

# Agonist-receptor efficacy

## II: agonist trafficking of receptor signals

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There is evidence to suggest that receptors with seven transmembrane domains can exist in G protein-activating conformations. It is not known how many activated receptor forms exist for each receptor. Furthermore, if there are multiple forms, does the chemical structure of the agonist determine which form dominates, and therefore, which response pathway is activated? This latter scheme is referred to as agonist-receptor trafficking, and is discussed in this, the second of two articles by **Terry Kenakin**. One way to approach these questions is to study receptors that couple to more than one G protein and, in essence, to try to allow the G protein to indicate the receptor state.

It is known that in many physiological, and heterologous, expression systems, agonists can activate numerous biochemical-response pathways. In general, there are two possible mechanisms for such pleiotropic agonist responses: one depends on the strength of the original signal, and the second results from selective G protein activation (Box 1).

The activation of different biochemical pathways in cells by different agonists acting on the same receptor does not in itself constitute evidence for multiple active receptor states since there are many biochemical pathways in cells that can trigger the activation of other pathways. For example, activation of bradykinin receptors in cultured rat mesangial cells causes a decrease in cAMP, but through a pathway involving phospholipase C (Ref. 1). Furthermore, the entry of  $Ca^{2+}$  from the extracellular space is known to trigger the release of intracellular  $Ca^{2+}$  from internal stores. In addition, it is now known that the activation of G proteins by receptors releases two sets of active effectors: the  $\alpha$  subunit and the  $\beta\gamma$  subunits. Under these circumstances, the measurable response to an agonist may depend upon the types of effector present, that is, one cell may contain effectors responsive to the  $\beta\gamma$  subunits and one may not.

### Differential strength of signalling

A general mechanism whereby agonists can variably activate multiple cellular pathways is through differences in the strength of the stimulus. This can be shown in a simulation where biochemical cascades are modelled by successive hyperbolae. Given two sequential hyperbolic functions, the product of one feeding into the other, it is

a mathematical consequence that the result of the multiple function will be an amplification of the product of the first function. Therefore, if one second messenger triggers the activation of a second measurable response, the magnitude of the second response necessarily will be more sensitive to the strength of the original receptor signal. Under these circumstances, there are numerous possible combinations of agonists of differing intrinsic efficacy that could produce measurable amounts of one of the hyperbolic products but not the other. For example, the effects of two agonists on sequential hyperbolic response systems are represented in Fig. 1. Agonist I has a high efficacy and produces measurable effects from both stimulus cascades. However, Agonist II has only 3% of the intrinsic efficacy of Agonist I and produces a measurable amount of the second (amplified) cascade product, but extremely low amounts of the first. Thus, it would appear that Agonist I produces a pleiotropic response while Agonist II produces only a single response. These data would not be evidence for differential production of receptor active states by the two agonists but rather would be the result of differential strength of signals and selective dissection of the stimulus-response cascade in the cells.

A useful method to delineate such mechanisms is to observe the effect of signal decline of the responses to an agonist of high efficacy. For example, Costa and colleagues have shown that the opioid receptor agonist [DAla<sup>2</sup>-DLeu<sup>5</sup>]enkephalin (DADLE) produces stimulation of high-affinity GTPase and also inhibition of basal adenylate cyclase in NG 108-115 cells<sup>2</sup>. Upon decline of the receptor stimulus through receptor alkylation, it was shown that the least sensitive response (GTPase response) was eliminated and the most sensitive response remained. Thus, by manipulation of receptor number, agonists could produce multiple or single biochemical responses<sup>2</sup>. Similarly, agonists of differential intrinsic efficacy could do the same: an agonist of high efficacy would activate multiple biochemical cascades while an agonist of low efficacy would only activate the most sensitive cascade. This would not constitute evidence for selective receptor active states for the two agonists.

### Receptor-G protein systems

A variant of this idea applies to receptor-G protein systems. If agonism is a function of selective affinity of an agonist for the active receptor state ( $R^*$ ) over the inactive receptor state ( $R$ ) as in the two-state theory for ion channels, then an array of agonists could be tested with varying differential affinity for  $R$  and  $R^*$  (denoted by the factor  $\alpha$  where  $\alpha$  = affinity for  $R^*$  / affinity for  $R$ ). Concentration-response curves for the chemical reaction between receptors (activated by three agonists) and a fixed amount of G protein are shown in Fig. 2a. All three agonists promote ternary complex formation but not to the same extent, i.e. the agonist that produces the highest level of  $R^*$  is most efficient at ternary complex formation. Thus, the value of  $\alpha$  dictates the location of the concentration-response curve between steady-state amounts of

activated receptor and G protein. If this reaction was fixed at a given receptor level (i.e.  $[R] = 5$ ) the quantity of the G protein would exceed that of the receptor. This situation is apparently seen in a number of physiological systems, although the imposition of cellular factors controlling receptor-G protein access (for example, cytoskeletal elements) make the use of these estimates doubtful<sup>3</sup>. With values of  $[R] = 5$  and  $[G] = 10$ , the agonist that produces the least amount of  $R^*$  ( $\alpha = 1.2$ ) produces the least amount of ternary complex (Fig. 2b).

These three different agonists produce very different amounts of ternary complex yet these involve the identical active state of the receptor. The difference in the amount of ternary complex arises from the fact that differing amounts of  $R^*$  exist at equilibrium in the presence of the agonist. This has been observed experimentally (Fig. 2c). Graded concentrations of acetylcholine produce, as expected, graded quantities of ternary complex with muscarinic acetylcholine receptors and G proteins in ventricular membranes<sup>4</sup>. The same effect is seen with maximally saturating concentrations of agonists of differing intrinsic efficacy. For example, in cardiac membranes there is a differential production of maximal amounts of ternary complex produced by the agonists carbachol, pilocarpine and McNA343 (Fig. 2d)<sup>4</sup>. Hence, from both simulations and experimental data, it can be seen that even with one receptor activating a single G protein and

## Box 1. Pleiotropic agonist response

Multiple biochemical cascades can be activated in cells in response to an agonist by a number of mechanisms. These can be generally divided into those dependent on the strength of the signal and those involving agonist trafficking.

### Strength of signal

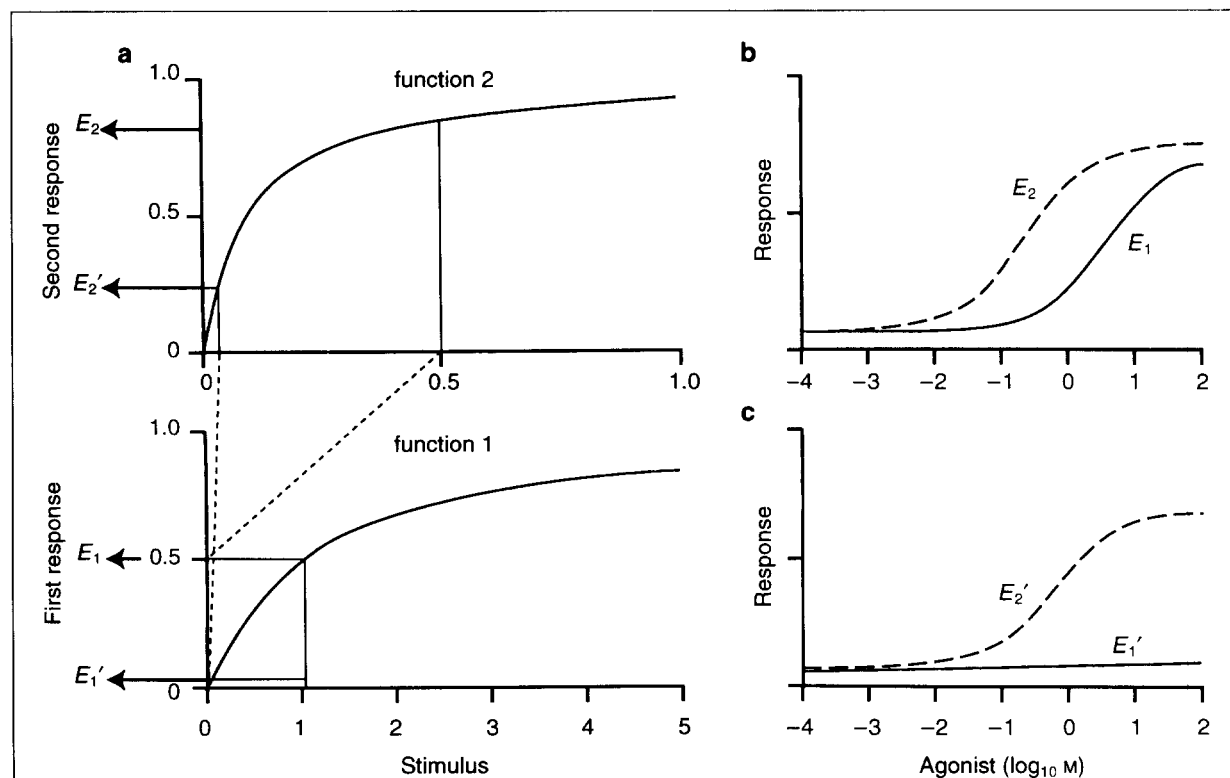
In this case, only one active receptor state is required. There are two possible schemes to explain the responses:

(1) A single receptor couples to one G protein to activate a biochemical cascade that then activates other cascades.

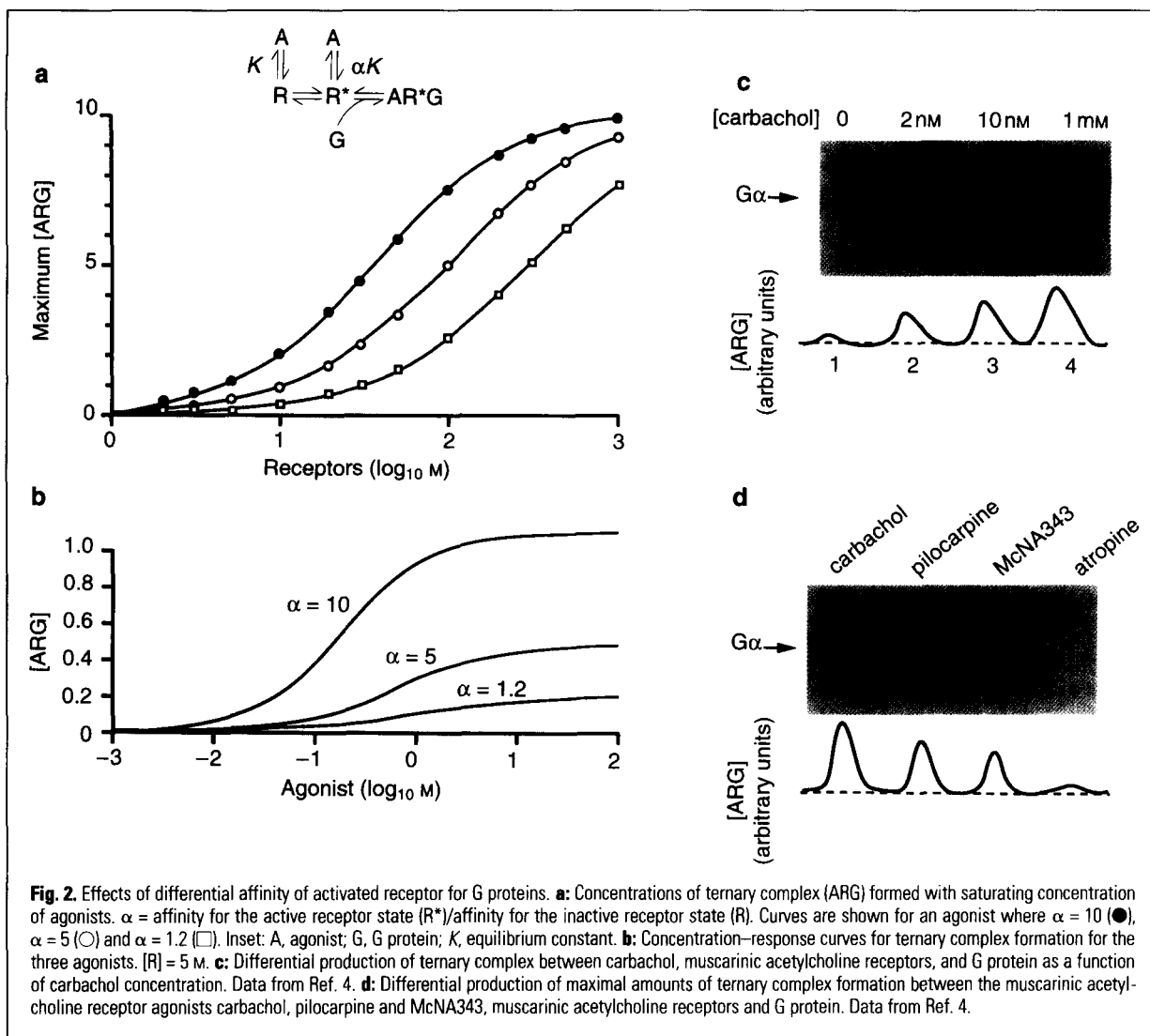
(2) A single receptor differentially couples to multiple G proteins to activate multiple cascades. Agonists of high efficacy activate multiple G proteins, whereas agonists of low efficacy only the most efficiently coupled G protein. This is known as receptor promiscuity, and is exacerbated by receptor overexpression. In this situation, heterologous systems are created that promote aberrant receptor-G protein couples.

### Agonist trafficking

This involves receptor promiscuity and separate agonist-specific active receptor states. These states selectively promote G protein coupling in response to activation by different agonists.



**Fig. 1.** First and second responses of two agonists (I and II) of differing efficacy according to two hyperbolic stimulus-response functions. **a:** The two hyperbolic responses according to  $E_1 = S/(S+1)$  (Function 1) and  $E_2 = S/(S+0.1)$  (Function 2) where  $E_1$  and  $E_2$  are the first and second responses, respectively, and  $S$  is the concentration of stimulus. **b:** Agonist I produces strong responses ( $E_1$  and  $E_2$ ) from both Function 1 and 2. **c:** Agonist II has 3% of the efficacy of Agonist I therefore the initial response  $E_1'$  according to Function 1 is nearly negligible; however, the amplification of  $E_1'$  by Function 2 produces a measurable secondary response ( $E_2'$ ) to this agonist.



producing a single active receptor state, apparently different responses from different agonists can result.

### Receptor promiscuity overlaps into active receptor states

There is a fundamental increase in complexity of the behaviour of receptors when they can activate multiple G proteins in membranes. There are examples of this type of behaviour in physiological and reconstituted systems<sup>5</sup>. However, while receptor promiscuity can occur, it should be put into stoichiometric perspective. The probability that any biochemical reaction can go forward is controlled by the magnitude of the equilibrium dissociation constant of the resulting complex and the relative molar quantities of the reactants. Receptors and individual G proteins have intrinsic association constants and it is a logical assumption that receptors differentiate between different G proteins to offer selectivity at this level of signalling. Promiscuous receptor coupling is becoming a more commonly observed phenomenon as more receptors are studied in heterologous expression systems under conditions of high levels of receptor expression<sup>5</sup>. Whether receptor promiscuity occurs physiologically or because of aberrant

stoichiometry is not relevant to the discussion of the basis of agonist efficacy. The point of this discussion is that when receptor promiscuity does occur, it can sometimes be used as a 'looking glass' into receptor activation states and indicate whether the receptor exists in a single activated state or one that changes with the type of agonist.

In systems where multiple ternary complexes are formed by agonists, it theoretically should be possible to test whether certain agonists selectively favour receptor coupling to one G protein over another (i.e. agonist trafficking). However, the influence of strength of signal must be considered here as well. For example, a single activated state of the receptor may have a high affinity for one G protein and a lower affinity for another. An agonist of high efficacy that produces a large amount of the activated receptor may produce enough activated receptor to couple to both G proteins while an agonist of low efficacy may promote coupling only to the most efficiently coupled G protein. Under these circumstances, it would appear that the former agonist activates two G proteins while (the latter agonist) activates only one. For example, the activation of the G proteins G<sub>s</sub> and G<sub>i</sub> has been investigated in Chinese hamster ovary (CHO) cells transfected

with human  $\alpha_2$ -adrenoceptors by adrenaline and oxymetazoline<sup>6</sup> (Fig. 3). When each G protein response is isolated by respective treatment of cells with pertussis or cholera toxin, adrenaline activates both  $G_s$  and  $G_i$  while oxymetazoline activates only  $G_i$ . This may suggest that oxymetazoline produces a unique activated receptor form that couples only to  $G_i$ , while adrenaline produces another form (or two forms) that activate(s) both G proteins. However, it also is possible that the strength of signal phenomenon may be the crucial factor: oxymetazoline may produce less activated receptor thereby producing coupling only to the most susceptible G protein.

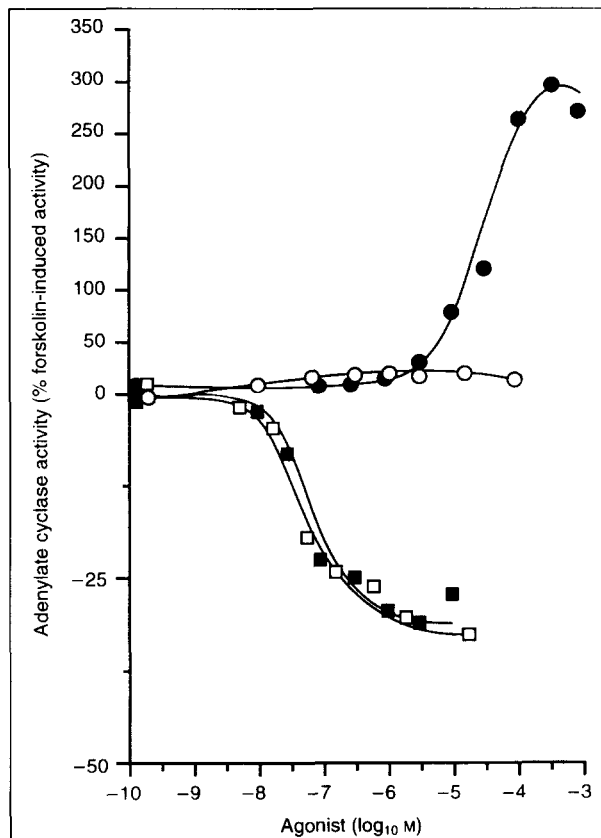
### Reversal of agonist potency

The most easily interpretable evidence of agonist-specific active receptor states would theoretically be obtained from actual reversals of relative agonist potency with different G proteins. Under these circumstances, the selectivity would not result from a simple case of high versus low efficacy agonists, but rather one agonist would be a strong promoter of one complex and the other agonist would be a strong promoter of the other complex (Fig. 4a). There are selective examples of this phenomenon.

The production of cAMP and inositol phosphate mediated by a splice variant of the pituitary adenylate cyclase activating polypeptide (PACAP) receptor transfected into LLC PK1 cells has been measured<sup>7</sup> (Fig. 4b). PACAP<sub>1-27</sub> is more active than PACAP<sub>1-38</sub> for the production of cAMP, but it is considerably less active in the production of inositol phosphates. This reversal of potency suggests that a simple difference in the strength of signal cannot account for the differential G protein activation.

Another example of such a reversal in an expression system is the *Drosophila* octopamine–tyramine receptor in CHO cells<sup>8</sup>. This receptor mediates attenuation of cAMP and Ca<sup>2+</sup> transients via different coupling mechanisms. A clear difference in agonist potency is seen for these two responses. Whereas tyramine is almost two orders of magnitude more potent than octopamine for cAMP attenuation, octopamine is more potent than tyramine when the kinetics of Ca<sup>2+</sup> responses are compared<sup>8</sup>.

One method of delineating agonist trafficking effects is to compare the relative efficacies of agonists in receptor systems. Agonist activity is a composite of the properties of affinity and intrinsic efficacy and the efficacy component is directly related to the nature of the G protein coupled to the receptor. Therefore, a measure of relative efficacy is a measure of a receptor–G protein interaction and not just a receptor<sup>9,10</sup>. For example, it has been found that the two dopamine receptor agonists quinpirole and 3-(3-hydroxyphenyl)-N-n-propylpiperidine [(+)-3-PPP] demonstrate reversed relative efficacies for activation of dopamine D<sub>2</sub> receptors in rat anterior pituitary and striatum<sup>11</sup> (Fig. 4c). The simplest account for such a reversal of potency for a single receptor is that the receptor is present in a milieu of different G proteins in each tissue and that the respective agonists produce activated receptor complexes of differing sensitivity to each G protein.



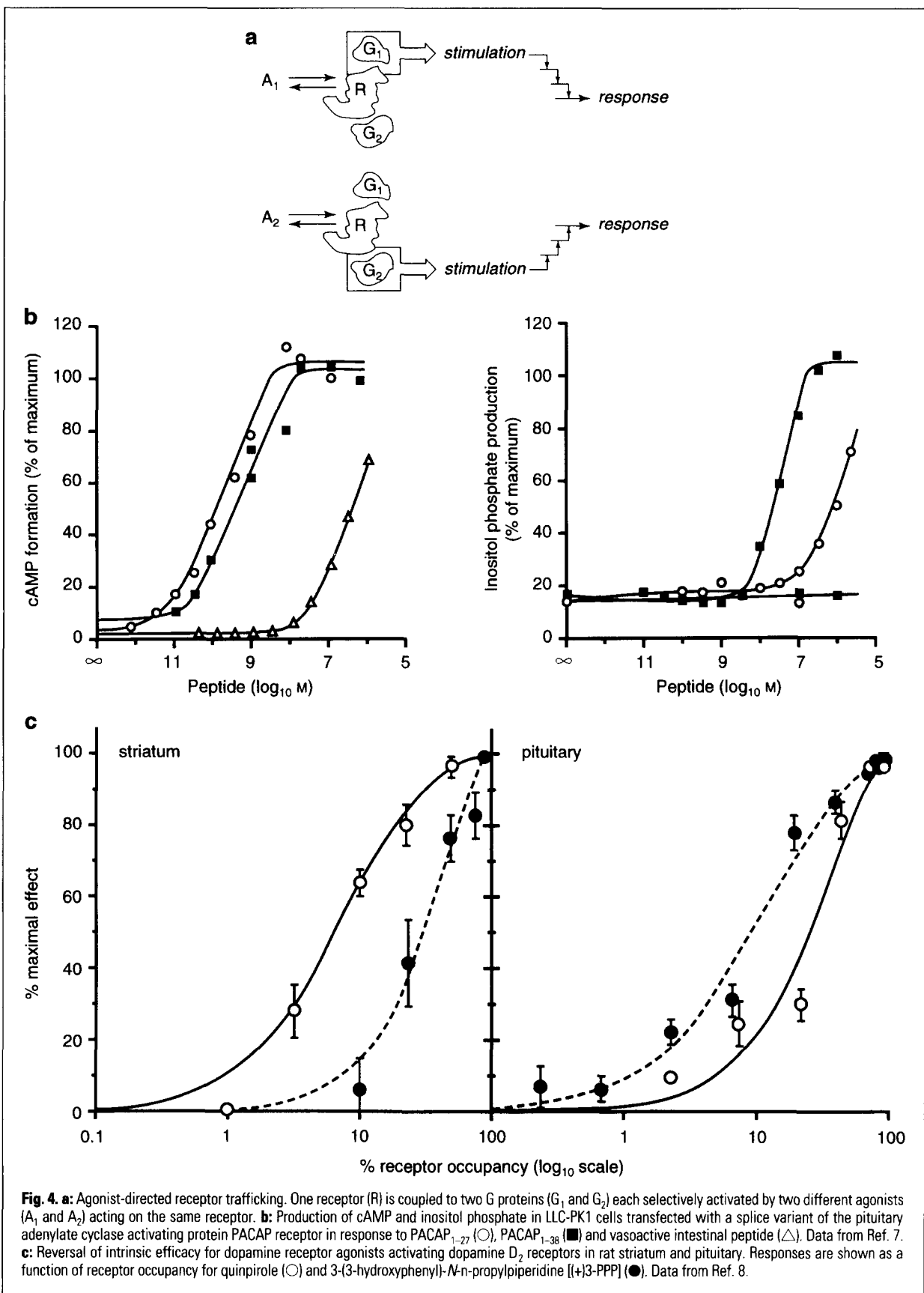
**Fig. 3.** Selective activation of G proteins  $G_i$  and  $G_s$  by agonist-activated  $\alpha_2$ -adrenoceptors in transfected Chinese hamster ovary cells. The effect of adrenaline is shown in the presence of pertussis and cholera toxin (● and ■, respectively), and the effect of oxymetazoline is shown in the presence of pertussis and cholera toxin (○ and □, respectively). Data from Ref. 6.

### Selective agonist blockade

An increasingly observed phenomenon is the differential sensitivity of different agonists to antagonists for the same receptor. For example, this has been observed for the tachykinin NK<sub>1</sub> receptor agonists substance P and an analogue septide with the antagonist RP67580 (Ref. 12), and other agonists that are analogues of substance P with a number of antagonists<sup>13,14</sup>.

### Receptor heterogeneity

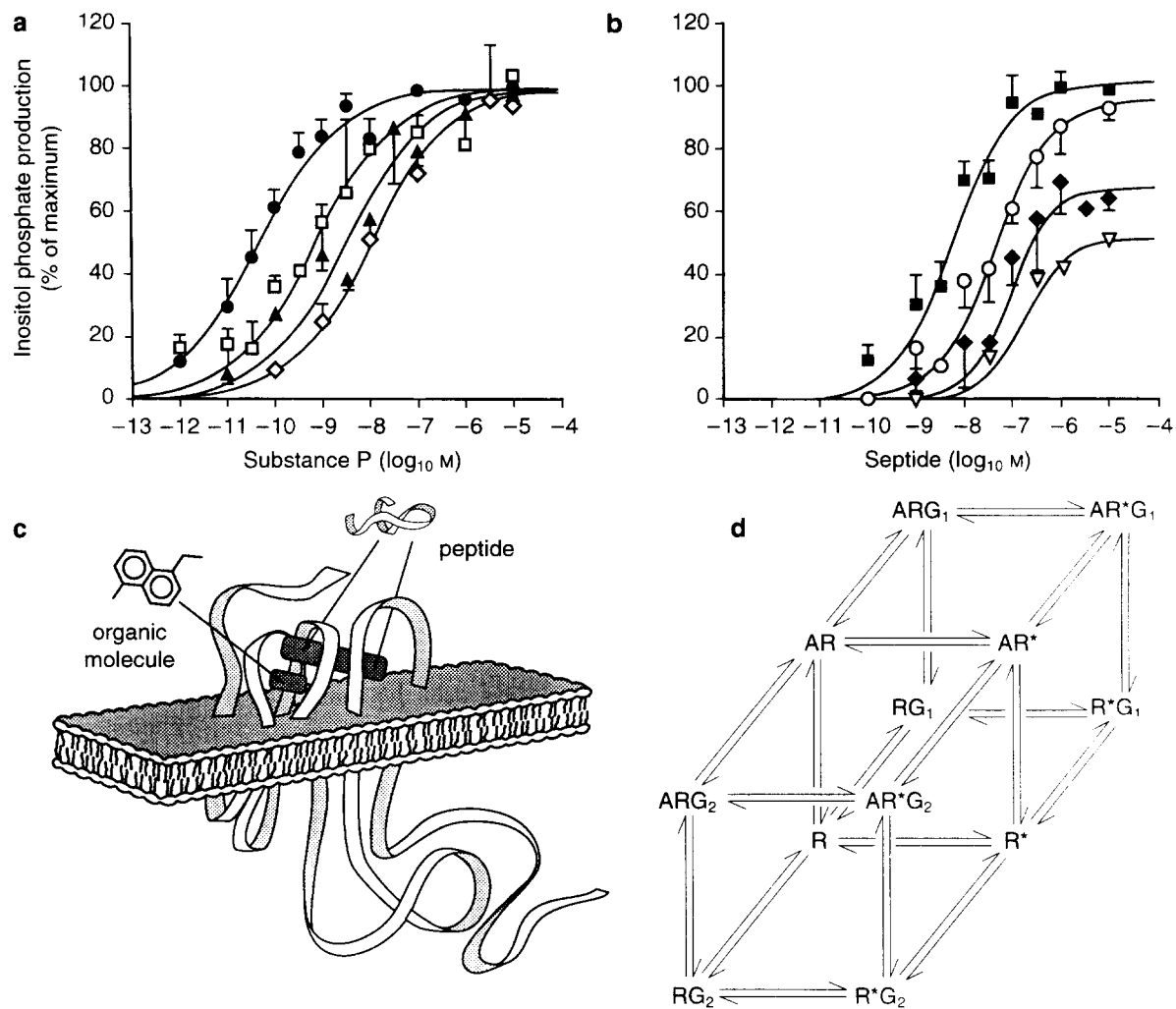
The most straightforward explanation for this behaviour is that the agonists activate a heterogeneous receptor population in the tissue with differential affinity for the antagonist. In tissues, receptor heterogeneity is almost always a possibility, and thus it is extremely difficult to differentiate between a mixture of receptors and a mixture of receptor coupling. This situation can be somewhat clarified in an expression system where a single receptor protein is expressed in a surrogate cell. Under these circumstances, the postulate of heterogeneous receptors can be excluded and the mechanism of differential blockade of agonists can be explored. In COS cells transfected with the NK<sub>1</sub> receptor and stimulated with either substance P (Fig. 5a) or septide (Fig. 5b), the potency of RP67580 and pattern of antagonism is different for the two agonists<sup>15</sup>. Thus, while RP67580 is an apparent competitive antagonist of NK<sub>1</sub> receptors with a pK<sub>b</sub> of 7.86 in the presence



of substance P, it is an apparent uncompetitive antagonist with an apparent  $pK_b$  of 8.9 in the presence of septide.

When the possibility of heterogeneous receptors has been eliminated, it can then be proposed that the involvement of

different binding sites on the receptor for the two agonists may account for selective agonist blockade. In essence, one is still dealing with a heterogeneous receptor situation except that the 'receptors' in this case are binding regions



**Fig. 5.** Production of inositol phosphate in COS-1 cells transfected with tachykinin NK<sub>1</sub> receptors. **a:** Concentration–response curves to substance P in the absence (●) and presence of the NK<sub>1</sub> receptor antagonist RP67580 100 nM (□), 400 nM (▲), and 800 nM (◇). Schild analysis indicated a pK<sub>i</sub> of 7.86. (Data from Ref. 15.) **b:** Concentration–response curves to septide in the absence (■) and presence of RP67580 10 nM (○), 30 nM (◆) and 60 nM (▽). **c:** A seven transmembrane receptor housing two separate binding regions for peptides and organic molecules. **d:** Hypothetical scheme for a single receptor in two states (R and R\*) coupling to two G proteins (G<sub>1</sub> and G<sub>2</sub>). A, agonist. Species coloured red theoretically can produce response.

on the same receptor macromolecule (Fig. 5c). Studies with site-directed mutagenesis in some receptor systems indicate disparities in the binding behaviour of peptides and small molecule antagonists. The most common interpretation of these data is that different portions of the receptor are primarily used for the binding of large peptides and small organic structures. There is an increasing database to support heterogeneous binding regions on the receptor macromolecules<sup>16</sup>.

### Selective trafficking of receptors to different G proteins

An alternative possibility to explain selective agonist blockade is that the differential antagonist potencies relate to the selective trafficking of receptors to different G proteins by agonists. This is a more complex hypothesis and requires that all of the ligands, including the antagonist, discern conformational states and coupling states of the receptor. For example, there are a number of

possible relevant receptor species capable of producing a response for a single expressed receptor interacting with two G proteins (Fig. 5d). If two agonists directed the receptor to form different ternary complexes then a complicated scenario could ensue with differential blockade by antagonists that possess negative or positive efficacy. Under these circumstances, agonist-directed trafficking could make the response differentially sensitive to antagonism by ligands that have either positive (weak partial agonists) or negative intrinsic efficacy (inverse agonists).

An apparent weakness in this hypothesis is the requirement for coupling effects on antagonists, i.e. the antagonist would require either some positive or negative efficacy. However, there is precedent for ligands with seemingly no efficacy in quiescent systems to have negative efficacy in constitutively active receptor systems. Theoretically, if conformational selection is a primary mechanism for efficacy, then some efficacy (either positive or negative) for all ligands should be expected. This is

because the ligand would need to have identical affinity for R and R\* not to exert some influence on subsequent coupling behaviour of the system. Under these circumstances, ligand efficacy must be defined as the property of a molecule that affects subsequent interaction of the receptor with other proteins<sup>17</sup>.

### *Differentiating between the two hypotheses*

Technically there are ways to differentiate the hypotheses of separate binding sites on the receptor and multiple G protein coupling. However, it is difficult to study these effects functionally because stimulus-response mechanisms allow very small populations of receptor species to produce large responses; even though two agonists are both full agonists, they could be operating at different levels of receptor occupation. Therefore, binding studies are required. Radiolabelled versions of both agonists are necessary to allow comparison of saturation curves. If different sites on the same receptor were involved, then the maximal binding capacity for both agonists would be the same, whereas if different coupling were involved, the  $B_{\max}$  values might well differ. The latter hypothesis might indicate the existence of different sized pools of agonist ternary complexes since different pools of G protein competing for a single pool of receptor would be involved. Another approach to this would be to study the system under varying levels of receptor expression since this would change the receptor to G protein ratios. This would not affect a system in which the agonists bind to different portions of the same receptor, but it could affect a coupling mechanism since the stoichiometries would vary with changing expression level.

### **Implications of agonist trafficking**

It is premature to conclude that agonists do or do not generally select multiple active receptor states. The bulk of the evidence indicates that many cases of selective stimulus production by agonists can be accounted for by the hypothesis of a single active receptor state produced in varying quantities by agonists of different intrinsic efficacy. However, the cases of reversal of relative potency of stimulus production cannot be explained by such an idea and the possibility of the more complex phenomenon of agonist-specific active receptor states must be considered. Such mechanisms have broad implications for the classification of receptors and drugs, and the screening of new chemical entities for therapeutic advantage.

As discussed earlier, the possibility of agonist-selective receptor states suggests the possibility of agonist-selective potency of antagonists, if those antagonists have either positive or negative efficacy. If a situation is considered where two agonists such as acetylcholine and oxotremorine traffic muscarinic receptors to different G proteins, then it is possible that partial agonists for muscarinic receptors would show differential potency in terms of antagonism of the responses to these two agonists. This differential could vary across biological systems if the relative quantities of the different G proteins involved varied with cell

type. Thus, it would be conceivable that a given drug could be a more potent inhibitor of oxotremorine responses than acetylcholine responses in this situation, or vice versa. It is obvious that misleading results could be obtained if the wrong agonist is chosen for screening purposes.

As with general statements regarding agonist trafficking, there are insufficient data to conclude whether this is only a theoretical, or a real phenomenon. However, if this effect is operable in some receptor systems, it may be possible to design more selective agonists. If some agonists produce unwanted side-effects because of multiple G protein activation, then those side-effects could be potentially avoided by discovering agonists that direct signalling to more constructive pathways<sup>18</sup>.

### **Concluding remarks**

In conclusion, it can be shown that selective patterns of agonism can be produced from receptor selection by an agonist that produces a uniform active state that then interacts with a complement of membrane G proteins in a homogeneous fashion. However, there are also pharmacological data that cannot be reconciled with such a scheme. These latter systems are of interest since they cannot easily be explained by simple receptor activation theories and thus may hold a key to a new understanding of the phenomenon of efficacy.

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#### **Chemical names**

**McNA343:** 4-(*m*-chlorophenylcarbamoyloxy)-2-butanyltrimethylammonium

**RP67580:** 2-[1-imino-2-(2-methoxyphenyl)-ethyl]-7,7-diphenyl-4-perhydroisoindolone (3*a*R, 7*a*R)