1988).

Likewise many of the inferences from mutation studies (and from much earlier electrophysiological studies) concerning the permeation pathway in potassium channels have been verified by the determination of crystal structure (Doyle *et al.*, 1998). But none of these studies involved our main concern here, which is how to disentangle effects on the ligand-binding site from effects on conformation change.

It has to be said from the outset, that single channels recordings will usually be essential for any sort of rigorous analysis, though much can also be achieved by using very fast concentration jumps. There is little chance of coming to firm conclusions from equilibrium concentration response curves and binding curves alone, except in rather extreme cases (for example, the very large potency changes seen in the NMDA study discussed below allowed a strong case to be made even before single channels were investigated; see Figure 7). It is precisely because the former methods are available only for ion channels, that it has been possible to carry the analysis so much further with them, compared with, for example, G protein-coupled receptors. As so often, it is just a question of methods. For example, during the 1970s, the methods that were available then (like noise analysis) proved inadequate to distinguish between binding and gating (the history of this era has recently been discussed by Colquhoun & Sakmann, 1998).

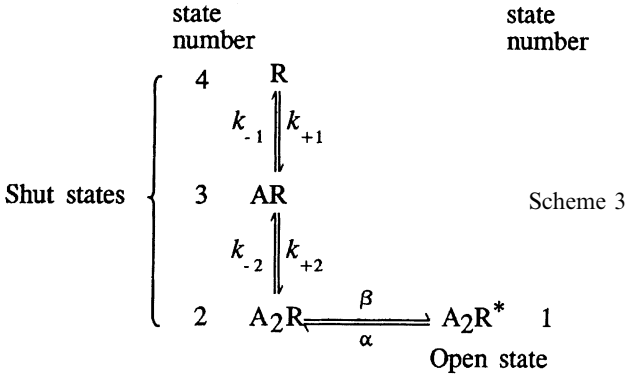
We shall next consider some examples of what can be done to solve the problems that were outlined above. First some general principles will be discussed, and then three case studies will be examined.

## Mechanisms for ion channels

The muscle type nicotinic acetylcholine receptor has two agonist binding sites, as inferred from Hill slopes and subsequently confirmed by the demonstration that it contained two $\alpha$ subunits. This channel (unlike many others) has few sub-conductance levels, and almost all suggested mechanisms suppose that the channel can exist in only two conformations, shut and open. In other words it is assumed that all five subunits flip together, in a concerted manner, from one conformation to the other. Some other possibilities are considered briefly below.

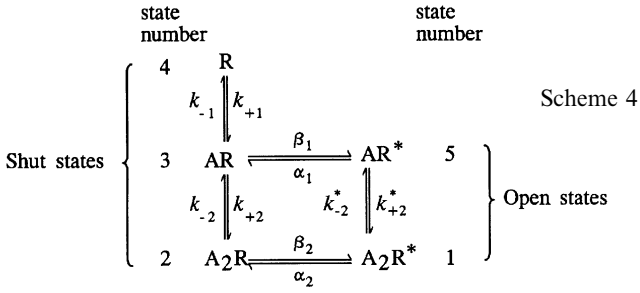
### *Two state (concerted) mechanisms*

A simple sequential mechanism would be appropriate if both sites had to be occupied for the channel to open, namely



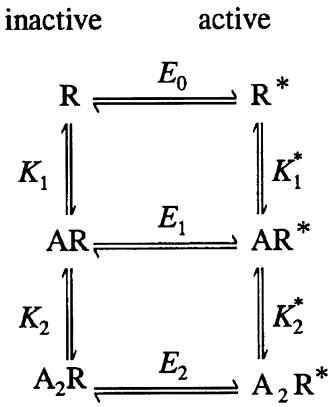

Scheme 3

When it was found that the channel could open (though rarely and briefly) with only one agonist molecule bound, this state had to be inserted too (Colquhoun & Sakmann, 1981).




Scheme 4

Spontaneous openings in the absence of agonist have been observed in a few cases with native receptors (e.g. Jackson 1994), and more commonly in some mutants. These observations necessitated the addition of an unliganded open state (R*), to give




Scheme 5

In the case where the first and second bindings are supposed to be the same ($K_1 = K_2$), this is the Monod-Wyman-Changeux scheme. The application of this sort of mechanism to receptors was considered by Karlin (1967) and Colquhoun (1973).

### *Macroscopic and microscopic constants*

The rate constants and equilibrium constants used here (e.g. in Schemes 3–5) are all microscopic constants, i.e. the constant per binding site. For example in Scheme 4, the actual (macroscopic) rate constant for the first binding will be $2k_{+1}$, because there are two vacant sites, either of which may become occupied. Similarly the macroscopic rate constant for the first dissociation from $A_2R$ will be $2k_{-2}$, because either of the two bound agonist molecules may dissociate. Thus the macroscopic equilibrium constant for the first binding is $K_1/2$, and the macroscopic equilibrium constant for the second binding is $2K_2$. Thus, if the two sites are equivalent, then the macroscopic equilibrium constant for the second binding will be four times greater than that for the first binding.

### *Sources of cooperativity*

All mechanisms, like those above, that involve more than one binding site with a conformation change show cooperativity, and the extent of this (e.g. the steepness of response or binding curves) can provide useful information about the action of agonists and the effect of mutations.

It is common practice to fit concentration-response curves with the Hill equation (it has become common, regrettably, for

this to be called the logistic equation, a term that is historically, mechanistically and mathematically inappropriate). The Hill equation has the property that when plotted as a Hill plot ($\log[(y-y_{min})/(y_{max}-y)]$ plotted against log([A])), it produces a straight line with a slope that is the Hill coefficient, or Hill slope, denoted $n_H$. The Hill equation is an empirical description that does not describe any known physical mechanism. Therefore no conclusions about binding and gating can be drawn directly from such fits. All real mechanisms predict curves which, when plotted in Hill coordinates, are not straight lines and which therefore have Hill slopes that vary with concentration. This is potentially informative, but experiments are rarely sufficiently precise to exploit this information.

The Hill slope (at any concentration) must be less than the number of agonist molecules that are needed to elicit the response, and may be much less (e.g. Wyman & Gill, 1990). For example, haemoglobin has four binding sites but the Hill slope (at the midpoint) is around 2.4.

The steepness of the curve (value of the Hill slope) is influenced by two separate factors. One is the concerted change in conformation. This will result in a Hill slope greater than one even if the binding itself shows no cooperativity at all, i.e. even if the binding has exactly the same affinity for the first and second binding to either conformation (i.e. $K_1 = K_2$ for the inactive conformation, and, in scheme 5, $K_1^* = K_2^*$ for the active conformation). But the Hill slope will be steeper if the binding itself shows cooperativity (higher affinity for the second binding than the first), and conversely will be reduced if the second binding shows lower affinity than the first (see Figure 4, below).

### *The 'Hill slope' for voltage-dependent ion channels*

It is worth pointing out, in passing, an analogy that seems often to have gone unnoticed. In the case of an agonist-activated channel we would commonly plot (ideally) probability of the channel being open ($P_{open}$) against log(concentration). When investigating multimeric voltage-dependent ion channels, such as potassium channels, it is common practice to plot the probability of the channel being open ($P_{open}$) against membrane potential ($E$). This is then usually fitted with the appropriate form of the Boltzmann equation (e.g. Hille, 1992, p. 55). This equation has exactly the same form as the logistic equation (see, for example, Colquhoun, 1971), so a plot of $P_{open}$ against voltage (not log voltage) has precisely the same form as a Hill equation plotted against log(concentration). This makes perfect sense because voltage and log(concentration) are analogous (the general expression for electrochemical potential is proportional to both potential and to log concentration, the former being what matters for voltage-dependent channels, and the latter for agonist-activated channels). In the context of voltage-dependent channels 'voltage-sensitivity' of the channel, or 'voltage-dependence of gating' is measured as the steepness of this curve ($P_{open}$ against $E$). (The usual measure is actually the reciprocal of the steepness, the 'the number of millivolts needed to produce an e-fold change'.) For example, mutations that reduce the number of positive charges in the S4 segment of a potassium channel reduce, in this sense, the voltage dependence of gating, so these charges are therefore thought to constitute the voltage sensor of the channel. However the voltage sensitivity so defined is precisely what would be called the Hill coefficient if we were dealing with chemical rather than electrical potential. As discussed below, anything that impairs the conformation change will reduce the Hill slope, so the problem of locating voltage sensors has problems that are directly analogous to the binding-gating problem for agonist-activated channels. This is not surprising because in this case voltage is the 'agonist' - it is what makes the channel open.

### *Can subunits change conformation independently?*

From the 1970s onwards the possibility has been considered that the conformation change may not be concerted (all or nothing), as implied by all of the mechanisms above. At the opposite extreme, for example, the subunits could behave entirely independently (as a naïve interpretation of the Hodgkin-Huxley potassium channel might suggest). The channel might then open only when a sufficient number of subunits had flipped to the active conformation, or it might open wider as more subunits flipped. There are far more distinct states in this sort of mechanism than in those mentioned above, which is probably one reason that they have received relatively little attention. If subunits were independent then no cooperativity at all would be expected in agonist binding, and only low Hill slopes are predicted for the response. Therefore it is unlikely that most ion channels have totally independent subunits. It may well be, though, that some intermediate situation exists. Perhaps the best evidence that this can happen comes from work on the ion channels that are activated by intracellular cyclic GMP. Ruiz & Karpen (1997) used as irreversible agonist an analogue of cyclic GMP that labelled subunits covalently (after UV irradiation). They succeeded, remarkably, in distinguishing between channels with 0, 1, 2, 3 and 4 agonist molecules bound to the homomeric tetramer, and found very low rates of opening with 0 or 1 agonist bound ($P_{open}$ around $10^{-5}$). With two agonist molecules bound the channel was open about 1% of the time, with three it was open about 30% of the time and when fully-liganded it was open for nearly all the time. These values are not in the ratios predicted by the MWC mechanism, but even more interestingly, the predominant channel conductance depends on the number of agonist molecules that are bound. Could it be that in this case the flipping of each subunit opens the channel wider? There is no direct evidence that this is what is happening, but it remains an intriguing possibility (see Miller, 1997). In another study of this sort of channel, Liu *et al*., (1998) also concluded that the transition change was not concerted, but their results suggested that the subunits could not change conformation independently either. Their results were fitted best by a scheme in which the tetrameric receptor was made up of two independent dimers, with the two subunits in a dimer undergoing a concerted transition between inactive and active states.

Another example of possibly similar behaviour was found by Rosenmund *et al*. (1998) in a chimaeric AMPA-kainate channel. Again it appeared that the single channel conductance was dependent on the number of agonist molecules bound, which suggests that the conformation change was not concerted.

Despite these examples, it has not usually been found that the single channel conductance depends on agonist concentration. Some channels, such as the adult nicotinic endplate receptor have very few subconductance states anyway. Most, however, have quite a lot. They are present in NMDA receptors (e.g. Stern *et al*., 1992; Wyllie *et al*., 1996), and are even more prominent in GABA and glycine receptors. The molecular basis for these multiple conductance states is not known, and they have not generally been found to be dependent on agonist concentration. Nevertheless, insofar as they represent different conformations of the receptor, their

existence is inconsistent with a simple two-state concerted transition mechanism. The simplicity of the MWC scheme, which has only three independent equilibrium constants regardless of the number of subunits, is aesthetically appealing, but the available evidence suggests that it is too simple to describe any known receptor.

## What can be inferred from concentration-response curves?

The classical methods for investigation of agonist action are concentration response curves and binding experiments. Apart from their inherent limitations, their usefulness for ion channels is severely limited by the phenomenon of desensitization. Almost all ion channels show profound desensitization with quite low concentrations of agonist, and for many this occurs rapidly, e.g. 50–100 ms for nicotinic receptors (Franke *et al*., 1993) and only a few ms for some glutamate receptors (e.g. Colquhoun *et al*., 1992). This means that accurate concentration-response curves can be obtained only in cases where the agonist can be applied very rapidly, and makes binding experiments almost impossible (except, of course, for investigation of desensitization).

The exception to this rule is when single channel methods are used to determine the probability of a channel being open, as a function of agonist concentration (e.g. Colquhoun & Ogden, 1988). This not only allows desensitization to be eliminated (by cutting out the desensitized bits from the record), but also gives a response ($P_{open}$) on an absolute scale from 0 to 1. It still does not, however, overcome the problem that 'high efficacy' agonists are indistinguishable from each other–all give a maximum $P_{open}$ near one (see Figure 2a and eq. 2). For $E = 50$ the maximum $P_{open}$ is 0.98 and for $E = 10000$ the maximum $P_{open}$ is 0.9999, and these are indistinguishable, so it is impossible to estimate $E$ from them. Therefore even this sort of concentration-response curve has limited ability to solve the binding-gating problem. But there is one sort of potentially useful information, and that stems from the fact that all agonist-activated ion channels seem to need to bind more than one agonist molecule to open efficiently, so their concentration-response curves are steep–they have Hill slopes greater than one.

### *What can be inferred from the maximum response?*

A reduced maximum response was the original definition of a partial agonist, but this nomenclature does disguise the crucial fact that not all 'full agonists' are the same. Efficacy (e.g. $E$ in eq. 1) has no upper limit. The binding-gating problem occurs in its most difficult form when it becomes necessary to distinguish between one 'full agonist' and another (e.g. between the curves for $E$ greater than 20 or so in Figure 2a), because in this region the effects of altering binding and gating are indistinguishable (from equilibrium data).

The information that can be obtained from the maximum response is limited in two other ways.

- Measurements of maximum response are obviously particularly susceptible to errors that result from desensitization. If two agonists desensitize to different extents, or if a mutated receptor desensitises to a different extent from the wild type, the potential for error is obvious. Inspection of published dose-response curves often shows that the points at the highest concentration (when they are shown) begin to decline, presumably as a result of desensitization (and/or channel block), so the observed maximum is in doubt.
- There is a particularly difficult problem when mutated receptors are being tested. When two agonists are compared on the same receptor preparation, we know that the number of receptors that are exposed to agonist is the same for both. But when we compare a mutated receptor with the wild type receptor, the two receptors have to be transfected separately into different batches of cells. The efficiency of transfection can very vary considerably between one batch of cells and another, but even when consistent maximum responses can be obtained, there is no guarantee that the efficiency of the construct used for expression is the same for wild type and mutant. If it is not, then the results may be consistent but wrong. What is really needed is a way to measure the number of functional surface receptors for both wild type and mutant. For example, this has been done by measuring binding (to intact cells) of α-bungarotoxin to nicotinic receptors (e.g. Campos-Caro *et al*., 1996), or of strychnine to glycine receptors (e.g. Lynch *et al*., 1997). Of course there is no guarantee that all receptors so measured are functional, but it is a great deal better than guessing.

### *What can be inferred from the Hill slope?*

Everything is simple if the two binding reactions have the same affinity ($K_1 = K_2$) in Schemes 3–5 (in which case Scheme 5 becomes the MWC mechanism). In this case the cooperativity arises entirely from the conformation change, and a pure change in the binding site does not change the Hill slope. Thus a reduction in Hill slope indicates an impairment of gating (though, as shown in Figure 3, it is possible for quite a large change in gating to produce only a small change in slope). But things get a bit more complicated if the two binding reactions are not the same (the two binding sites differ, or occupancy of one affects the other). For the muscle type nicotinic receptor there is (unfortunately for simplicity) a lot of evidence that the two binding sites are not equivalent (see, for example, Sine *et al*., 1990). For other sorts of ion channel there is still no substantial evidence, one way or the other. In this case the binding reactions will also contribute to the observed cooperativity. If the affinity for the second binding is greater than that for the first then the Hill slope is increased, as illustrated in Figure 4, and conversely if the second binding is weaker then the Hill slope is reduced. This may be referred to as cooperativity of binding to distinguish it from the cooperativity that arises from the conformation change. Of course, the latter alone will cause the binding curve to be steep. When we say 'the affinity for the second binding is greater than that for the first' we are referring once again to the microscopic affinity for one of the conformations (i.e. we are saying $K_1 > K_2$); we are not referring to what would be seen in a binding experiment (in which the total binding to both conformations is measured).

If a mutation reduces the ability to change conformation, or if a low efficacy agonist is tested, the Hill slope is expected to fall. This is illustrated in Figure 3, which shows the effect of a mutation that changes only the conformation change (the initial binding is unchanged) for an ion channel with two agonist binding sites.

In Figure 3a and b the conformation change constant ($E = \beta/\alpha$ in Scheme 3) is reduced from 20,000 in the wild type to 100 in the mutant (with $K_1 = K_2 = 100$ μM for both). The Hill slope is reduced only slightly when $E$ is still quite large in the mutant. Figure 3c and d show the same thing but with $E = 200$ for wild type, and $E = 1$ for the mutant. In this case the Hill slope (at the $EC_{50}$) is obviously reduced in the mutant (for the

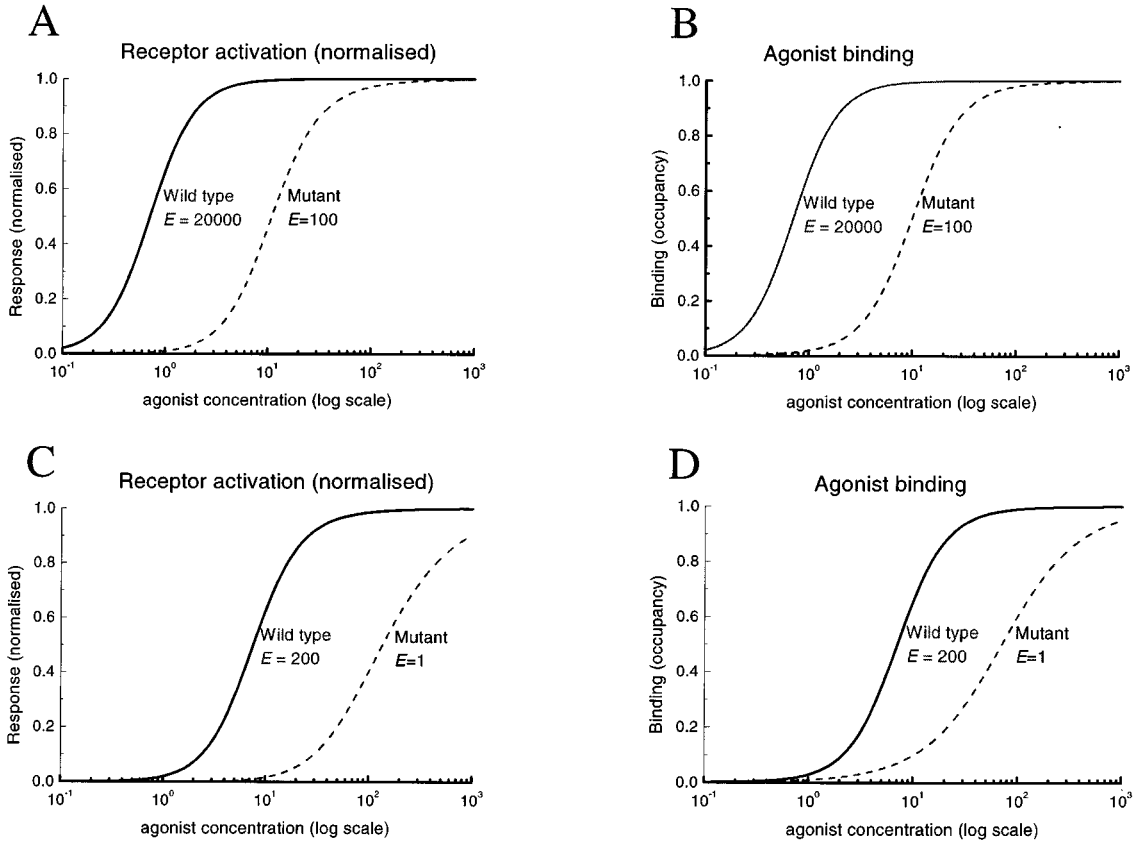



**Figure 3** Effect of impairment of gating on response and on binding. Illustration of the effect of a pure change in gating for a receptor that needs two agonist molecules to be bound to open the channel (Scheme 3). The affinity for the binding site ($K_1 = K_2 = 100$ $\mu$M) is exactly the same for wild type and mutant. The graphs show normalized response (left) and agonist binding (right). The upper curves show the case where the 200 fold reduction in efficacy still leaves the mutant as a full agonist. The lower curves also illustrate a 200 fold reduction in efficacy but in this case the wild type had lower efficacy and the mutant is a partial agonist. Only in the latter case is the reduction in Hill slope very obvious.

response it is reduced from 1.9 to 1.3). The maximum response ($P_{open}$) would also be reduced, from 0.98 to 0.5, but the problems of expression systems (mentioned above) would make this hard to detect with any certainty.

This sort of effect has proved useful in interpretation of experiments (see example of the glycine receptor, below). It is illustrated more systematically in Figure 4, which shows a plot of Hill slope (measured at the $EC_{50}$) against the value of the gating constant, $E$. The centre (solid) curve shows the case where the binding itself shows no cooperativity ($K_1 = K_2$ in Scheme 3, as for Figure 2). When the binding shows positive cooperativity too the upper dashed curve ($K_1 = 10K_2$) shows the same trend, but the Hill coefficients are larger. When the binding itself shows negative cooperativity (lower dashed curve, $K_1 = 0.1K_2$), the Hill coefficients are smaller, but change more with $E$. Clearly the observation of a reduced Hill slope in a mutant receptor cannot be interpeted unambiguously unless we have evidence that the cooperativity of the binding reaction itself ($K_1/K_2$) has, or has not, changed. Once again, the effects of changes in binding and conformation change are not easily disentangled.

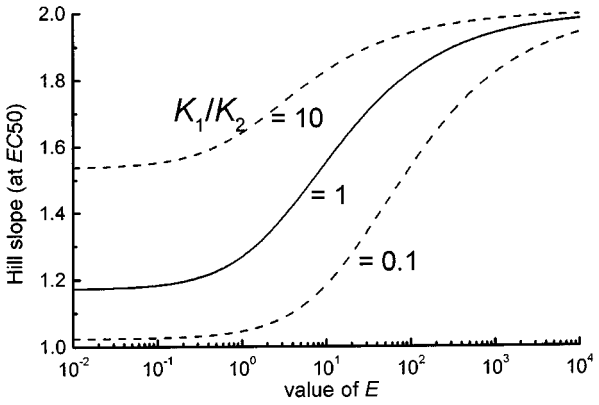



**Figure 4** Effect of gating constant on the Hill slope. The dependence of the Hill slope (measured at the $EC_{50}$) on the efficiency of gating (the 'efficacy', $E = \beta/\alpha$, in Scheme 3), when two agonist molecules are needed. The Hill slope increases with efficacy. The middle (solid) curve is the simple case where the binding itself makes no contribution to cooperativity, the upper curve shows the case when the second binding has higher affinity than the first; the lower curve shows the converse case.

### *How much shift can gating changes produce?*

Another useful property of receptors that show cooperativity can be seen by comparing Figures 1 and 3C,D. In both cases the gating constant, $E$, was reduced from 200 in the wild type to 1 in the mutant. In Figure 1, for a monomeric receptor, this produced a parallel shift of both response and binding curves by a factor of about 100. In Figure 3, the curves are not only not parallel, but the shift is much smaller; measured at the $EC_{50}$ it is 18 fold for response (Figure 3c) and 10 fold for

binding (Figure 3d). In other words, for a receptor that shows cooperativity, a much bigger change in gating is needed to produce a given shift of the curves. This too has proved useful (see NMDA receptor study, below). The effect is shown graphically in Figure 5. The relative $EC_{50}$ for response and binding are plotted against the gating constant for the mutant (solid curves). The assumptions are as for Figure 3, with the gating constant for the wild type taken as $E=1000$ (high efficacy). The upper dotted curve shows the relationship that would be found (for both response and binding) with a monomeric receptor (Scheme 1), while the lower dotted line shows the relationship $\sqrt{(E/E_{mut})}$, which is seen to give a reasonable approximation over much of the range. For the case in Figure 3c, d, we have $\sqrt{200}=14.1$.

### *What can be inferred by using competitive antagonists?*

The problems of interpretation of the action of agonists all arise from the fact that the occupancy of the binding site by an agonist is linked, *via* a conformation change, to subsequent events which result in a response. These complications can, therefore, be avoided if we use a ligand that produces no conformation change, i.e. a pure competitive antagonist. As a way of locating the agonist-binding site, this obviously helps only insofar as the agonist and antagonist occupy the 'same' site. It also seems that pure competitive antagonists are rare; many are actually inverse agonists, but this should not make much difference when there is little activity in the absence of agonist (Colquhoun, 1973).

As always, the aim has to be to estimate real physical equilibrium constants, so the binding of antagonists should be determined either by the Schild method, or by a ligand binding experiment. Determination of $IC_{50}$ values should give a rough guide, but since they inevitably depend on all sorts of local variables (especially on the agonist concentration that is used), they cannot be compared between different labs. It has been well-known at least since 1949 (Schild, 1949; Arunlakshana & Schild, 1959) that it is impossible to estimate real equilibrium constants from $IC_{50}$ curves, and consequently such measurements cannot be used to compare receptors in different tissues or expression systems. Leff & Dougal (1993) tried to resuscitate the $IC_{50}$, but their attempt to modify the Hill equation to allow for competitive antagonism is, like the Hill equation itself, entirely empirical; it cannot be derived from any physical mechanism (try it!). It merely describes parallel-shifted Hill curves, and as such is useful only as a means of implementing the Schild method. The 'general Cheng-Prusoff equation' which they derive from it has no sound physical basis, and so cannot be relied upon to estimate real equilibrium constants, though it has been seized upon by some authors as a convenient short cut. In effect the Hill equation refers to the impossible case of infinitely strong cooperative interactions between binding sites such that no intermediate states of ligation are possible. If a competitive antagonist is present there is no sensible way to allow for the obvious possibility that some receptors may be occupied by agonist, some by antagonist, some by both, and some by neither, or to incorporate the necessary assumptions about which of these states can elicit a response.

It is for these reasons that it is futile to attempt to compare receptor subtypes (or the same receptor in different tissues or expression systems) by comparing $IC_{50}$s, though this is not infrequently attempted (e.g. Sucher *et al*., 1996).

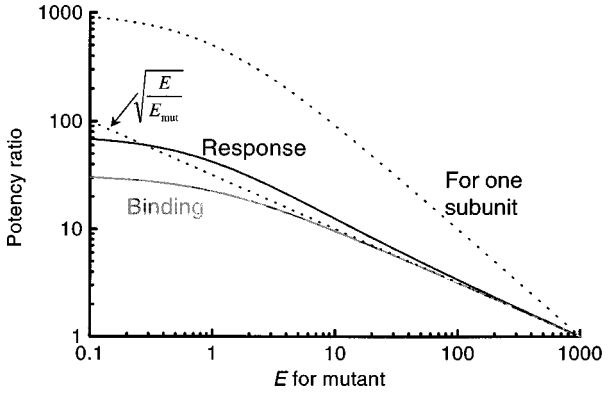



**Figure 5** Dependence of potency ratio on $E_{mutant}$. The shift (relative $EC_{50}$) of response and binding curves (solid lines) produced by impairment of gating only, for a receptor that needs two agonist molecules (Scheme 3). The relative $EC_{50}$ is plotted against the efficacy of the mutant, with $E=1000$ for the wild type.

## Two more direct approaches to the binding site

### *The substituted cysteine accessibility method*

This method uses mutations that introduce cysteine residues into the putative binding site (see Karlin & Akabas, 1995). This substitution may, of course, affect the receptor itself, but the method depends on the (further) change that can be produced by covalent linking of bulky reagents to the sulphydryl groups that have been introduced. If the presence of an agonist retards this change, then it is assumed that the cysteine in question must be in the binding site. In general, this argument is not immune to the binding-gating problem, because the agonist that is being used in the protection experiment will cause a global conformation change, by activating and/or desensitizing the receptor. This change would extend far beyond the binding site itself, and so could influence access of the sulphydryl reagent to sites that are remote from the binding site. This method was used by Wood *et al.* (1997) to investigate the binding site for the co-agonist glycine on the NR1 subunit of the NMDA receptor, and after introduction of an A714C mutation, glycine, but not glutamate, was found to protect against inhibition by a sulphydryl reagent. In this case glutamate was not present during the glycine protection experiment, and glycine alone should produce no major conformational change, so the binding-gating problem was largely avoided.

### *Photo-affinity labelling of the binding site*

Perhaps the most direct method of all for location of an agonist binding site is to use an agonist that can label the site covalently. This is hard work of course, because it means that the protein has to be sequenced, rather than DNA, and suitable reagents may be hard to find. Furthermore only a few amino acids (e.g. tryptophan, tyrosine or phenylalanine) can be labelled in this way, so one is reliant on one of them occurring in the binding site. Nicotine itself has been used in this way on the Torpedo nicotinic receptor, and was found by Middleton & Cohen (1991) to label mainly Tyr-198 of the $\alpha$ subunit, though Tyr-190 and Cys-192 (one of the two adjacent cysteines) were also labelled. In addition Tyr-55 of the $\gamma$ subunit is also labelled Chiara *et al*., 1998). In similar experiments on the $GABA_A$ receptor, Smith & Olsen (1994) found the primary site of photo-labelling by the agonist muscimol to be Phe-64 of the $\alpha$1 subunit.

These methods seem to show a fair degree of specificity, at least as judged by the retardation of covalent labelling by antagonists that are supposed to be competitive, but the nature of the reactions that are activated by UV irradiation are poorly understood. When the label is an agonist, the position of the label is likely to tell us about the nature of the binding site in the desensitized conformation rather than its structure in the resting or active conformations (see discussion above concerning 'what is the binding site?'). The results could, therefore, be different from those found in mutation studies in which efforts have been made to isolate the microscopic binding constant for the inactive state. Clearly the rate at which covalent labelling occurs could well be influenced by the ability of the receptor to change conformation to the active and desensitized conformations, but this approach is nevertheless probably, of all known methods, the least likely to be invalidated by the binding-gating problem.

## Single channel conductance – how constant is it?

It has been found, in almost every case that has been tested, that the single channel conductance is the same whichever agonist is used to open the channel (e.g. Gardner *et al*., 1984 for nicotinic receptors; Howe *et al*., 1991 for NMDA receptors, but see Swanson *et al*., 1997, for a possible exception). This suggests that the open conformation may be very similar, regardless of which ligand is bound. In other words, it is good evidence that a two-state approach is reasonable. The effect of using one agonist rather than another is merely to stabilise the active (open) conformation to different extents.

Many mutations in receptors are found to leave the conductance properties of the channel entirely unchanged. This is true, to a good approximation, in all of the experimental studies that are discussed below. This suggests that the active conformation of the mutant receptor is much the same as that of the wild type, though the stability of this conformation, or the ease with which agonist binds to it, may be drastically changed.

Other mutations, especially those in or near the M2 region, can have very drastic effects on conductance. For example, the NMDA receptor (NR1/NR2A) normally has a 50 pS main level and 40 pS sublevel (in 1 mM calcium). The T671A mutation in NR2A, which appears to affect mainly the binding of glutamate (see below) affects neither main nor sublevel at all (Anson *et al*., 1998), but the N598R mutation in NR1 reduces conductance drastically, to 2.6 pS, with a 1.2 pS sublevel (Béhé *et al*., 1995).

A change in single channel conductance is essentially a change in the ability of an agonist to produce a response once bound, i.e. a change in efficacy. This is one sort of change in efficacy that can, unlike most, be detected and measured very easily.

In fact the measurement of single ion channel currents provide very direct evidence (perhaps the most direct that there has ever been) that molecules do indeed exist in two (or a few) discrete conformations, between which they switch very rapidly. This is an assumption that underlies all chemical kinetics, and it is good to know there is some truth in it.

## The muscle nicotinic acetylcholine receptor

As almost always, more is known about this receptor than any other. It was the first receptor for which a serious attempt was made to separate the binding steps from the gating steps (Colquhoun & Sakmann, 1981, 1985), and is still probably the only one for which the analysis is considered reasonably reliable.

### *Effect of different agonists on the frog endplate receptor*

At this receptor, the agonists suberyldicholine (SubCh) and DecCh (decan-1,10-dicarboxylic acid dicholine ester) are three to five times more potent than acetylcholine (ACh), whereas carbachol is about ten time less potent. Are these differences caused by differences in the ability to bind to the receptor in the first place, or are they caused by differences in the ability to open the channel once bound? This is the classical affinity-efficacy question.

The analysis depends on the observation of repeated openings, separated by short shut times, during a single activation of the receptor (see Edmonds *et al*., 1995). This can give estimates of the opening and shutting rate constants ($\beta_2$ and $\alpha_2$ in Scheme 4), and the dissociation rate constant, $k_{-2}$. The results were analysed in terms of Scheme 4, rather than Scheme 5, for the simple reason that spontaneous openings were not detectable. The results were as in Table 1. They are consistent with results obtained from equilibrium $P_{open}$ curves (Colquhoun & Ogden, 1988) and from fast concentration jump experiments (Franke *et al*., 1993).

It is apparent that SubCh and DecCh, although more potent than ACh, are actually less efficacious at opening the channel once bound (lower $E_2$). Their potency results from their higher binding affinity. Notice also both afffinity and 'efficacy' equilibrium constants are much the same for SubCh and DecCh, but the rate constants $\beta$ and $\alpha$ for DecCh are about half those for SubCh, so channel openings are longer for DecCh. This illustrates the incomplete description provided by equilibrium descriptions of affinity and efficacy.

The analysis in Table 1 assumed that the two binding sites do not interact so $K_1 = K_2$. A slightly better fit could be obtained if the two sites were assumed non-identical, or non-equivalent. The opening rate of the channel is fast, around 30,000 $s^{-1}$ even at 11°C (Colquhoun & Sakmann, 1985), and around 60,000 $-80{,}000\ s^{-1}$ at room temperature (e.g. Maconochie & Steinbach, 1998).

Improved methods of analysis for single channel data have been now been developed (e.g. Sine *et al*., 1990; Colquhoun & Sigworth, 1995; Colquhoun *et al*., 1996; Qin *et al*., 1997). In addition, it is now possible to exploit information from non-stationary single channel recordings (Wyllie *et al*., 1997, 1998; Colquhoun *et al*., 1997).

**Table 1** Rate constants for frog endplate receptor at 11°C, in Scheme 4 (Colquhoun & Sakmann, 1985)

| *Agonist* | $\beta$ ($s^{-1}$) | $\alpha$ ($s^{-1}$) | $E_2 = \beta/\alpha$ | $k_{-2}$ ($s^{-1}$) | $k_{+2}$ ($M^{-1}\ s^{-1}$) | $K_2$ ($\mu$M) | $EC_{50}$ ($\mu$M) |
|---|---|---|---|---|---|---|---|
| ACh | 30600 | 714 | 43 | 8150 | $10^8$ | 80 | 14.4 |
| SubCh | 18000 | 625 | 29 | 2410 | $1.6 \times 10^8$ | $\approx 15$ | $\approx 3.4$ |
| DecCh | 9500 | 303 | 31 | 2420 | $1.6 \times 10^8$ | $\approx 15$ | $\approx 3.4$ |

*Mutant nicotinic receptors*

Some of the most detailed analyses of mutant ion channels have been done on the 'slow channel' mutants of the human muscle nicotinic receptor, which cause certain congenital myasthenic syndromes (see Vincent *et al*., 1997).

An interesting example is provided by a glycine to serine mutation (G153S) in the N-terminal part of the $\alpha$ subunit. This subunit was expressed, together with wild type $\beta$, $\delta$ and $\varepsilon$ subunits, by Sine *et al*. (1995). The single channel results were analysed according to Scheme 3, and to a more complicated version in which the two agonist-binding sites were supposed to be non-equivalent. The outcome clearly showed that the mutation had little effect on the gating rates. The opening rate constant $\beta$ was about 60,000 $s^{-1}$ for both wild type and G153S, and $\alpha$ was reduced only slightly in G153S, so the 'efficacy' $E$ was high for wild type (26–32 depending on assumptions), and increased slightly in G153S (to 43–51). In contrast the affinity for acetylcholine (especially for one of the sites) was greatly increased in the mutant. For the site most affected, the association rate constant increased from $1.8 \times 10^7$ $M^{-1}s^{-1}$ to $9.5 \times 10^7$ $M^{-1}$ $s^{-1}$, whereas the dissociation rate constant fell from 16,500 $s^{-1}$ to 970 $s^{-1}$, so the microscopic equilibrium constant for this step was reduced about 90 fold in the mutant. Individual openings are of similar length but the slow dissociation causes the receptor to re-open many more times during a single activation. These results provide strong evidence that the binding, rather than conformation change, is altered in the mutant receptor, and, by implication, that G153 forms part of the binding site.

In another slow-channel mutant, $\alpha$N217K, Wang *et al*. (1997) found a very similar change in binding, with little change in gating, so again the mutation seemed to affect the binding site. This example is interesting because it shows that even with the best analysis there may still be problems in identifying the physical location of the binding site by mutation studies. The N217 residue is in the first transmembrane region, which is, or so it is generally supposed, not part of the acetylcholine-binding site.

Other mutations affect mainly the conformation change. For example Akk *et al*. (1996) used a similar analysis on the $\alpha$D200N mutant (in mouse receptor), which is in the extracellular N-terminal region, separated by only six amino acids from the adjacent cysteine residues at 192, 193 which are thought to form part of the binding region. It has relatively little (2–4 fold) effect on binding, but reduces the channel opening rate constant ($\beta$) 100 fold (adult receptor), or 400 fold (embryonic receptor), while the closing rate is somewhat speeded, resulting in a reduction in the gating equilibrium constant of up to 1,200 fold. In the mutant, $E$ is less than 0.2, so acetylcholine becomes a weak partial agonist in the mutant receptor, as was clear for equilibrium concentration-response curves in which the absolute $P_{open}$ was used as response.

Many other mutations produce effects on both binding and gating. For example Chen *et al*. (1995) investigated mutations at tyrosine 190 in the $\alpha$ subunit of mouse receptor. This residue, which is likely to be close to, or in, the acetylcholine-binding site, affects both the binding stage and the conformation change.

## The glycine receptor – a gating mutation?

The glycine receptor, unlike the muscle nicotinic receptor, has no generally accepted kinetic mechanism. As a result, it is much harder to interpret the effects of mutations in any rational way. The rare 'jumping lumberjack' syndrome (startle disease, or hyperekplexia) is known to result from mutations in the glycine receptor (reviewed by Rajendra & Schofield, 1995). One such mutant, which causes an unusually severe form of the disease, is K276E in the $\alpha$1 subunit of the human receptor, which is in the loop between M2 and M3. Rajendra *et al*. (1995) found that when this mutant was expressed homomerically in HEK293 cells, $\beta$-alanine and taurine behaved as antagonists, whereas they are partial agonists on the wild type receptor. This alone is sufficient to suggest that the mutation had hindered the ability to change conformation. Figure 6 shows equilibrium concentration-response curves for both homomeric and heteromeric (probably the native type) receptors (Lewis *et al*., 1998). It is immediately obvious that the $EC_{50}$ is increased for the mutant, and more importantly, there is an obvious reduction in the Hill slope.

This reduction in Hill slope suggests that the mutation has affected the ability of the receptor to open, i.e. impaired gating as discussed above (see Figures 3 and 4), though there was really no evidence to show that the inherent binding cooperativity was unchanged in the mutant (see above). Single channel measurements showed that the single channel conductance was not reduced in the mutant (if anything it was bigger), but openings were, on average, 8.4 fold briefer, which again suggests that the gating is impaired. A reduction of 'efficacy' of the sort needed to reduce the Hill slope would be expected (see Figures 3 and 4) to reduce the maximum response to an extent that should, with luck, be measurable. The curves in Figure 6 have been normalised to have the same maximum response, but the original data showed an average maximum response that was reduced (by a factor of about 10 fold in the heteromer). However, in the absence of any certain knowledge of the number of functional receptors in the oocyte membrane (as well as of the effect of desensitization on the measurements), this number is rather uncertain.

It is not so obvious whether or not this impairment is sufficient to cause the 29 fold increase in $EC_{50}$ seen in the mutant heteromer (as illustrated in Figure 6), or whether binding of the agonist has also been affected. The equilibrium constant for the binding of the competitive antagonist, strychnine, was found (by the Schild method) to be essentially the same in wild type and mutant receptors. Insofar as glycine and strychnine share the same binding site this provides

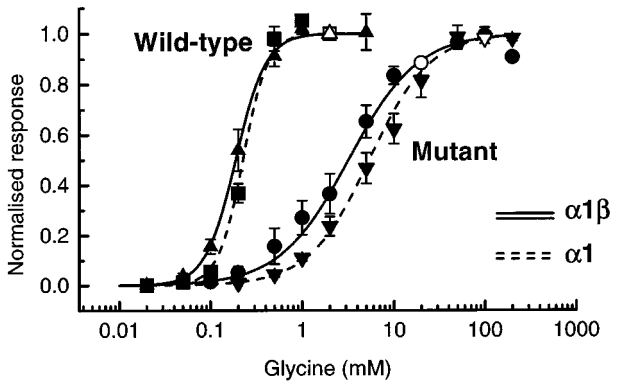



**Figure 6** Effect of the mutation K276E in the $\alpha$1 subunit of the human glycine receptor. Equilibrium concentration response curves, normalized to the same maximum, determined on wild type and mutant receptors expressed in oocytes. Curves are shown both for the homomeric $\alpha$1 receptor, and the heteromeric $\alpha 1\beta$ receptor. From Lewis *et al*. (1998).

evidence that the mutation has not affected the binding site very much, as discussed above.

In the absence of any well-defined kinetic mechanism it is difficult to take the analysis much further than this at present. Lynch *et al.* (1997) showed that the homomeric mutant expressed as well as the wild type in cell lines. If this were regarded as sufficient reason to take literally the observed the 10 fold reduction in maximum response, then it is possible to fit to the results a number of different plausible mechanisms. When this was done by Lewis *et al.* (1998), the results did indeed suggest, roughly speaking, that the observed effects could result largely from a change in gating, though they did not fit the idea that the sole effect was to change the 'allosteric constant' (like $E_0$ in Scheme 2).

In summary, there is good reason to think that the mutation affected gating, but the extent to which it may also have affected binding will remain far from certain until better mechanisms are worked out for the glycine receptor.

## The NMDA receptor – a binding site mutation?

The kinetic behaviour of NMDA receptors is very much more complicated than that of muscle nicotinic receptors (Gibb & Colquhoun, 1992; Wyllie *et al.*, 1998), and as for the glycine receptor, there is no accepted kinetic mechanism yet.

The location of the glutamate-binding site has been investigated by making mutations in the NR2 subunit by Laube *et al.* (1997) and by Anson *et al.* (1998). Once again we face the binding-gating problem – is there good evidence that the mutated residues are in the binding site? Mutations in the pre-M1 and the post-M3 regions (the so-called S1 and S2 domains) were found to produce large reductions in the potency of glutamate. Equilibrium concentration-response curves for the wild type, and for the three most effective mutations found by Anson *et al.* (1998) are shown in Figure 7. The most effective was T671A, which produced a 1000 fold increase in the $EC_{50}$ for glutamate, but how can we be sure that this was not a result of an impaired conformation change, as in the example in Figure 1? The relevant points are as follows: (1)

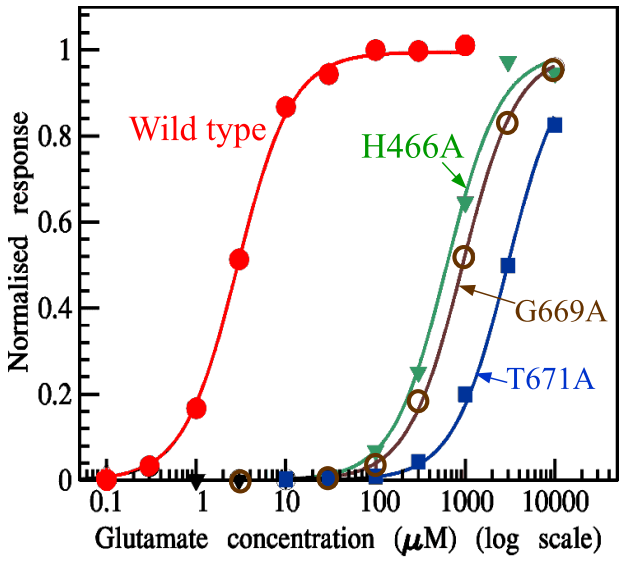



**Figure 7** Mutations in the NR2A subunit of the NMDA receptor. Equilibrium concentration-response curves for wild type NMDA receptors (NR1a/NR2A) expressed in oocytes, and for NR1 co-expressed with each of three mutant NR2 subunits. From Anson, *et al.* (1998).

An impairment of gating should produce a reduction in maximum response, but this would be measurable only if the gating constant ('efficacy', $E$) was reduced below 10 or so, as is obvious from Figure 2a. In any case, the curves in Figure 7 are normalised. Although no gross changes in maximum response were observed, uncertainties about the relative expression efficiencies of the mutants prevent any precise statement being made; (2) The reduction in potency is very large. Given that (at least) two binding sites must be occupied by glutamate to open the channel effectively, this means that a 1000 fold reduction of potency would require something like a million fold reduction in the gating constant (insofar as the square root approximation shown in Figure 5 is valid). If this were not to produce a gross reduction in maximum response, glutamate would have to be enormously efficacious on the wild type receptor ($E$ above $10^6$ or so), which is most unlikely in view of the fact that the channel is open for only a third or so of the time for which the binding sites are occupied (Gibb & Colquhoun, 1992; Wyllie *et al.*, 1998); (3) The curves in Figure 7 are parallel, i.e. there is no detectable reduction of the Hill slope. Again, unless the efficacy were enormously high in the wild type, a reduction in Hill slope would be expected if the effects were caused by changes in gating (see Figures 3 and 4); (4) The equilibrium constant for binding of a competitive antagonist (APV) was reduced 250 fold in the most effective mutant (T671A). Insofar as such an antagonist can be assumed to have zero efficacy ($E=0$) (see above) the binding-gating problem disappears when competitive antagonists are used. Therefore this observation constitutes strong evidence that the binding site is affected by the mutation, as long as the antagonist and agonist do indeed bind at the 'same' site; (5) Single channel measurements were made on wild type, and the most effective mutant (T671A). They showed that both the main and the subconductance level, and even the frequency of transitions between them, were quite unaffected by the mutation. The nature of the open state appeared to be little affected. The mean open lifetime of mutant channels appeared to be slightly longer than that of the wild type, which might imply a slight change in gating (though in the wrong direction to explain the large reduction in glutamate potency). In any case, the apparent lengthening of the mean open time could easily result from failure to detect short shut times, which would be expected to be briefer (and rarer) for a low-affinity agonist; (6) Brief concentration jumps on the T671A mutant shows that the current decays, after removal of glutamate, about ten times faster than for wild type, a result that is consistent with the channel re-opening fewer times while it was occupied by agonist, as expected for a low affinity agonist, though again other explanations are possible.

In summary, these lines of evidence, taken together, constitute strong evidence that the affinity for the initial binding of glutamate is reduced in the T671A mutant, despite the fact that it is still not possible to do a complete analysis of the sort that has been achieved for muscle nicotinic receptors.

# Experiments on G protein-linked receptors

## The nature of the problem

Up to now, the binding-activation problem has been discussed in terms of ion channels. Now we move on to the far larger group of receptors whose effects are transduced by G proteins.

The questions to be considered are very much the same as for ion channels, and, as before, the same problem occurs in

two guises: (a) Structure-activity relations: how do we interpret the effects of changes of agonist structure – is the binding changed or the ability to activate (the affinity/efficacy problem); (b) Receptor mutants: how do we interpret the effects of changes in receptor structure (the binding/activation problem), e.g. how can we find evidence that a mutated residue is in the binding site?

In this group of receptors the problems of interpretation are greater than for ion channels, for several reasons. Firstly, and perhaps most importantly, it is not yet possible to observe directly the conformation change in the receptor itself, which communicates the effect of agonist binding on one side of the membrane to the G protein on the other side. Secondly, the system is far more complex, and even responses such as GTPase activity are far 'further' from agonist binding than is channel opening in the case of an ion channel. Thirdly, the fact that these receptors are not oligomers, which in some ways makes them simpler than ion channels, actually deprives us of potentially useful information from changes in the steepness of the concentration-response curves (see above).

It is clear from what has already been said about ion channels that it is futile to hope that the effects of changes of agonist structure (structure-activity relations), or effects of changes in receptor structure (mutant receptors), can be interpreted sensibly without any knowledge of mechanisms. Stephenson's ideas were, of course, evolved in the context of G protein-coupled receptors, though nothing was known about transduction mechanisms at the time. Since his black box methods simply do not work, we must next consider what can be said about mechanisms. First, though, a brief summary of some of the experimental facts will be given.

## Some experimental evidence

The phenomenon of partial agonism was first observed with G protein-coupled receptors, and it is what stimulated both Stephenson and Katz to revise earlier theories. Far later, it was discovered that some agents (inverse agonists) could suppress the basal response level. These observations led to the idea that the receptor might be active in the absence of agonist (as envisaged in the MWC mechanism, see above). According to this view, a ligand was an agonist if it bound more tightly to the active than to the inactive conformation of the receptor, a competitive antagonist if it bound equally to both, and an inverse agonist (or 'active antagonist') if it preferred the inactive conformation. These simple 'two-state' ideas could describe, qualitatively, most of the observed phenomena. But what they could not do was to allow clear inferences to be made about whether a change in the structure of the agonist, or of the receptor, caused a change in the initial binding of the agonist, or in the ability of the receptor to change conformation. To get further, it has turned out to be essential to know about the transduction mechanism.

One of the earliest observations that was made after investigation of transduction mechanisms began, was that the measured equilibrium binding of agonist to the receptor (in the absence of GTP) was not described by a simple hyperbolic (Langmuirean) curve, but required two (or more) such components to fit it. Addition of GTP (or a non-hydrolyzable analogue such as GTP$\gamma$S) caused the agonist binding to revert mostly to the low-affinity form (Birdsall *et al*., 1978; Hulme *et al*., 1978). (Only the latter is physiologically relevant because cells contain more than enough GTP and GDP to keep the system in the latter form.) There is still no universally agreed explanation for this 'biphasic' binding, but some ideas are mentioned below.

After cloning of the receptors it was soon found that many mutant receptors were active in the absence of any agonist (e.g. Kjelssberg *et al*., 1992; Lefkowitz *et al*., 1993), and of course the action of inverse agonists became much more obvious on these mutant receptors. In fact it turned out that agents which had previously been classified as competitive antagonists were actually active inverse agonists (e.g. Costa & Herz 1989; Costa *et al*., 1992; Burstein *et al*., 1997). In the context of the two-state approach, this was, of course, hardly surprising. The chance of finding two ligands that have exactly the same affinity for active and inactive conformations of the receptor must be vanishingly small.

After a great deal of work, the following picture of the transduction mechanism has emerged. The agonist binds to the receptor, and so alters the equilibrium between its inactive and active conformations. The active receptor combines with a G protein in its resting state (G-GDP), or, if the G protein is pre-coupled to the receptor, produces a conformation change in G-GDP. This accelerates the rate of (catalyses) GDP dissociation, leaving R*G with the nucleotide binding site transiently vacant. GTP very soon occupies the site and the G trimer then dissociates into G$\alpha$-GTP and G$\beta\gamma$, both of which can then act as second messengers, producing a variety of complex effects in the cell which eventually lead to the response that is observed (e.g. Clapham, 1996; Bourne, 1997).

Within this picture, one qualitative explanation of the observations on agonist binding is that the R*G form has a high agonist affinity and this is what is measured in the absence of GTP. When GTP (or an analogue such as GTP$\gamma$S) is present the processes above cause the receptor to revert to its resting state (R dissociated from G-GDP) which is presumed to have low agonist affinity. This argument has not been made quantitative, and does not really explain why a low affinity component is seen in the absence of GTP. Alternative (or complementary) explanations have been proposed based on the depletion of free G protein molecules in the vicinity of the receptor as they become bound (see Appendix), or on compartmentalization of the reacting components.

In recent years a great deal has been learned about the structure of the three G protein subunits, because they have been crystallized (see Bourne, 1997), but much less is known about the receptor. It is the latter which still constitutes the biggest unknown, since much depends on the nature of the conformation change that it undergoes when a ligand binds to it.

## Mechanisms for G protein-coupled receptors

It may be noticed that I avoid the term model, which is commonly used to describe reaction mechanisms such as that in Figure 8. This usage is deliberate; it is intended to emphasize the fact that reaction schemes are likely to be helpful only insofar as they describe (to an adequate approximation) real physical events, a real mechanism. An empirical model with no physical basis is likely to be more of a hindrance than a help.

The first attempts to consider G protein interactions in a quantitative way were essentially Clark's classical scheme, extended to include binding of the G protein as well as agonist to the receptor to produce an active ternary complex (e.g De Lean *et al*., 1980; Birnbaumer, *et al*., 1980). Eventually, though, it became clear that it was essential to postulate explicitly a conformation change in the receptor itself, just as had been done much earlier for ion channels (del Castillo &

Katz, 1957). Without some large-scale conformation change in the receptor itself, how could the G protein on the inside of the membrane 'know' about the binding of an agonist on the outside of the membrane? This was first done by Samama *et al.* (1993), and Lefkowitz *et al.* (1993), and a more complete version, the 'cubic ternary complex' scheme was proposed by Weiss *et al.*, (1996a, b, c). This is illustrated in Figure 8.

The mechanism involves an agonist (A), a receptor (R) and a G protein (G). In the absence of G protein it is simply a del Castillo-Katz scheme, extended by the existence of an active receptor conformation (R*) in the absence of the agonist, in the spirit of Monod-Wyman-Changeux. This is the front face of the cube in Figure 8. The back face consists simply of the same four states, but with G protein bound to them. Thus the four vertical sides represent binding of agonist, the four front-to-back edges represent binding of G protein, and the four left-to-right edges represent the change in conformation between resting (R) and active (R*) conformations. There are 12 equilibrium constants shown in Figure 8, but because of the constraints of microscopic reversibility only seven free constants are needed to describe the mechanism (see Appendix).

Weiss *et al.*, (1996a, b, c) present a complex analysis of this scheme, but most of their mathematics does not really bear on the features that are essential in the present context. These properties can be summed up very simply:

- If the concentration of G protein is constant (excess G) then: (a) The fraction of active receptors; (b) the binding of agonist, and (c) the binding of G protein are all related to agonist concentration in a simple hyperbolic fashion. All three follow a simple Langmuir curves with a Hill slope of one.
- The concentration of agonist needed for a 50% effect ($EC_{50}$ or $K_{eff}$) is *exactly the same for all three*. The information provided by measurement of response, agonist binding and (if it were possible) G protein binding is therefore, in principle, the same.
- The single $EC_{50}$ value for all three sorts of measurement depends on all of the seven independent equilibrium constants that describe the reaction. It therefore has no simple interpretation. It is this value that would be found by the Furchgott method.

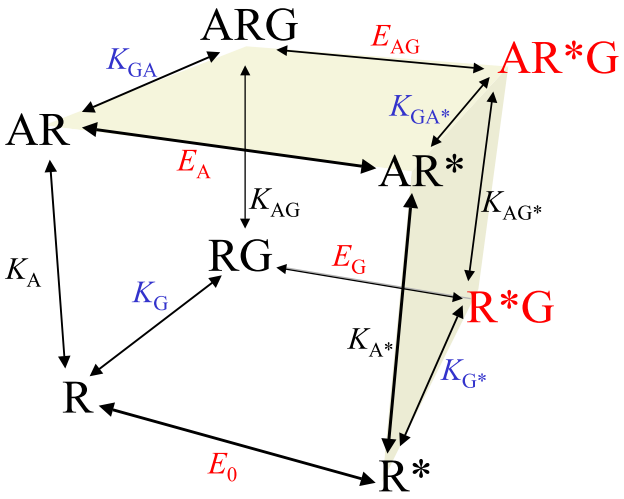



**Figure 8** The 'cubic ternary complex' mechanism (Weiss *et al.*, 1996a). The receptor is represent as R, the G protein as G, and the agonist as A.

- If the supply of G protein is limited, so its concentration falls as it becomes bound (see Jenkinson, 1989, 1996), the agonist binding curve (but not the response curve) is no longer hyperbolic, but takes on a biphasic appearance, reverting to a low affinity component as G protein becomes exhausted.
- Only one of the seven constants in the mechanism can be expected to tell us about the agonist-binding site. All of the other six are likely to be influenced by the ability of the receptor to change conformation. Without the ability to measure the constants separately, inferences about the binding site must inevitably lack a rational basis.

Thus, just as for the Castillo-Katz problem illustrated in Figure 1, it is futile to imagine that binding experiments will resolve the affinity/efficacy problem or, in the context of mutant studies, the binding/activation problem.

## Which equilibrium constants tell us about the binding sites?

In practice, what we need is a measure of affinity that tells us only about the binding site, and is unaffected by changes in the ability of the receptor to change conformation. Inspection of Figure 8 shows that there is only one equilibrium constant that comes into this category, namely $K_A$, for the binding of A to the inactive receptor. All the other six free constants (except perhaps $K_{AG}$, see below) depend on the ability of the receptor to undergo the global conformation change from R to R* (and are therefore 'efficacy-like', in the same sense as $E$ in the del Castillo-Katz mechanism; see above). What this means is that, if a mutation in the receptor changes only $K_A$ then we can reasonably suppose that there has been a change in the binding site itself. And insofar as most point mutations produce only short-range effects (Shortle, 1992), we might infer that the amino acid in question was part of the binding site. But if any of the other six equilibrium constants are changed, then it is entirely possible that the mutation has changed the ability of the receptor to change conformation (e.g. the mutation could be in a hinge region); the observed changes in response and binding would result from this, rather than from any change in the binding site.

At first sight it might be thought that a change in $K_{AG}$ (for the binding of A to the inactive receptor that has G bound to it) might be interpreted similarly. But there is no obvious reason why $K_A$ and $K_{AG}$ should differ at all. If the receptor is still in the R rather than the R* conformation, how could the G protein 'know' that A has been bound? Thus $K_A$ and $K_{AG}$ should be the same, unless binding itself produces a large conformation change before the R→R* transition (but this is not included in this reaction scheme). In any case, if such a conformation change existed it might be influenced by changes in any part of the molecule, so a change in $K_{AG}$ only would not necessarily suggest a change in the agonist binding site. In fact $g = K_A/K_{AG}$, like all the parameters apart from $K_A$, behaves like an efficacy-like parameter insofar as it influences the maximum attainable response (at low G concentrations).

If there is only one active conformation (R*) which is much the same for all agonists (a question that is discussed further below), it is worth noticing that the only thing that the G protein can 'see' is the fraction of time that the receptor spends in R*. The G protein can have no other 'knowledge' of which ligand is bound to the receptor. This means that the phenomena of full, partial and inverse agonism must depend on properties of the receptor itself (and the nature of the

agonist), not on subsequent steps in the transduction pathway. Of course later processes that follow after the activation of the G protein can obviously limit the size of the response, so making a partial agonist look like a full one, and affecting the relative potencies of different agonists (see below). This is, to a good approximation, what happens with ion channels, but in the case of G protein-coupled receptors the question is controversial (see below).

## Limitations of ternary complex mechanisms

The sort of mechanism shown in Figure 8 would be very useful for interpretation of agonist structure-activity relationships, and of the effects of receptor mutations, if (a) it were a good approximation to the real mechanism, and (b) the equilibrium constants in it could be estimated. In this case, for example, a receptor mutation that affected only $K_A$, but not the other six constants, could reasonably be interpreted as having affected the binding site. Sadly, though, it is unlikely to be sufficiently near the real mechanism and even if it were, methods do not exist for estimating the constants. The problems that are not fully solved include the following. (a) Not much is known about the change in receptor conformation ($R \rightarrow R^*$), and it is controversial whether one active conformation suffices to explain the facts (see below); (b) Mechanisms such as that in Figure 8 all require one to specify the concentration of G protein, and it is not even clear what this term means for something that is membrane-bound (and perhaps compartmentalized). Receptor 'concentrations' can be 'normalized out' by expressing all species as a fraction of the total number of receptors, but G protein concentrations cannot be so eliminated. (c) In the scheme in Figure 8, the observed response is taken to be proportional to the number of receptors that are in either the R*G or the AR*G states at equilibrium. Even when the response is measured as closely as possible to the receptor (GTPase activity or binding of GTP$\gamma$S), the evidence for this being a good approximation is slim; (d) Most reaction mechanisms that have been proposed so far do not include a realistic description of the GTP hydrolysis cycle (GTP and GDP do not appear at all in Figure 8). It seems likely that this step will have to be incorporated to get usable results; (e) Most treatments consider the reaction only at equilibrium. Although it would be nice to consider the rate of approach to equilibrium too, a more important deficiency is that the GTP-hydrolysis cycle is inherently not an equilibrium process, and some sort of (quasi-) steady-state treatment is likely to be essential, as a minimum.

A good start on the last two questions has been made (e.g. Shea *et al*., 1997), but more experimental results will be needed to pin down the numbers.

## What is known about the conformations of the receptor? Fusion of the receptor and G protein

Quite a lot is known about the structure of G proteins (see Bourne, 1997), but very little is known about the conformation change in the receptor itself. This is unfortunate, because it is probably in the receptor conformation change that the main secrets of agonism (full, partial and inverse) lie, and since it is essential for the proper interpretation of mutation experiments.

In the case of an ion channel, this conformation change can be 'seen' very directly as a channel opening. For a G protein-coupled receptor, no such direct approach is possible. If we were able to measure the fraction of receptors that were in the active conformation, life would be a lot easier. And it would be better still if this could be done on an absolute scale (in the way that, under favourable circumstances, $P_{open}$ can be measured for ion channels), rather than relative to an observed maximum.

It has been mentioned that one of the largest uncertainties in attempts to be quantitative about mechanisms lies in our ignorance of how many G protein molecules the receptor has easy access to. An ingenious approach to this problem has been the construction of molecules in which the N-terminus of the receptor is fused to the C-terminus of the G$\alpha$ subunit, to make a single big molecule (Bertin *et al*., 1994, Wise & Milligan, 1997). The result is, of course, not very physiological, but the fixed stoichiometry between receptors and G proteins has been exploited by Milligan's group to cast light on the nature of partial agonism (Wise *et al*., 1997). A complication arose when it was found that the fused receptor-G$\alpha$ protein was able to activate not just the G protein that was fused to the receptor, but, surprisingly, also endogenous G proteins as well (Burt *et al*., 1998). For this reason a mutant $G_{i1}$ was used, which is resistant to inhibition by pertussis toxin. By using pertussis toxin-treated cells, the measured response (GTPase activity) was limited to that produced by the fused G protein. Furthermore, it was verified that the Michaelis constant for GTP hydrolysis was not dependent on the nature of the agonist, which suggests that the interaction between G protein and GTP is similar for all agonists. This has the important consequence that the measured response is likely to reflect the activation of the receptor itself. The maximum responses were measured (relative to that for adrenaline) for a series of agonists, as an index of the extent to which they were partial agonists. It was found that all of the agonists produced a smaller maximum response (relative to adrenaline) than they did in experiments in which the G protein was not fused to the receptor (the latter being measured by separate co-transfection of the same mutant $G_{i1}$). However, the rank order of the agonists was the same in both experiments. This provides a clear confirmation of the view that partial agonism is essentially a property of the receptor itself. These experiments, strictly speaking, do not measure agonist efficacy, because if that term is to have any useful meaning it cannot (as in these experiments) have an upper limit, but when all the agonists tested are obviously partial, this distinction will not affect the conclusions that were drawn.

The use of fused receptor-G protein proteins should also settle the question of whether the two-component agonist binding curves that were discussed above can be explained by depletion of G protein. Seifert *et al*. (1998) used a $\beta_2$ receptor fused with $G_s\alpha$, and found more or less normal two-component agonist binding and reversion to the low affinity form on addition of guanine nucleotides. Since the G protein is fused on, it cannot be depleted. This appears to rule out the depletion explanation. However the finding by Burt *et al*. (1998), that the fused receptor can also activate endogenous free G proteins, makes this conclusion insecure.

## What is known about the conformations of the receptor? Fluorescence changes

The only other experiments in which direct measurement of the receptor conformation has been attempted are those in which receptor fluorescence changes have been measured. Gether *et al*. (1995) labelled cysteine residues in a purified $\beta_2$ catecholamine receptor with a fluorescent molecule which is sensitive to the polarity of its environment. It was found that

agonists caused a decrease in fluorescence, which was reversed by antagonists, and that inverse agonists could increase fluorescence. Although the results have yet to be incorporated in a quantitative mechanism, they certainly looked as though the changes were measuring qualitatively something that correlates with the physiological response. There is only one problem, and that is that the measured changes in fluorescence developed astonishingly slowly. The half-time for development of the response is in the range of hundreds of seconds, whereas it is well known that responses to $\beta_2$ receptors, though slower than responses mediated *via* nicotinic receptors, still develop in seconds (Nargeot *et al*., 1983; Pott, 1979). How, then, could such a slow conformation change be the cause of a response, when it is something of the order of 100 fold slower than the response it is supposed to cause? Some discrepancy might be expected in cases where a small fraction only of receptors is needed to elicit the response, but it would be hard to account for a 100 fold discrepancy in this way. In any case, the fluorescence change is just as slow for partial agonists, and for them there is abundant evidence that all receptors must be occupied for a maximum response. At present this remains a mystery. Of course these results were obtained with a soluble purified receptor in the absence of G protein or GTP. It will be interesting to see whether the mystery goes away in more complete systems.

## How many receptor conformations are there?

Up to now it has been supposed that the receptor can adopt only two conformations. In other words, it is assumed that the active conformation is much the same whichever ligand is bound to it (or if no ligand at all is bound). The only effect of the ligand is to alter the fraction of time that the receptor spends in the active conformation. Many experiments are compatible with this view (e.g. Wise *et al*., 1997; Burstein *et al*., 1997). Recently, it has been suggested that there may be many such conformations, and that each different ligand induces a different conformation, or distribution of conformations (see Kenakin, 1997b; Tuček, 1997, Leff *et al*., 1997, 1998). The evidence cited in favour of this view is all indirect (direct effects on activation of specific G proteins have not yet been measured); it is of three sorts. One sort concerns mutant receptors – these certainly may not have the same active conformation as the wild type, just as some mutations change the conductance of ion channels. A second sort concerns the action of agonists that work at two different sites on the receptor; some complex observations have been made with such agonists that are not easy to explain if there is only one active conformation (e.g. Hulme *et al*., 1990; Tuček, 1997). But the most interesting case concerns different agonists that bind to the same site on the receptor. It has been suggested that each agonist may induce a different conformation or set of conformations, but the evidence for this is quite thin. Most of the evidence comes from selective activation of different transduction pathways by different ligands, but this sort of observation does not necessitate the existence of different activation conformations of the receptor itself, as a simple example shows. Figure 9 shows normalized concentration-response curves for two different agonists.

Agonist 1 shows a large degree of selectivity for the second type of response (Figure 9b), compared with the first (Figure 9a). Agonist 1 (solid line) has highish efficacy at the receptor itself, agonist 2 (dashed line) has low efficacy. Figure 9a shows curves for a response that is almost proportional to the amount of activated receptor-G protein complex (R*G), and

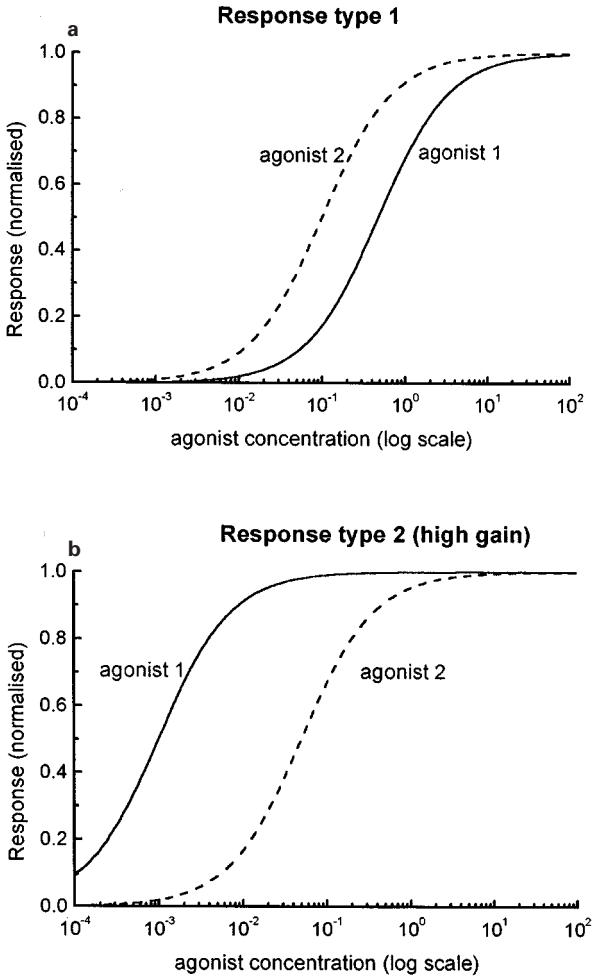



**Figure 9** Illustration of effects of two agonists that work on the same receptor. Both agonists produce the same active conformation, but the activated receptor is coupled, with different gains, to two different responses. The curves assume $A+R==AR==AR^*$, with equilibrium constants $K$ and $E$ for the two steps. Coupling of the response (R) to $p_{AR^*}$, $K=10$, $E=10$, and for the partial agonist 2, (dashed line) $K=0.1$, $E=0.001$. For the response 1 shown in part A, $e=1$, whereas for the high gain response shown in B, $e=1000$.

these do not differ greatly from what would be seen if AR* was taken as the response. Figure 9b shows curves for the same two agonists, with the same effects on the receptor itself, but in this case the response is one that is very tightly-coupled to the amount of activated receptor-G protein complex, so little of it is needed to produce the response (there is a large gain). It is seen that the relative potencies of the two agonists are reversed for the two responses, though their effects on the receptor itself are the same; and there is only one active state of the receptor. This is not intended to be a physical mechanism for the receptors, but merely to show what can happen even with the simplest assumptions. Contrary to what has often been suggested, there is no necessity to postulate more than one active state to explain agonist-specific transduction.

It seems that there is little precedent for ligands which bind to the 'same' site producing different global conformation changes. Consideration of other systems, about which more is known, suggests that this sort of explanation would be rather unusual. The whole reason for the development of the two state approach (see, for example, Wyman & Allen, 1951) was the observation that the structure of oxy- conformation of the haemoglobin molecule was much the same whether oxygen or

carbon monoxide was the ligand. In the case of ion channels, it has been the (almost) universal rule that the structure of the active conformation (the open channel), as judged by its conductance, is very much the same, whichever agonist is used to open the channel (e.g. Gardner *et al*., 1984; see discussion above). Again, all that the nature of the ligand affects is the fraction of time that is spent in the open state. In general, it is found that the nature of the ligand that is bound to enzymes makes only the most subtle difference to the crystal structure of the molecule (Shortle, 1992). In principle, it is possible for proteins to exist in a near-infinite number of conformations, but in actual fact it is usually observed that one or two, rarely more, are sufficiently more stable than the others that the protein spends most of its time in (or close to) these few conformations.

Of course every ligand must produce a slightly different complex, at least in the region where the ligand is bound. If it were not different, the lifetime of ion channels could not depend on the nature of the agonist. And the existence of subconductance levels in ion channels is direct evidence for the existence of different active states (of a presumably trivial sort). The point is that these differences are largely confined to stabilization, to varying extents of a single active conformation (as judged by its conductance). The question for G protein-coupled receptors is not so much whether each ligand produces a distinct active state – that must be the case. The question is, rather, are the differences in conformation for different agonists sufficiently great at the far end of the receptor molecule that interacts with the G protein, for the G-protein to 'know' which agonist is bound. The evidence that this is the case seems slim at the moment. The simpler idea that the G protein does not 'know' which agonist is bound is supported by experiments with a series of G protein-coupled glutamate receptors (the type commonly designated by the totally inappropriate description 'metabotropic'). Parmentier *et al*. (1998) swapped the large extracellular N-terminal domains of these receptors, and found that this domain determined the agonist specificity of the resulting chimaeric receptor, but did not affect which G protein the intracellular side of the receptor interacted with.

## Summary

It seems that there is still insufficient knowledge of mechanisms of G protein-coupled receptors to allow the unambiguous inferences about whether receptor mutations affect the primary binding reaction or later conformation changes. Therefore many inferences about the nature of the agonist-binding site lack any rational basis. Until we have detailed structures, we shall not know how many of them are wrong. It will be very interesting to find out.

# Conclusions

## A definition of efficacy?

It is arguable whether, at this stage, it is worth attempting to define formally the terms efficacy and affinity. These terms are rarely used in the field (agonist-activated ion channels) in which they have most successfully been measured. Nevertheless, the terms are still widely used in the context of drug development, and I still find them useful for indicating a general problem, uncluttered by details of particular mechanisms. There are two other motives for attempting a formal definition. One is that no satisfactory definition can be found in textbooks. The other is that many people still seem to find the idea of efficacy elusive (e.g. Clarke & Bond, 1998), largely because they try to base arguments on Stephenson's incorrect formulation, or because they confuse the macroscopic definition of affinity, as observed in binding experiments, with Stephenson's microscopic concept.

There is nothing elusive about efficacy as long as one recognises that agonist cannot be analysed by a black-box approach, but that specific mechanism must be proposed, which provides a sufficiently accurate description of the actual physical mechanism that transduces binding into response. Once we have this, then affinity (in the Stephenson sense) is simply the microscopic equilibrium (or rate) constant(s) for binding to the inactive state(s). Efficacy is everything else. So efficacy is simply the set of all of the other microscopic equilibrium (or rate) constants, which describe all the transduction events that follow the initial binding reaction. Note that the efficacy constants must include those for binding of agonist to the active states, those for binding of G proteins, as well as all of those for conformation changes. In addition, they must include quantities such as single channel conductances, and other such later parts of the transduction pathway. The definition is perfectly simple. It is measuring the values that is often hard.

## The way forward

The effects of mutations on receptors can clearly be quite complicated. But the few cases that have been analysed in detail (largely mutants of the nicotinic receptors) do give hope that functional domains of receptors can, at least sometimes be identified clearly. Some mutations at least seem to have effects that are restricted to the binding site, whereas others affect mainly the conformation change (though many others seem to affect both). It is equally clear (see Figure 1) that serious mechanistic studies are needed to sort out these effects. Eyeballing the results will not suffice. Even for ion channels, there is a long way to go for most receptors other than the muscle nicotinic subtype.

In the case of G protein-coupled receptors, it is probably true to say that nobody has yet succeeded in estimating a constant (like the affinity, $K_A$, in Figure 8) which could say whether or not an effect is on the binding site. Although sensible inferences can be made about obviously partial agonists, there is no firm basis for distinguishing between different degrees of efficacy among agonists that can all produce a maximum response (a distinction that is essential for the interpretation of mutations and for rational drug design).

These problems do not mean, of course, that all the inferences that have been made about the binding site are wrong. But it does mean that we are not sure which of them are right. At present we have to rely rather heavily on convergent lines of evidence from different approaches, each insecure, but with luck converging on something close to the right answer.

A new era in ion channel work has been opened recently by the determination of the first high-resolution crystal structure of an ion channel (Doyle *et al*., 1998), and by the structural work of Unwin (1995). Such work will, in the future, resolve many of the questions about the location of binding sites. This does not mean, however, that we shall no longer need to bother about problems of mechanism, or determining rate and equilibrium constants. Structures are static but receptors are not. Both approaches will continue to be essential.

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# Appendix

## Some properties of the cubic ternary complex mechanism

The scheme in Figure 8 has eight receptor species, and twelve equilibrium constants. However, if it were at equilibrium the constraint of microscopic reversibility would imply that there are not twelve independent equilibrium constants, but only seven.

The active species in the scheme are supposed to be R*G and AR*G, so the comments made here suppose that a response, $p_{act}$, can be measured that is proportional to $p_{R*G} + p_{AR*G}$, the sum of fractions of receptor in these two states. The fraction of active receptors is, say, $p_{act}(0)$ at zero concentration, and $p_{act}(\infty)$ (the maximum response) at very high agonist concentration. If the response is normalized so that it lies between 0 and 1, i.e. we define

$$p_{norm} = \frac{p_{act} - p_{act}\ (0)}{p_{act}\ (\infty) - p_{act}\ (0)}, \qquad \text{(A1)}$$

then the $EC_{50}$ referred to above is the agonist concentration for $p_{norm} = 0.5$.

The fractional binding of agonist is the total fraction of the receptor in the four agonist-bound states (those on the top surface of the cube in Figure 8). This increases from 0 to 1 as agonist concentration is increased, according to a simple hyperbolic curve which has exactly the same $EC_{50}$ as that for response (see Figure A1). It has often been supposed that, because the scheme contains several states with different affinities for the agonist that the binding curve will contain several components, and that this might explain the multi-component agonist binding curves that have often been observed in experiments. This is not so (at equilibrium anyway), and some other explanation is needed for these observations.

The fraction of receptors that have G protein bound is, similarly, the sum of the fractions in the four states on the back face of the cube in Figure 8. When normalized as above, this too has the same $EC_{50}$, as illustrated in Figure A1.

**Table 2**

| *Notation in Figure 8* | *Microscopic equilibrium constant* | *Notation in Weiss* et al. *(1996a)* |
|---|---|---|
| $K_A$ | $K_A$ | $K_A$ |
| $K_{A*}$ | $K_A/a$ | $a\ K_A$ |
| $K_{AG}$ | $K_A/g$ | $\gamma\ K_A$ |
| $K_{AG*}$ | $K_A/dag$ | $\delta\alpha\gamma\ K_A$ |
| $K_G$ | $K_G$ | $K_G$ |
| $K_{G*}$ | $K_G/b$ | $\beta\ K_G$ |
| $K_{GA}$ | $K_g/g$ | $\gamma\ K_G$ |
| $K_{GA*}$ | $K_G/dbg$ | $\delta\beta\gamma\ K_G$ |
| $E_0$ | $E_0$ | $1/K_E$ |
| $E_A$ | $a\ E_0$ | $1/\alpha K_E$ |
| $E_G$ | $b\ E_0$ | $1/\beta K_E$ |
| $E_{AG}$ | $d\ a\ b\ E_0$ | $1/\delta\alpha\beta K_E$ |

## Definition of equilibrium constants

There are only seven independent constants, so the twelve macroscopic equilibrium constants in Figure 8 can be written as follows.

The seven independent constants are $K_A$, $K_G$ and $E_0$, $a$, $b$, $g$ and $d$ (the last five are all defined as the reciprocals of the corresponding constants in Weiss *et al.*, 1996a, so that they increase as 'efficacy' increases). $K_A$ and $K_G$ are dissociation equilibrium constants for binding of agonist and G protein, respectively, to the inactive receptor (R), and $E_0$ measures the tendency of the vacant receptor to activate (the equilibrium ratio [R*]/[R]). The constant $a$ is the factor by which affinity for A increases when the receptor activates (see $K_{A*}$), and also the factor by which the tendency to activate increases when A is bound (see $E_A$). Similarly $b$ measures the increased tendency of G protein to bind to the active conformation rather than the inactive (see $K_{G*}$), and also the increased tendency to enter the active conformation when G (but not A) is bound (see $E_{AG}$). The constant $g$ measures the increased tendency of A to bind when A is bound (see $K_{AG}$), and equally the increased tendency of G protein to bind when A is bound (see $K_{GA}$). Finally the constant $d$ occurs in all three equilibrium constant that lead to the main active species, AR*G, so the bigger the value of $d$, the more efficacious the agonist.

The maximum 'response', as used in (equ. A1), is given by

$$p_{act}\ (\infty) = \frac{E_{AG}\left(\frac{[G]}{K_{GA}}\right)}{1 + E_A + (1 + E_{AG})\left(\frac{[G]}{K_{GA}}\right)}. \qquad (A2)$$

The maximum response is seen to depend not only on the 'efficacy' for the receptor itself ($E_A$); in fact it depends primarily on (a) $E_{AG}$, the equilibrium constant for conformation change in the receptor that has both agonist and G protein bound to it (see Figure 8, and Table above), and (b) the 'concentration' of G protein. This means that it is quite possible for an agonist that is quite weak (small $E_A$) on the receptor itself to be a full agonist at the level of the measured response (as defined here).

If the G protein concentration is treated as constant (i.e. it is present in excess) then, as stated above, binding of agonist, and of G protein, as well as the (normalized) response, are all simple hyperbolic (Langmuirean) functions of agonist concentration, as illustrated in Figure A1. (These examples are merely to show the qualitative behaviour of the mechanism – the numbers are not based on real data.)

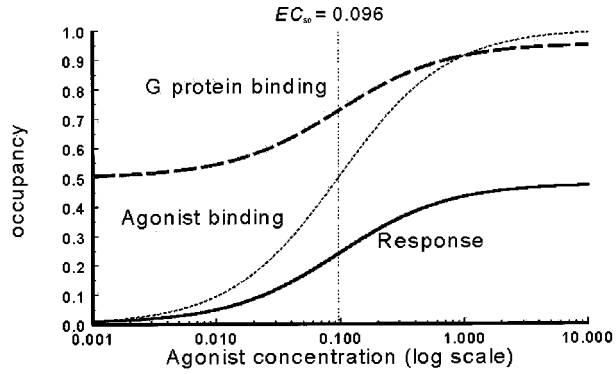


**Figure A1** Behaviour of cubic ternary mechanism when G protein concentration is constant (no depletion, excess G). Response (solid curve), binding of agonist (dotted curve, and binding of G protein (dashed curve), as function of [A] ($\mu$M). This is for the cubic ternary mechanism (Figure 1), with $K_A = 1\ \mu M$, $K_G = 1\ \mu M$, $E_0 = 0.001$, $a = b = g = d = 10$. The *EC50*, or $K_{app}$, from eq.A3, is 0.096 $\mu$M.

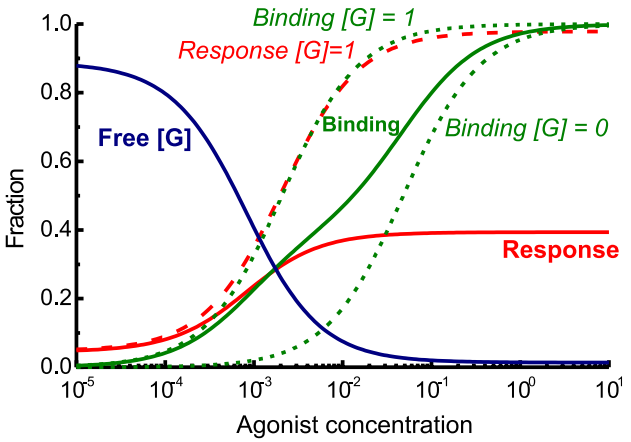


**Figure A2** Effect of depletion of the G protein concentration as a result of its binding to the receptor. Response, agonist binding and free G concentration (solid curves), in the case where G protein is depleted. For comparison, the dotted curves show what happens at constant [G] = 1 $\mu$M, and at [G] = 0. The initial receptor concentration is $[R]_0 = 2.5\ \mu M$, initial G concentration is $[G]_0 = 1\ \mu M$, with $K_A = 1\ \mu M$, $K_G = 1\ \mu M$, $E_0 = 0.01$, $a = 2000$, $b = 10$, $g = 1$, $d = 5$.

The $EC_{50}$, or apparent affinity, $K_{app}$, is the same for all three curves and is

$$EC_{50} = \frac{K_A\left[1 + \frac{[G]}{K_G} + E_0\left(1 + \frac{[G]}{K_G^*}\right)\right]}{1 + \frac{[G]}{K_{GA}} + E_A\left(1 + \frac{[G]}{K_{GA}^*}\right)\Bigg]}. \qquad (A3)$$

Notice that this is a function of all seven parameters. Just as in the simpler del Castillo-Katz case, it has no simple interpretation. If we want to know about the binding site, we need to know about $K_A$, not about $K_{app}$. In the example in Figure A1, $K_A$ is 1 $\mu$M, but the $EC_{50}$, or $K_{app}$, is 0.096 $\mu$M. They are not the same, and it is the latter value, not the former, that would be given by the Furchgott method, which, as in the simpler ion channel case, therefore fails to make the required distinction between binding and activation.

If the number of G protein molecules is regarded as constant, then each binding reduces the number of unbound G molecules and the 'concentration of G' (whatever that means) must fall. This is illustrated in Figure A2. It can be seen that the response curve is normal in shape, and 'high affinity'. But the agonist binding curve looks biphasic, starting off at the higher affinity that is found when [G] is not depleted (left dotted line), but ending at the lower affinity found when [G] = 0 (right dotted line), as free G protein becomes exhausted. This is roughly as observed, though it is far from certain that depletion is the true reason for the observations.

In this example, the $EC_{50}$ for the response, when G protein depletes, is 0.00082 $\mu$M (solid response line in Figure A2). If there is excess G protein, so [G] stays constant at its initial value of 1 $\mu$M the $EC_{50}$ for response and for binding is actually higher, 0.0021 $\mu$M (left dashed lines in Figure A2). If the G protein concentration is zero, there is no response, but the $EC_{50}$ for agonist binding is 0.048 $\mu$M (right dashed line in Figure A2). However none of these is close to the constant that tells us about the binding site alone, which is $K_A = 1$ $\mu$M. That is the heart of the problem.

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