Pergamon

*Biochemical Pharmacology*, Vol. 50, No. 1, pp. 17–26, 1995.

0006-2952/95 $9.50 + 0.00

0006-2952(95)00137-9

# COMMENTARY

# ON THE IMPORTANCE OF THE "ANTAGONIST ASSUMPTION" TO HOW RECEPTORS EXPRESS THEMSELVES

TERRY KENAKIN,*† PAUL MORGAN‡ and MICHAEL LUTZ‡

Departments of *Cellular Biochemistry and ‡Research Computing, Glaxo Research Institute, Research Triangle Park, NC 27709, U.S.A.

*Key words:* receptor classification, drug affinity, efficacy, receptor theory, heterologous receptor expression

Drug and drug–receptor classifications are based on the identity of equilibrium dissociation constants of drug–receptor complexes (designated $K_d$). It is axiomatic in this system that $K_d$ estimates are chemical terms unique only to the drug and receptor pair. It is now accepted that agonists may display observed $K_d$ values that are not dependent solely upon the nature of the agonist and receptor but also upon the process of stimulus transduction. Thus, the expression of agonism may serve to "isomerize" the agonist-bound receptor to perturb the equilibrium between agonist and receptor and, thus, distort the observed $K_d$ [1]. For example, the formation of ternary complexes between agonists, receptor and G-proteins can decrease significantly the magnitude of the observed $K_d$ from the true $K_d$ by a factor dependent upon the concentration of the G-protein and the equilibrium dissociation constant of the ternary complex [2]. For this reason, pharmacologists base receptor classifications primarily on antagonist $K_d$ values and use these to classify and compare receptors cloned and expressed in surrogate systems with those present in natural physiological systems. The cornerstone upon which all of these classifications rest is the assumption that the reference antagonists are indeed "antagonists" in the sense that they bind only to the receptor and do not, in any way, alter the receptor to isomerize it. This can be called the "antagonist assumption," and recent data suggest that it is worthy of critical examination. This paper discusses the mechanistic basis for the definition of antagonists, where pure antagonists fit into a theoretical spectrum of negative to positive efficacy, and the implications of these ideas on the classification of drugs and receptors in surrogate receptor expression systems.

## SPECTRUM OF EFFICACY

Drug receptors are special proteins in that they incorporate the dual properties of *recognition* and *transduction* [3]. Thus, they exist to recognize endogenous agonists and transmit the messages they contain to their cellular hosts. Receptors also may be unique from the point of view of interactions with foreign ligands. A hypothesis put forth by Weinstein [4] suggests that receptors, in general, are more malleable than most proteins and modify their tertiary structure in accordance with ligand binding more readily than do most enzymes. Thus, a receptor is a receptor because it responds to ligand binding more efficiently than other proteins. Given this license, the binding of ligands to receptors (as "malleable" proteins) suggests a potential predisposition to allotopic protein–protein interactions. As stated by Colquhoun [1], under these circumstances drug affinity and efficacy may be difficult to dissociate since the forces that cause a molecule to bind to a receptor also may tend to disrupt the three-dimensional structure of the receptor. If these disruptions extend to the cytosolic domains of the receptor, then subsequent binding to membrane-bound proteins may be affected. In general, it may be more appropriate to consider that biologically active ligands have both the ability to bind to receptors and the potential ability to modify receptors for subsequent interaction with membrane proteins. If the binding of the ligand increases the probability of receptor binding to the transduction protein to activate it, then the ligand will promote cellular response and be defined as having positive

**Taxonomy of Drugs**

| Classification | Response | Distinguishing Feature |
|---|---|---|
| Agonists | • Produce Response<br>• Augment Basal Activity | Stabilize Receptor Complexes |
| Neutral Antagonists | • Do NOT Produce Response or Affect Basal Activity | Do NOT Affect Receptor Complexes |
| Inverse Agonists | • Do NOT Produce Response<br>• Depress Basal Activity | Destabilize Receptor Complexes |

Fig. 1. Classification of drugs by criteria relating to observed effects on response and membrane-based mechanism. The class of negative antagonists is often not apparently distinct from neutral antagonists in systems where basal receptor activity is not elevated.

† Corresponding author: Dr. Terry Kenakin, Department of Cellular Biochemistry, Glaxo Research Institute, 5 Moore Drive, P.O. Box 13358, Research Triangle Park, NC 27709. Tel. (919) 990-6192; FAX (919) 990-6465.

intrinsic efficacy, i.e. be classified as an agonist. If the ligand decreases the probability of receptor–transducer association or destabilizes pre-existing spontaneous association of these entities, then the ligand can be thought to possess *negative efficacy* [5–7]. These types of compounds will be referred to in this paper as "inverse agonists." An analogous situation exists in ion channels where ligands can shift pre-existing equilibria between spontaneously opened or closed ion channels [8]. Thus, the binding of a ligand could produce a spectrum of effects on receptor–transducer association from stabilization to destabilization of receptor–transduction protein complexes. In this scheme, "neutral" antagonists, which bind to the receptor and *do not* affect receptor–transducer association, would be at the midpoint of the spectrum (Fig. 1). The important aspect of this idea is that neutral antagonists are special cases in the world of ligands and not necessarily the majority, as has been generally assumed. "Assumed" may be the key word here, since most classifications of antagonists are based on incomplete data, i.e. only the non-observance of agonist response upon binding. This may be a poor indicator of the absence of ligand efficacy, since it is known that tissue stimulus–response mechanisms control observed agonism and that ligands can be seen to be full agonists to full antagonists by activating the same receptor in different tissues with differing efficiencies of receptor coupling [9–11]. This can be especially true in over-expressed systems. For example, the standard antagonists for serotonin receptors, such as dihydroergotamine and yohimbine, are *agonists* for 5-$HT_{1B}$ receptor-mediated adenylyl cyclase inhibition in transfected Y-1 cells [12–14]. Perhaps of more relevance is the possibility that negative efficacy will be undetected because of low levels of basal responses. Inverse agonism is made manifest in receptor pre-coupled systems in which basal responses are high and can be decreased by negative antagonists. In the absence of such activity, inverse agonists are indistinguishable from neutral antagonists, i.e. some ligands currently thought to be pure antagonists may, in fact, be inverse agonists.

The tacit assumption is made that the antagonist occupies a binding site on the receptor, thus forming a complex that does not predispose further interaction of the bound receptor with other membrane proteins. However, antagonism is not necessarily an intrinsic property of a drug, but rather a label pharmacologists put on a drug in accordance with a set of predetermined guidelines. Thus, if a chemical does not produce a response but rather blocks the response produced by a known agonist, by the classical definition it is *labeled* an antagonist and by *implication* it interacts only with the native receptor (i.e. it does not predispose formation of complexes with other membrane-bound proteins). The defining criterion is the lack of observation of a response.

Recent models of receptor–transducer interaction suggest that ligand binding may be more complicated than that initially envisioned within the confines of classical occupation theory. Data are available now from reconstitution studies to show that receptors can spontaneously couple to G-proteins in the absence of agonist to elevate GTPase activity. For example, Fig. 2 shows the effects of increasing concentrations of $G_{i2}$ protein in a system reconstituted with $D_2$ dopamine receptor [15]. These data show that, in reconstituted systems with 1.5 and 2.5 pmol $G_{i2}$ protein, a *basal* elevation of GTPase activity can be observed in the absence of the agonist. This suggests that the receptors and G-proteins in this system couple spontaneously in the membrane to produce G-protein activation. There are other examples of reconstituted systems where this is thought to occur (e.g. adenosine $A_1$ receptors [16]).

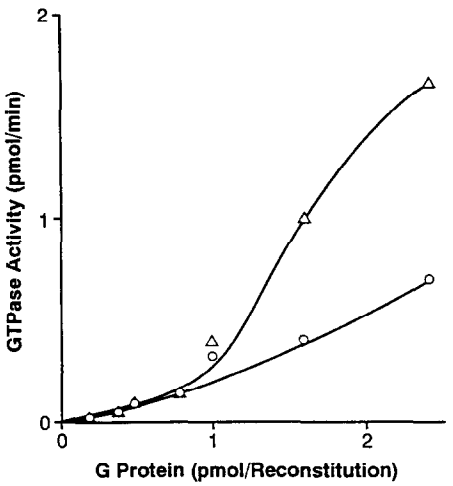
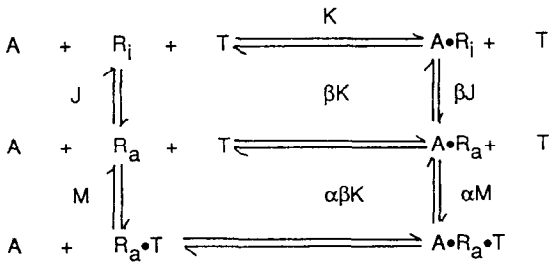



Fig. 2. Spontaneous activation of $G_{i2}$ protein upon reconstitution with $D_2$ dopamine receptor. Curves with $G_{i2}$ protein reconstituted with ≈75 fmol $D_2$ dopamine receptor alone (○) and in the presence of dopamine (△). Reprinted with permission from *J Biol Chem* **265**: 4507–4514, 1990. Copyright (1990) The American Society for Biochemistry and Molecular Biology, Inc. [Ref. 15].

There also are data from natural membrane systems that suggest that spontaneous receptor/G-protein coupling may occur [5, 17–30]. One thermodynamic scheme for these types of interactions has been presented by Samama and colleagues [31, 32] as an extension of the well-known ternary complex model of receptor–effector interaction:




This model is based on experimental data that indicate that receptors can exist in so-called active [$R_a$] and inactive [$R_i$] states with respect to predisposition to association with membrane-bound transduction proteins [T]. The equilibrium between [$R_a$] and [$R_i$] is described by an affinity constant designated *J*. The ligand receptor complex ([A·R])

is controlled by an affinity constant, $K$. Ligands affect the distribution of the receptor population between $[R_a]$ and $[R_i]$ by a multiplicative factor, $\beta$. The activated receptor ($[R_a]$) also can form a complex with a transduction protein ([T]) spontaneously with an equilibrium dissociation constant for the product designated $M$. When an agonist ([A]) is present, ternary complex ($[A{\cdot}R_a{\cdot}T]$) formation from the $[R_a{\cdot}T]$ complex is facilitated by a multiplication of M by the factor $\alpha$. An important aspect of the scheme is that a ligand may have two different binding states of the receptor to choose from, namely $[R_i]$ and $[R_a]$ (and $[R_a{\cdot}T]$). If $\alpha$ is greater than unity, ligand binding will promote ternary complex formation and the ligand will be an agonist. If $\alpha < 1$, the ligand will *destabilize* pre-existing receptor–transducer protein complexes ($[R_a{\cdot}T]$) and have a higher affinity for $[R_a]$ over $[R_a{\cdot}T]$. Under these circumstances, such *inverse agonists* would have a lower observed affinity for the receptor in systems where significant receptor pre-coupling exists (i.e. substantial quantity of $[R_a{\cdot}T]$) *or* when they are used to displace agonist radioligands utilized in concentrations sufficient to produce significant steady-state $[A{\cdot}R_a{\cdot}T]$ complexes. A neutral antagonist would have equal affinity for $[R_a]$, $[R_i]$ and $[R_a{\cdot}T]$ ($\alpha = 1$). Under these circumstances, the observed affinity would be affected by the same factors as those that affect agonist affinity.

## DIFFERENTIATION BETWEEN NEUTRAL ANTAGONISTS AND INVERSE AGONISTS

Given the possibility that an inadequate screening system for true antagonists may be in use, a class of ligands comprised of neutral antagonists and inverse agonists may be thought to be a homogeneous population. The obvious question then arises: how can inverse agonists be differentiated from pure antagonists? For G-protein linked receptors, there are two potential experimental approaches available to answer this question: (1) the observation of a dose-dependent decrease in basal activity in systems where receptors and G-proteins spontaneously pre-couple, and (2) the demonstration of receptor–transducer protein (G-protein) effects on the binding of the antagonist. It is worth discussing these two approaches to the study of receptor ligands.

There are known receptor systems that possess basal G-protein activity as a result of a spontaneous coupling of receptors to G-proteins. The addition of an inverse agonist to a spontaneously coupled system will produce antagonism of the basal activity. Figure 3A shows the effects of the $\delta$-opioid receptor inverse agonist ICI 174864 on GTPase activity in membranes from NG 108-15 cells. In this system, ionic conditions can be manipulated to decrease or increase spontaneous association of receptors to G-proteins [5, 6]. In low basal activity membranes, a small decrease of spontaneous activity is produced by ICI 174864 (Fig. 3A). When basal activity is increased, the negative effects of this inverse agonist become clearly evident. Another potentially useful type of system for the detection of negative intrinsic efficacy is a receptor over-expression system. There is evidence to show that as receptor numbers are increased, there is a concomitant increase in the likelihood of spontaneous receptor coupling to G-proteins. For example, 2- to 4-fold increases in adenylyl cyclase activity are observed in CHO cells transfected with $\beta_2$-adrenoceptors, with 2- to 3-fold increases in expressed receptor levels [31]. Therefore, in theory, cells in which receptors have been over-expressed may serve as sensitive indicators of inverse agonism by ligands with negative intrinsic efficacy. It should be noted that the functional readouts would be more sensitive indicators of such activity if it is assumed that cellular biochemical cascades amplify the effects of low levels of spontaneous receptor/G-protein complexes. Such negative effects on basal activity would not be observed with a neutral antagonist that is only bound to the receptor and has no discerning affinity for [R] or the $[R{\cdot}T]$ complex. Of note is the fact that in the absence of detectable basal activity, inverse agonists and neutral antagonists would be indistinguishable in terms of their direct effects on the receptor system.

Another way of detecting complex binding of ligands to receptors and G-proteins is to determine possible sensitivity of ligand affinity to GTP in binding experiments. It is well established that ligands which predispose ternary complex formation (i.e. agonists) demonstrate high affinities in systems where ternary complexes are allowed to form under steady-state conditions. The addition of an excess of GTP (or non-hydrolyzable analogue), which destabilizes receptor/G-protein complex formation, reduces the steady-state concentration of the ternary complex and transforms the complex two-stage binding of agonists to a single lower affinity binding reaction. Under these conditions, the well-known "GTP-shift" is observed with the observed affinity of the agonist decreasing upon the inclusion of GTP in the medium. For example, Fig. 3B shows the GTP-induced decrease in binding of the dopamine receptor agonist [$^3$H]*N*-propylapomorphine in porcine pituitary gland membranes [18]. In general, any experimental intervention that interferes with receptor/G-protein interaction can cause the same effect. Thus, the treatment of cells with pertussis or cholera toxin inactivates some transducer proteins in terms of specific binding with receptors. Figure 3C shows the effects of pertussis toxin treatment on the binding of the $\delta$-opioid agonist DADLE. As expected, pertussis toxin treatment produced the rightward shift of the binding curve for the agonist.

These data have been reproduced in many laboratories and clearly indicate the loss of observed affinity upon reduction of G-protein and receptor interaction in agonist binding studies. Such experiments also have yielded extremely interesting data to suggest that some apparent antagonists are affected by ternary complex formation as well. One example is the *opposite* effect of GTP on the binding of the dopamine receptor antagonist [$^3$H]-spiroperidol. As shown in Fig. 3B, the binding of this antagonist is increased by GTP. This suggests that spiroperidol could be an inverse agonist that destabilizes ternary complex formation. Another example is the effect of pertussis toxin treatment of NG 108-15 cells on the affinity of the $\delta$-opioid inverse agonist ICI 174864. In this case, the affinity of ICI

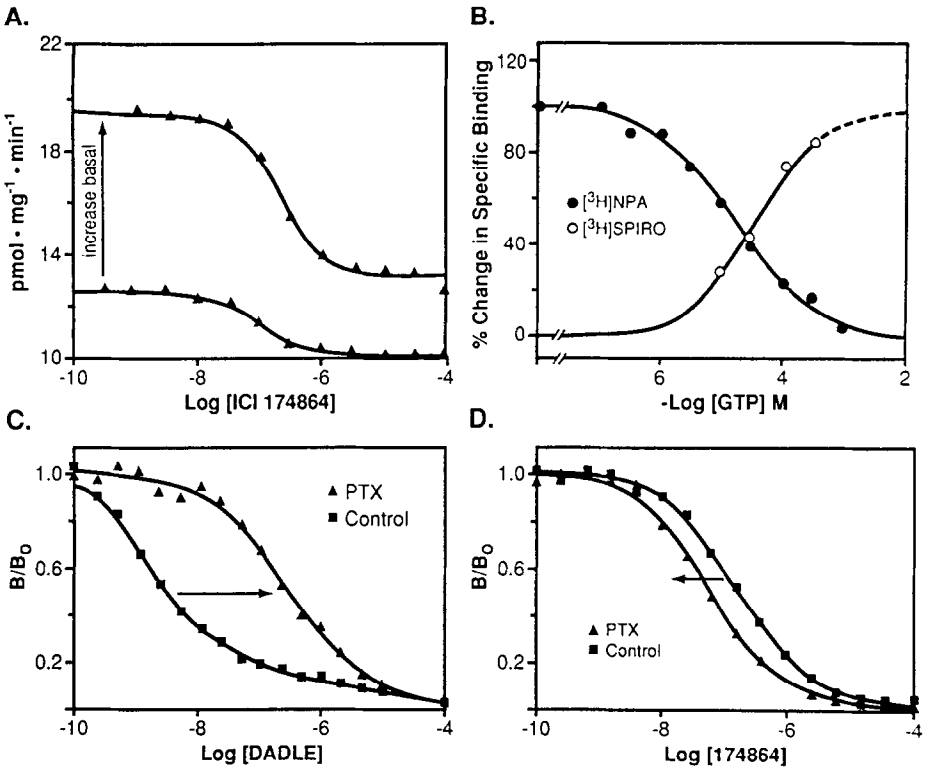



Fig. 3. Effects of agonists and negative antagonists on receptor systems. (A) Effects of the negative antagonist ICI 174864 ([*N,N'*-diallyl-Tyr$^1$,Aib$^{2,3}$]Leu$^5$-enkephalin) on GTPase activity in membranes from NG108-15 cells. Basal GTPase activity increased by substitution of $K^+$ for $Na^+$. (B) Effect of GTP on the binding of the agonist [$^3$H]*N*-propylapomorphine ([$^3$H]NPA, 155 pM) and the antagonist [$^3$H]spiroperidol ([$^3$H]SPIRO, 160 pM) in porcine anterior pituitary gland membranes. Specific binding is expressed as a percentage of binding in the absence of GTP. Reprinted with permission from *Mol Pharmacol* **22**: 290–297, 1982. Copyright (1982) The American Society for Pharmacology and Experimental Therapeutics. [Ref. 18]. (C) Effects of inactivation by pertussis toxin of G-protein on displacement of [$^3$H]diprenorphine (0.25 nM) in membranes from NG108-15 cells by the agonist DADLE ([D-Ala$^2$,D-Leu$^5$]enkephalin). Pertussis toxin treatment decreases the observed affinity of DADLE. (D) Effects of pertussis treatment on displacement of [$^3$H]diprenorphine (0.25 nM) by the inverse agonist ICI 174864. Pertussis treatment increases the affinity of the inverse agonist. Data for panels A, C and D reprinted with authors' permission from *Proc Natl Acad Sci USA* **86**: 7321–7325, 1989. [Ref. 5].

174864 is *increased* by removal of receptor/G-protein coupling ("reverse GTP-shift"—see Fig. 3D). Presumably, pertussis toxin removes pre-existing [R·T] complex, thereby removing a lower affinity state of the receptor for ICI 174864. The relevant point to these data is the fact that spiroperidol and ICI 174864 both appear to be simple antagonists in functional studies and in systems where spontaneous receptor activity is not observed. In general, sensitivity of ligand affinity to manipulations that affect receptor coupling should be a warning sign of complex ligand–receptor binding and the potential dependence of affinity on more than just the receptor type.

Treatment with GTP analogues assumes that a ligand will show its hand in either natural pharmacological or expression systems (by producing a shift in an affinity binding curve with GTP) or G-protein targeted membrane treatment such as pertussis or cholera toxin. However, this requirement sometimes can be problematic since the reduction of two-stage binding by GTP is a kinetic process known to differ in various systems. Experimental conditions can affect the magnitude of GTP-shifts, as seen with the binding of muscarinic agonists in membranes from rat heart [33]. There also are cases of systems in which the binding of agonists known to produce ternary complexes is not altered by GTP. For example, while Gpp(NH)p produces effects on muscarinic agonist binding in membranes from rat heart and cerebellum, no effect in rat cortex can be demonstrated [34]. In general, the GTP effect is a one-way experiment in that if the effect is observed, the data support the involvement of G-proteins with receptor binding. However, if the effect is not observed, it cannot automatically be assumed that G-proteins are not involved. It may be that the experiment was inadequate to demonstrate the effect.

## DEFINITION OF A NEUTRAL ANTAGONIST

In mechanistic terms, the definition of a "neutral"

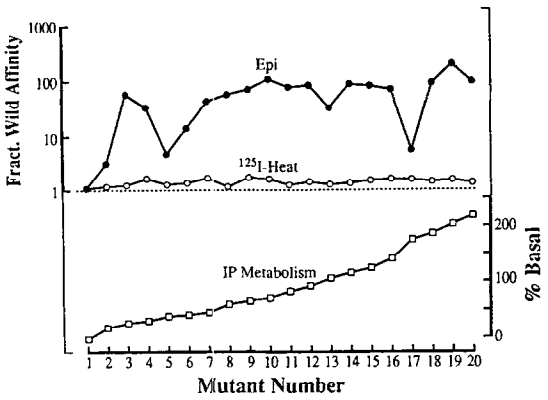



Fig. 4. Affinities and basal activities of mutants of $\alpha_{1B}$-adrenoceptors expressed in COS-7 cells. Numbers on the abscissa refer to different mutations of $\alpha_{1B}$-adrenoceptors. The right-hand ordinate refers to percent basal elevations of phosphoinositide hydrolysis produced by the mutant receptors. The left-hand ordinate (logarithmic scale) shows the ratios of the equilibrium dissociation constants of epinephrine (●) and the antagonist $^{125}$I-HEAT (○) to that obtained for the wild-type receptor. Note that the affinity of the mutants for epinephrine is selectively elevated. Constitutive activity graph from Kjelsberg *et al.* [37]; affinities were drawn from the table values in the same paper. Reprinted with permission from *J Biol Chem* **267**: 1430–1433, 1992. Copyright (1992) The American Society for Biochemistry and Molecular Biology, Inc.

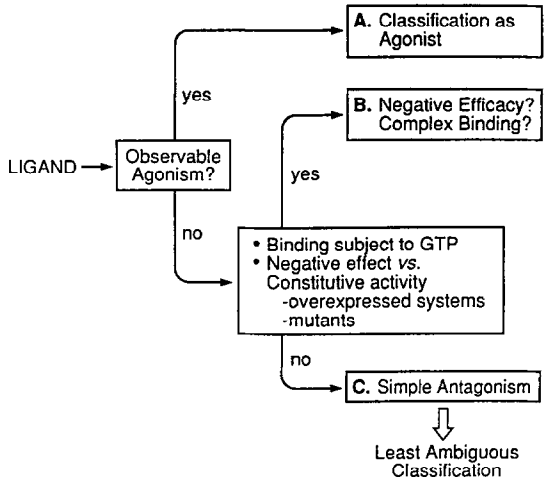



Fig. 5. Schematic diagram of a serial approach to the use of ligands for receptor classification.

antagonist is different from the standard functionally based definition. It is worth describing the molecular behavior of neutral antagonists in the membrane societal realm of receptors, transduction proteins and effectors. Two mechanisms by which an agonist can transfer information to a receptor have been described by Burgen [35], namely conformational induction (agonist binding produces a change in the tertiary structure of the receptor) or conformational selection (the agonist selectively binds to one or more pre-existing forms of the receptor). The idea that receptors exist in two affinity states (conformational selection) is implicit in the ternary complex model described by De Lean *et al.* [36] and explicitly defined in the extended ternary complex model by Samama *et al.* [31], namely, the allosteric interaction between the receptor and the G-protein and the concept of an active (high affinity for the G-protein) and inactive (low affinity for the G-protein) state for receptors. Recent data with mutants of the $\alpha_{1B}$-adrenoceptor support the existence of high- and low-affinity receptor states for ligands [37]. Specifically, substitution of any amino acid residue at position 293 of the $\alpha_{1B}$-adrenoceptor results in mutants that spontaneously elevate GTPase activity (see Fig. 4). Of particular relevance to the two-state idea for receptors is the fact that these mutants also have selectively higher affinities for the agonist epinephrine over the wild-type receptor (filled circles, Fig. 4), but show no concomitant change in the affinity for the antagonist HEAT (open circles, Fig. 4). These data suggest that the point mutations resulted in the production of tertiary conformations that resemble the active state of the receptor. Similar effects have been observed in studies on $\beta_2$-adrenoceptors [31] and $\alpha_2$-adrenoceptors [38].

In the conformational induction scenario, a neutral antagonist binds to sites necessary for agonist binding but in doing so does not alter the tertiary structure of the receptor. For conformational selection, the antagonist has *identical* affinities for each of the pre-existing forms of the receptor. In both of these schemes, a pure antagonist would be neutral with respect to its binding to the receptor. If the premise is accepted that receptors are predisposed to change their tertiary conformation upon ligand binding or predisposed to existing as two or more tertiary conformations in the membrane, then a neutral ligand becomes a special class of drug with great value in receptor classification. Given this, the burden is on the pharmacologist interested in drug and receptor subtyping to prove that an apparent neutral antagonist does *not* perturb receptor/G-protein interaction. Figure 5 shows a scheme for the use of ligands for receptor classification. The hypothesis is that the classification data with ligands in class C would be least subject to variation across receptor expression systems.

## RELEVANCE TO RECEPTOR EXPRESSION SYSTEMS

Technological advances in molecular biology have ushered in an era of control of receptor expression in pharmacology. Thus, the genes coding for receptor proteins may be introduced into cellular systems to produce high levels of receptors in membranes. A theoretical advantage of this approach is the ability to produce pure populations of receptors in cells, presumably avoiding potential confusion in receptor classification studies with heterogeneous receptor mixtures. An obvious shortcoming of this approach is the failure of such systems to assess correctly the

Table 1. Binding parameters for M1 muscarinic receptors in a series of transfected B82 cells

| Clone | $B_{max}$ (fmol/$10^6$ cells) | [$^3$H]QNB $K_d$ (pM) | Carbachol* | | |
|---|---|---|---|---|---|
| | | | $K_H$† ($\mu$M) | %H‡ | $K_L$§ ($\mu$M) |
| LK3-1 | 12 ± 0.98 | 100 | 7.7 | 100‖ | |
| LK3-4 | 18 ± 1.5 | 110 | 1.9 | 43 ± 7.3 | 46 |
| LK7-6 | 30 ± 4.4 | 97 | 3.8 | 68 ± 10 | 34 |
| LK3-7 | 96 ± 15 | 150 | 5.8 | 62 ± 12 | 41 |
| LK7-2 | 140 ± 19 | 150 | 7.1 | 47 ± 10 | 53 |
| LK3-3 | 240 ± 15 | 160 | 4.8 | 40 ± 7.6 | 45 |
| LK3-8 | 260 ± 48 | 190 | 2.4 | 42 ± 5.9 | 53 |

Data are from Mei *et al.* [39]. Reprinted with permission from *J Pharmacol Exp Ther* **251**: 90–97, 1989. Copyright (1989) The American Society for Pharmacology and Experimental Therapeutics.

* Displacement of [$^3$H]QNB by carbachol.

† Equilibrium dissociation constant of carbachol with high affinity site.

‡ Percentage of sites apparently in the high-affinity form.

§ Equilibrium dissociation constant of carbachol for the low-affinity site.

‖ Only one site detected.

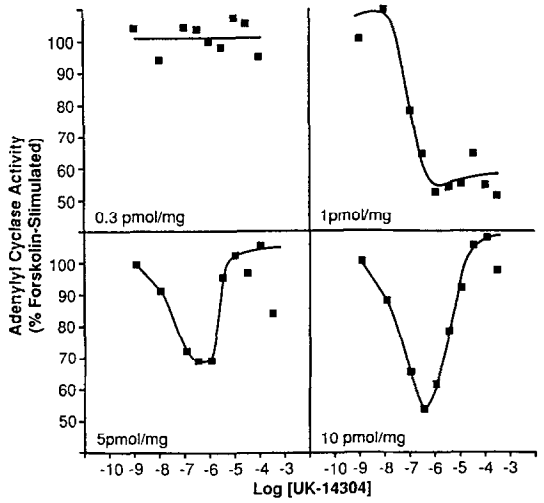



Fig. 6. Effects of UK-14304 on adenylate cyclase activity in transfected CHO cells with different expression levels of $\alpha_2$-C10 adrenoceptors. Membranes were prepared from cells expressing 0.3, 1, 5 and 10 pmol/mg $\alpha_2$ C10 receptors. Reprinted with permission from *J Biol Chem* **267**: 15795–15801, 1992. Copyright (1992) The American Society for Biochemistry and Molecular Biology, Inc. [Ref. 50].

affinity of ligands that require other natural membrane components, the most obvious such class being agonists.

In binding studies where agonists are used as the radiolabel (i.e. as is often the case with iodinated peptide ligands) and in systems where there is a significant amount of spontaneous receptor–transducer coupling in the absence of ligands, the affinity of a ligand is dependent upon the amount and/or the type of G-protein present in the cell membrane hosting the receptor. Under these conditions, the expectation of observing the same affinity in a surrogate expression system (i.e. COS cells) becomes a game of chance. This is because the receptor density of cultured cells is well known to vary with cell passage and other conditions, and the over-expression of receptors may take the receptor/G-protein ratio further away from physiological levels. It is well known that the relative quantities of receptor and G-proteins affect the relative predominance or absence of high-affinity ternary complex ligand binding. For example, Table 1 shows the variation in the relative proportion of high-affinity sites for the agonist carbachol in a series of transfected B82 cells in relation to the number of receptors expressed [39]. These effects can be exacerbated in systems where the receptors are promiscuous with respect to G-proteins and interact with more than one type. This is a common phenomenon in reconstituted systems, and there is much circumstantial evidence that this may occur in natural systems as well [40–46]. Recently, Munshi *et al.* [16] described a system where receptor promiscuity could be demonstrated in a natural membrane. Using an agonist affinity column for adenosine $A_1$ receptors, they found that the receptors in membranes from bovine brain co-purified with $G_{i1}$, $G_{i2}$ and $G_o$. These data and quantitative reconstitution experiments indicate that this receptor promiscuously binds to all three G-proteins in this native membrane system when activated by an agonist. A particularly graphic illustration of receptor G-protein promiscuity has been shown for agonist-activated opioid receptors in NG108-15 neuroblastoma X glioma cells [47]. Activation with the opioid agonist DADLE (D-Ala$^2$-D-Leu$^5$-enkephalin) produces a dose-dependent $^{32}$P incorporation into three distinct G-proteins. If such mechanisms are operative in cells, then both the type and the relative quantities of coupling proteins can control observed receptor affinities for agonists [48, 49]. Since the relative densities of receptors and coupling proteins are known to change in cell cultures (with cell passage number and other conditions), this results in a potentially unstable measuring system for agonist ligands binding to G-protein-linked receptors. In general, binding data for agonists are

known to be quite variable in cell culture systems. For example, Fig. 6 shows the effects of various expression levels of $\alpha_2$-C10 adrenoceptors in transfected CHO cells [50]. At a receptor expression level of 0.3 pmol/mg, the $\alpha_2$-adrenoceptor agonist UK-14304 produces no effects on adenylate cyclase (Fig. 6, top left panel). However, at 1 pmol/mg, a concentration-dependent inhibition of adenylate cyclase activity is observed (Fig. 6, top right panel). At higher levels of receptor expression (5 and 10 pmol/mg), this inhibition becomes a biphasic response to increased adenylate cyclase activity (Fig. 6, bottom panels). The respective inhibition and stimulation of adenylate cyclase activity can selectively be cancelled by pretreatment of the cells with pertussis and cholera toxin, indicating serial activation of two G-proteins ($G_i$ and $G_s$) by the expressed receptor in the membrane. The type and magnitude of the various responses are controlled by the expression level of the receptors.

These ideas are well known and generally steer classification-minded pharmacologists away from classifying receptors with agonists in cell culture receptor systems. However, the recent data with inverse agonists suggest that these same pitfalls could be encountered with some apparent antagonists. Thus, there may exist a class of "silent" ligands with affinity for receptors that is dependent upon G-proteins. In this sense, the term silent refers to the inability of the ligands to produce a measurable response. Recent studies with $\beta$-adrenoceptor apparent neutral antagonists in systems containing mutant constitutively active $\beta_2$-adrenoceptors [51] or over-expressed wild-type $\beta_2$-adrenoceptors [52] show marked inverse agonism for some previously classified $\beta$-blockers. Similar results for some $\alpha_2$-adrenoceptor blockers have been found in PC-12 cells expressing $\alpha_2$-adrenoceptors [53].

In artificial receptor expression systems, such ligands may yield affinity data that are variable due to receptor–transducer protein effects and not to subtypes of receptors. Some comfort can be taken in the fact that many systems where receptors have been over-expressed have yielded binding data comparable to that observed in functional systems. This is a positive finding; however, the unequal weight of negative data that show expressed and natural systems to be different should not be underestimated. As in Schild analysis, where the correspondence of a large number of antagonist affinities cannot be accepted as proof of receptor identity (i.e. two receptors are the "same" until one antagonist shows them to be different), apparent correspondence between data from expression systems cannot be weighted equally with data that show these systems to be different from natural ones. Also, the assumption is often made that the experimental test system (i.e. expressed receptors in cell lines) may be more simple than the natural physiological system, i.e. the receptor will be exposed for the simple binding protein that it is, since it cannot hide behind the membrane cacophony of interactions seen in the native cell. There are two potential problems with this idea. The first is that the receptor may require the cacophony to express its true impact on the control mechanisms of the organism and, second, the test system may introduce an alien player into the membrane symphony and, thus, give capricious data. For example, unexpected receptor coupling can occur. The mouse 5-$HT_{1C}$ receptor is known to couple to phospholipase C, but when this receptor was transfected into Syrian hamster tumor cells, an unexpected inhibition of adenylate cyclase was observed [54]. This second effect was blocked by pertussis toxin treatment indicating a receptor/G-protein source for the response. In general, the expression of a receptor in a host cell that normally does not express may lead to surprising results. For example, Duzic and Lanier [55] showed how $\alpha_2$-adrenoceptors expressed in NIH 3T3 and $DDT_1$ MF-2 cells mediated inhibition of cyclic AMP, whereas the expression of the same receptor in PC-12 cells mediated elevation of cyclic AMP. Other examples of diverse receptor coupling can be found for dopamine $D_2$ receptors in rat striatum and anterior pituitary tissue [56] and pituitary $GH_4C_1$ cells and $Ltk^-$ fibroblasts [57]. There may be special problems with receptor over-expression in that high densities of receptors may encourage interaction with G-proteins normally not involved in the physiology of the system. Also, receptor over-expression theoretically would favor the spontaneous coupling of receptors with G-proteins, thus producing potentially different binding species.

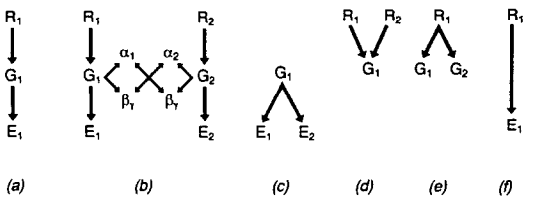



Fig. 7. Potential interactions between receptors (R), coupling proteins (G) and effector systems (E) in cell membranes. Receptors can monogamously interact with one G-protein and one effector system (a), or two separate receptors could interact with two G-proteins that would, in turn, generate two interchangeable sets of $\beta\gamma$ subunits that could cross-over in the signalling process (b). Alternatively, an activated transducer protein could interact with two effectors (c), two receptors could vie for a single G-protein (d), one receptor could activate more than one G-protein (e), or a single receptor could directly activate an effector (f). Reprinted with permission from *Biochem J* **272**: 1–13, 1990. Copyright (1990) The Biochemical Society. [Ref. 58].

There are considerable data to suggest that chemical intracellular communication networks are extremely complex and may achieve fine-tuning at the level of the cell membrane. Thus, receptors may be promiscuous with respect to the transduction proteins with which they interact and the effector systems they activate. Figure 7 shows a set of theoretical connections between receptors and effector systems possible in cells. These interconnections may be very important to the activity that a given receptor-targeted drug expresses *in vivo* [58]. Given this possibility, the expression of a bare

receptor in a foreign membrane and expecting the full activity to become evident may be unrealistic. Promiscuous interactions of receptors and receptor pre-coupling to G-proteins could preclude the existence of a gold standard affinity of some drugs for some receptors. Instead, their affinity may be protean, i.e. changing with the different systems in which they are expressed and studied. In recent attempts to establish a rational approach to the classification of drug receptors, the IUPHAR Committee on Receptor Nomenclature has debated the relative merits of classification by receptor structure and receptor function. It may be that classifying receptors by structure may prove to be an easier task than the classification of the pharmacology of receptors with foreign ligands. A structural classification would be a valuable taxonomy from the point of view of grouping receptors in families and looking for cross-over ligands to detect new drugs. However, in this process the basic assumption that all "antagonists" are equal must be tested. There are two practical reasons for this. The first is the obvious potential problem of using a ligand that is not a neutral antagonist to classify receptor systems and, thus, introducing erroneous classification information into the database. The second is the possibility of missing a potentially valuable therapeutic entity in not questioning the profile of a set of antagonists and looking for the one that does not fit into the general mold of the class. For example, it might be proposed that inverse agonists with negative efficacy could be more valuable than pure antagonists if spontaneous receptor activity (e.g. mutant receptors in cancer) is a factor in the pathology. Also, inverse agonists may be expected to produce a wider spectrum of activity in blocking various organs under basal hormonal and/or neurochemical control.

Insufficient data are available to specifically equate receptor expression systems with natural systems or indeed to evaluate whether the data from such systems will stand the test of prediction of like activity in humans. The promise is that such systems will greatly simplify receptor pharmacology. It is worth critically evaluating the tools used in these studies to ensure that ambiguity will not be introduced at an early stage in this process.

## REFERENCES


1. Colquhoun D, Affinity, efficacy, and receptor classification: Is the classical theory still useful? In: *Perspectives on Receptor Classification. Receptor Biochemistry and Methodology* (Eds. Black JW, Jenkinson DH and Gerskowitch VP), Vol. 6, pp. 103–114. Alan R. Liss, New York, 1987.
2. MacKay D, Use of null equations, based on classical and ternary models of drug action, to classify receptors and receptor–effector systems even if some of the agonists and antagonists available have additional actions on tissues. In: *Perspectives on Receptor Classification. Receptor Biochemistry and Methodology* (Eds. Black JW, Jenkinson DH and Gerskowitch VP), Vol. 6, pp. 193–206. Alan R. Liss, New York, 1987.
3. Kenakin TP, Bond RA and Bonner TI, Definition of pharmacological receptors. *Pharmacol Rev* **44**: 351–362, 1992.
4. Weinstein H, Classification based on ligand binding: On the chemical meaning of ligand affinity in studies of drug receptor interactions. In: *Perspectives on Receptor Classification, Receptor Biochemistry and Methodology* (Eds. Black JW, Jenkinson DH and Gerskowitch VP), Vol. 6, pp. 41–50. Alan R. Liss, New York, 1987.
5. Costa T and Herz A, Antagonists with negative intrinsic activity at δ-opioid receptors coupled to GTP-binding proteins. *Proc Natl Acad Sci USA* **86**: 7321–7325, 1989.
6. Costa T, Ogino Y, Munson PJ, Onaran HO and Rodbard D, Drug efficacy at guanine nucleotide-binding regulatory protein-linked receptors: Thermodynamic interpretation of negative antagonism and of receptor activity in the absence of ligand. *Mol Pharmacol* **41**: 549–560, 1992.
7. Schutz W and Freissmuth M, Reverse intrinsic activity of antagonists on G-protein coupled receptors. *Trends Pharmacol Sci* **13**: 376–380, 1992.
8. Colquhoun D, The relation between classical and cooperative models for drug action. In: *Drug Receptors* (Ed. Rang HP), pp. 149–182. University Park Press, Baltimore, 1973.
9. Kenakin TP, Prenalterol as a selective cardiostimulant: Differences between organ and receptor selectivity. *J Cardiovasc Pharmacol* **7**: 208–210, 1985.
10. Kenakin TP, Agonists, partial agonists, antagonists, inverse agonists, and agonist/antagonists. *Trends Pharmacol Sci* **8**: 423–426, 1987.
11. Hoyer D and Boddecke HWGM, *Trends Pharmacol Sci* **14**: 270–275, 1993.
12. Adham N, Ellerbrock B, Hartig P, Weinshank RL and Branchek T, Receptor reserve masks partial agonist activity of drugs in a cloned rat 5-hydroxytryptamine$_{1B}$ receptor expression system. *Mol Pharmacol* **43**: 427–433, 1993.
13. Schoeffter P and Hoyer D, 5-Hydroxytryptamine 5-HT$_{1B}$ and 5-HT$_{1D}$ receptors mediating inhibition of adenylate cyclase activity. *Naunyn Schmiedebergs Arch Pharmacol* **340**: 285–292, 1989.
14. Schoeffter P, Waeber C, Palacios JM and Hoyer D, The 5-hydroxytryptamine 5-HT$_{1D}$ receptor subtype is negatively coupled to adenylate cyclase in calf substantia nigra. *Naunyn Schmiedebergs Arch Pharmacol* **337**: 602–608, 1988.
15. Senogles SE, Spiegel AM, Pardrell E, Iyengar R and Caron MG, Specificity of receptor G-protein interactions. Discrimination of G$_i$ subtypes by the D$_2$ dopamine receptor in a reconstituted system. *J Biol Chem* **265**: 4507–4514, 1990.
16. Munshi R, Pang I-H, Sternweis PC and Linden J, A$_1$ adenosine receptors of bovine brain couple to guanine nucleotide-binding proteins G$_{i1}$, G$_{i2}$ and G$_o$. *J Biol Chem* **266**: 22285–22289, 1991.
17. Leung E and Green RD, Density gradient profiles of A$_1$ receptors labeled by agonist and antagonist radioligands before and after detergent solubilization. *Mol Pharmacol* **36**: 412–419, 1989.
18. De Léan A, Kilpatrick BF and Caron MG, Dopamine receptor of the porcine anterior pituitary gland: Evidence for two affinity states discriminated by both agonists and antagonists. *Mol Pharmacol* **22**: 290–297, 1982.
19. Cerione RAJ, Codina J, Benovic JL, Lefkowitz RJ, Birnbaumer L and Caron MG, The mammalian $\beta_2$-adrenergic receptor: Reconstitution of functional interactions between pure receptor and pure stimulatory nucleotide binding protein of the adenylate cyclase system. *Biochemistry* **23**: 4519–4525, 1984.
20. Korn SJ, Martin MW and Harden TK, *N*-Ethylmaleimide-induced alteration in the interaction of agonists and muscarinic cholinergic receptors of rat brain. *J Pharmacol Exp Ther* **224**: 118–126, 1983.

21. Wreggett KA and De Léan A, The ternary complex model: Its properties and application to ligand interactions with the $D_2$-dopamine receptor of the anterior pituitary gland. *Mol Pharmacol* **26**: 214–227, 1984.
22. Baron BB, Gavish M and Sokolovsky M, Heterogeneity of solubilized muscarinic cholinergic receptors: Binding and hydrodynamic properties. *Arch Biochem Biophys* **240**: 281–296, 1985.
23. Chatterjee TK, Moy JA, Cai JJ, Lee H-C and Fisher RA, Solubilization and characterization of a guanine nucleotide-sensitive form of the calcitonin gene-related peptide receptor. *Mol Pharmacol* **43**: 167–175, 1993.
24. Costa T, Lang J, Gless C and Herz A, Spontaneous association between opioid receptors and GTP-binding regulatory proteins in native membranes: Specific regulation by antagonists and sodium ions. *Mol Pharmacol* **37**: 383–394, 1990.
25. Jeffs RA, Cooper CL and Harden TK, Solubilization of a guanine nucleotide-sensitive form of the $P_{2y}$-purinergic receptor. *Mol Pharmacol* **40**: 85–92, 1991.
26. Law SF, Manning D and Reisine T, Identification of the subunits of GTP-binding proteins coupled to somatostatin receptors. *J Biol Chem* **266**: 17885–17897, 1991.
27. Matsui H, Asakura M, Tsukamoto T, Imafuku J, Ino M, Saitoh N, Miyamura S and Hasegawa K, Solubilization and characterization of rat brain $\alpha_2$-adrenergic receptor. *J Neurochem* **44**: 1625–1632, 1985.
28. Niznik HB, Otsuka NY, Dumbrille-Ross A, Grigoriadis D, Tirpak A and Seeman P, Dopamine $D_1$ receptors characterized with [$^3$H]SCH 23390. Solubilization of a guanine nucleotide-sensitive form of the receptor. *J Biol Chem* **261**: 8397–8406, 1986.
29. Sladeczek F, Bockaert J and Rouot B, Solubilization of brain alpha-2 adrenoceptor with a zwitterionic detergent: Preservation of agonist binding and its sensitivity to GTP. *Biochem Biophys Res Commun* **119**: 1116–1121, 1984.
30. Demoliou-Mason CD and Barnard EA, Characterization of opioid receptor subtypes in solution. *J Neurochem* **46**: 1129–1136, 1986.
31. Samama P, Cotecchia S, Costa T and Lefkowitz RJ, A mutation-induced activated state of the $\beta_2$-adrenergic receptor. Extending the ternary complex model. *J Biol Chem* **268**: 4625–4636, 1993.
32. Lefkowitz RJ, Cotecchia S, Samama P and Costa T, Constitutive activity of receptors coupled to guanine nucleotide regulatory proteins. *Trends Pharmacol Sci* **14**: 303–307, 1993.
33. Watson M, Yamamura HI and Roeske WR, [$^3$H]-Pirenzepine and (−)-[$^3$H]quinuclidinyl benzilate binding to rat cerebral cortical and cardiac muscarinic cholinergic sites. I. Characterization and regulation of agonist binding to putative muscarinic subtypes. *J Pharmacol Exp Ther* **237**: 411–418, 1986.
34. Vickroy TW, Yamamura HI and Roeske WR, Differential regulation of high-affinity agonist binding to muscarinic sites in the rat heart, cerebellum and cerebral cortex. *Biochem Biophys Res Commun* **116**: 284–290, 1983.
35. Burgen ASV, Conformational changes and drug action. *Fedn Proc* **40**: 2725–2728, 1981.
36. De Léan A, Stadel JM and Lefkowitz RJ, A ternary complex model explains the agonist-specific binding properties of the adenylate cyclase-coupled $\beta$-adrenergic receptor. *J Biol Chem* **255**: 7108–7117, 1980.
37. Kjelsberg MA, Cotecchia S, Ostrowski J, Caron MG and Lefkowitz RJ, Constitutive activation of the $\alpha_{1B}$-adrenergic receptor by all amino acid substitutions at a single site. Evidence for a region which constrains receptor activation. *J Biol Chem* **267**: 1430–1433, 1992.
38. Ren Q, Kurose H, Lefkowitz RJ and Cotecchia S, Constitutively active mutants of the $\alpha_2$-adrenergic receptor. *J Biol Chem* **268**: 16483–16487, 1993.
39. Mei L, Lai J, Yamamura HI and Roeske WR, The relationship between agonist states of the $M_1$ muscarinic receptor and the hydrolysis of inositol lipids in transfected murine fibroblast cells (B82) expressing different receptor densities. *J Pharmacol Exp Ther* **251**: 90–97, 1989.
40. Kenakin TP, Drugs and receptors: An overview of the current state of knowledge. *Drugs* **40**: 666–687, 1990.
41. Chakraborthy M, Chatterjee D, Kellokumpu S, Rasmussen H and Baron R, Cell cycle-dependent coupling of the calcitonin receptor to different G-proteins. *Science* **251**: 1078–1082, 1991.
42. Ewald DA, Pang I-H, Sternweis PC and Miller RJ, Differential G protein-mediated coupling of neurotransmitter receptors to $Ca^{2+}$ channels in rat dorsal root ganglion neurons *in vitro*. *Neuron* **2**: 1185–1193, 1989.
43. Gerhardt MA and Neubig RR, Multiple $G_i$ protein subtypes regulate a single effector mechanism. *Mol Pharmacol* **40**: 707–711, 1991.
44. Luthin DR, Eppler CM and Linden J, Identification and quantification of the $G_i$-type GTP-binding proteins that copurify with a pituitary somatostatin receptor. *J Biol Chem* **268**: 5990–5996, 1993.
45. Marbach I, Shiloach J and Levitski A, $G_i$ affects the agonist-binding properties of $\beta$-adrenoceptors in the presence of $G_s$. *Eur J Biochem* **172**: 239–246, 1988.
46. Rubenstein RC, Linder ME and Ross EM, Selectivity of the $\beta$-adrenergic receptor among $G_s$, $G_i$'s and $G_o$: Assay using recombinant $\alpha$ subunits in reconstituted phospholipid vesicles. *Biochemistry* **30**: 10769–10777, 1991.
47. Roerig SC, Loh HH and Law PY, Identification of three separate guanine nucleotide-binding proteins that interact with the $\delta$-opioid receptor in NG108-15 X glioma hybrid cells. *Mol Pharmacol* **41**: 822–831, 1992.
48. Kenakin TP, Are receptors promiscuous? Intrinsic efficacy as a transduction phenomenon. *Life Sci* **43**: 1095–1101, 1988.
49. Kenakin TP and Morgan PH, Theoretical aspects of single and multiple transducer receptor coupling proteins on estimates of relative potency of agonists. *Mol Pharmacol* **35**: 214–222, 1989.
50. Eason MC, Kurose H, Holt, BD, Raymond JR and Liggett SB, Simultaneous coupling of $\alpha_2$-adrenergic receptors to two G-proteins with opposing effects. *J Biol Chem* **267**: 15795–15801, 1992.
51. Samama P, Pei GP, Costa T, Cotecchia S and Lefkowitz RJ, Negative antagonists promote an inactive conformation of the $\beta_2$-adrenergic receptor. *Mol Pharmacol* **45**: 390–394, 1994.
52. Chidiac P, Hebert TE, Valiquette M, Dennis M and Bouvier M, Inverse agonist activity of $\beta$-adrenergic antagonists. *Mol Pharmacol* **45**: 490–499, 1994.
53. Tian W-N, Duzic E, Lanier SM and Deth RC, Determinants of $\alpha_2$-adrenergic receptor activation of G proteins: Evidence for a precoupled receptor/G protein state. *Mol Pharmacol* **45**: 524–531, 1994.
54. Lucaites VL, Nelson DL, Yu L and Baez M, Promiscuity of the cloned mouse 5-$HT_{1C}$ receptor for G-proteins as shown by effects on adenylate cyclase and phospholipase C. *J Cell Biochem* Suppl 0 (16 Part E), 228, 1992.
55. Duzic E and Lanier SM, Factors determining the specificity of signal transduction by guanine nucleotide-binding protein-coupled receptors. *J Biol Chem* **267**: 24045–24052, 1992.
56. Meller E, Puza T, Diamond J, Lieu H-D and Bohmaker K, Comparative effects of receptor inactivation, 17$\beta$-estradiol and pertussis toxin on dopaminergic inhibition of prolactin-secretion *in vitro*. *J. Pharmacol Exp Ther* **263**: 462–469, 1992.

57. Vallar L, Muca C, Magni M, Albert P, Bunzow J, Meldolesi J and Civelli O, Differential coupling of dopaminergic $D_2$ receptors expressed in different cell types. *J Biol Chem* **265**: 10320–10326, 1990.

58. Taylor CW, The role of G proteins in transmembrane signalling. *Biochem J* **272**: 1–13, 1990.