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The Classification of Seven Transmembrane Receptors in Recombinant Expression Systems

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'... our complicated experiments have no longer anything to do with nature in her own right, but with nature changed and transformed by our own cognitive activity.' Werner Heisenberg (1901–1976)

I. Introduction

The major premise of this review is that seven transmembrane receptors (7 transmembrane (TM) receptors, also referred to in literature as G-protein coupled receptors) are specialized proteins designed for chemical *recognition* of ligands and the subsequent *transduction* of the information encoded in those ligands to the machinery of the cell (Kenakin et al., 1992). The superfamily of 7TM domain G-protein coupled receptors interact with alkaloids, biogenic amines, peptides, glycoprotein hormones, light and odorants. They are unsurpassed as therapeutic targets and probably will continue to be so

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in the future. For example, it can be estimated that if only 5% of the human genome codes for 7TM receptors, then there is complementary deoxyribonucleic acid (cDNA) available for 5000 receptors. Less than 10% of these are known leaving a fertile area for drug development. To this may be added also the potential targets of 7TM receptor mutations in disease states.

To achieve information transfer, the ability to bind ligands to a recognition domain and allosterically transmit the presence of that ligand to an intracellular domain appears to be a specialized feature of 7TM receptors. The very properties that define receptors as such also impart unique protein behaviors to receptors, and these behaviors, in turn, affect drug activity.

Heterologously expressed receptors and combinatorial libraries of molecules represent the new technology of drug discovery (Luyten et al., 1991; Luyten and Leysen, 1993; Baum, 1994). Until the advent of molecular biology, the behavior of receptors was limited by the constrained stoichiometries of natural systems. However, the ability to insert receptors into foreign surrogate cells at various expression levels has led to an explosion of information regarding the activity of drugs and the behavior of receptors. This has led to better understanding of the effects of receptor-effector stoichiometry and the influence of the cellular host on receptors. The behavior of receptor proteins can be critical to what is observed as drug-induced activity. This review discusses the recognition of aberrant receptor behavior in foreign cells and the measurement of drug-receptor parameters that transcend this behavior.

II. Receptor Pharmacology in Drug Discovery

For most of the history of receptor pharmacology, the discovery of biologically active ligands has centered on the testing of chemicals on animal host systems containing teleologically 'similar' receptors. This similarity generally has stemmed from the fact that the endogenous chemicals to be recognized often are the same in both animals and humans (i.e., neurotransmitters such as norepinephrine, acetylcholine, etc.). It followed, therefore, that the recognition units for these chemicals (the receptors) could be similar enough to detect like activity that would transcend the gap between animal and human host systems. The science of pharmacology has been built upon this correspondence.

Recent advances in molecular biology have greatly reduced the need for reliance on animal receptor systems and allowed the critical testing of this approach. Thus, the steps toward total correspondence (i.e., the testing of drugs on the human receptor in the exactly correct tissue under the appropriate pathology) have been made with advances in molecular biology (see table 1). Currently, the state of the art mainly resides in systems where human receptor material (i.e., cDNA) coding for receptor is introduced into surrogate cells.

While animal receptor systems are available and can be considered 'physiological,' the obvious shortcomings of such systems are the fact that the receptors are still facsimiles of the human targets. Another problem with natural animal systems is related to their basic design. There is evidence to suggest that, perhaps as a response to the need to finely tune the control of cellular biochemistry, cells express mixtures of receptor subtypes in varying quantities to take advantage of endogenous agonist information. This results in the study of ligands on mixtures of very similar binding sites, leading to the obvious potential for misleading classification. The expression of human receptors in surrogate cell systems has eliminated these shortcomings, i.e., human receptors can be expressed in apparently (vide infra) pure populations in cells.

There is a good deal of circumstantial evidence available to suggest that receptors from animal sources are good templates for predicting drug activity on human receptors. However, there also is striking evidence that slight differences between human and animal receptors can have profound effects on drug activity. It is known that there are differences in affinity that result from relatively small sequence differences between human and animal receptors, as seen in the rat and human 5-hydroxytryptamine $(5\text{-HT})_{2A}$ receptor (Johnson et al., 1994). This is especially true for nonpeptide antagonists for peptide receptors where it appears that evolution has produced mutations that have not altered binding of

Abbreviations: TM, transmembrane; cDNA, complementary deoxyribonucleic acid; 5-HT, 5-hydroxytryptamine; mRNA, messenger ribonucleic acid; T, population of 7TM receptors that can exist in a so-called 'inactive' state T (following the convention for ion channels); R, population of 7TM receptors that can exist in a so-called 'active' state; L, allosteric constant (where L = [T]/[R]); A, a drug; M, ability of A to alter equilibrium; cAMP, cyclic adenosine monophosphate; CGRP, calcitonin gene related peptide; ASPET, American Society for Pharmacology and Experimental Therapeutics; PI, phosphoinositol; XAC, [^{3}]xanthine amine congener; PKC, protein kinase C; GTP, guanosine triphosphate; DADLE, [D-Ala^2, D-Leu^5]enkephalin; GnRH, gonadotrophin-releasing hormone; GDP, guanosine diphosphate; CCK, cholecystokinin; NECA, 5'-N-ethylcarboxamidoadenosine; m1AChR, m1 acetylcholine receptors; PACAP, pituitary adenylate cyclase-activating polypeptide; PTX, pertussis toxin; CTX, cholera toxin; SPAP, secreted human placental alkaline phosphatase.

TABLE 1
Pharmacological receptor testing systems

Animal receptors-animal tissues
↓
Animal genetic receptor material-animal surrogate cells
↓
Human genetic receptor material-animal surrogate cells
↓
Human genetic receptor material-human surrogate cells
↓
Human genetic receptor material-human target cells
↓
Human genetic receptor material-human target cells with appropriate pathology

natural peptides but do produce differences for foreign nonpeptide ligands (Jensen et al., 1994). Thus, two recently developed nonpeptide substance-P antagonists show marked differences in their affinity for human substance-P receptors as compared with the corresponding rat receptor (Fong et al., 1992b). In some cases, differences in affinity for ligands may result from very small differences in amino acid sequence as in the *single* amino acid difference between the human and rat 5-HT_{1B} receptor (Oksenberg et al., 1992; Metcalf et al., 1992). The presence of $threonine_{355}$ in the human 5-HT_{1B} receptor (as opposed to a corresponding asparagine in the rat receptor) accounts for a remarkably different pharmacology between the two receptors, despite a 95% amino acid sequence identity (Hamblin et al., 1992).

In general, it is not possible to prove that differences in amino acid sequences in receptors will *not* result in different pharmacology because the differences may be ligand-specific. For example a single point mutation in human 5-HT receptors (5-$HT_{1D\alpha}$, 5-HT_{1F}) increases the affinity for propranolol and pindolol by a factor of 100- to 1000-fold but leaves the affinity for 5-HT unchanged (Adham et al., 1994a).

There are specific cases in which receptors from animal sources would not be predictive to human disease. For example, polymorphic variations in human dopamine D_4 receptors, thought to be related to psychiatric disorders, result in receptors with variably sized third cytoplasmic loops. Because this region of the human receptor is not found in the rat homologue of the D_4 receptor, the rat receptor would not reflect ligand-specific effects in this human population (Van Tol et al., 1992). For these reasons, it is obvious that the testing of possible new drug entities on human receptors is preferred.

III. Translation, Expression, and Co- or Post-translational Modification

It must be assumed that the genetic material introduced into the surrogate cell can find its way to the appropriate locus, be translated correctly and the resulting product processed as in native systems. The correct transcription of the gene in the expression system may be critical to subsequent expression. For example, single site-directed mutagenesis has shown that prevention of a putative cysteine-cysteine disulfide bond in the γ-aminobutyric acid type A channel prevents the functional expression of that receptor subunit (Amin et al., 1994). Complete sequences must be expressed for correct receptor function. Truncates of receptors, when compared with full-length wild type receptor, have been shown to have lower affinity (Fong et al., 1992a), no differences in affinity (Rodriguez et al., 1992; Reneke et al., 1988) or increased affinity (Findlay et al., 1994). In general, there is considerable evidence that nonstandard translational events may affect the nature of expression products (Santos and Tuite, 1993).

Expression of multiunit receptors can be especially difficult because of the potential for incorrect assembly of subunits. The IR-A and IR-B isoforms of the insulin receptor are made by alternative splicing of exon 11 in the insulin receptor gene and are expressed in a tissue-specific manner (Moller et al., 1989; Mosthaf et al., 1990; Goldstein and Dudley, 1990). Although this is not a problem with 7TM receptors, there are cases in which alternative splicing of messenger ribonucleic acid (mRNA) from a gene results in receptor isoforms for dopamine receptors (Giros et al., 1989; Monsma et al., 1989; Dal Toso et al., 1989) and for rhodopsin (Tanabe et al., 1992; Fong et al., 1992b; Sugimoto et al., 1993). In chromaffin cells, alternative splicing of the mRNA from the single gene encoding for the prostaglandin EP_3 receptor yields four receptor isoforms that differ only in their C-terminal tails. These differences determine differences in G-protein coupling (Namba et al., 1993). Alternate splicing is responsible for the different isoforms of receptors such as the GHRH receptor (Mayo, 1992), dopamine D_{2A} and D_{2B} receptors (Dal Toso et al., 1989), and the pituitary adenylate cyclase-activating polypeptide (PACAP) receptor (Spengler et al., 1993). Although there are five cloned somatostatin receptor types, a further degree of diversification has been observed with the alternative splicing to produce mSSTR2A and mSSTR2B (i.e., Reisine et al., 1993). The two isoforms differ in the cytoplasmic C-terminus (Vanetti et al., 1992) and show different coupling efficiency to adenylate cyclase and propensity to desensitize (Vanetti et al., 1993b).

Posttranslational changes in dopamine D_2 receptors have been reported to account for differences in ligand affinity (Giros et al., 1989; Monsma et al., 1989). There are several biochemical modifications of receptor proteins that can be made including glycosylation, palmitoylation, terminal amino acid acylation, amino acid cyclization, carboxy-terminal amidation, sulfation (tyrosine residues), phosphorylation, hydroxylation, and methylation. Some of these are more important than others for various receptors. A key modification that may be important for receptor systems co-expressed with G-proteins (vide infra), is prenylation, both in terms of targeting to the membrane and the signaling from the receptor system (Casey, 1995). Receptor glycosylation can cause differences in the size of receptors. For example, a marked tissue difference in glycosylation has been noted for the angiotensin type 2 receptor in human myometrium, murine fibroblasts and rat PC-12 cells (Servant et al., 1994) and for opioid receptors in various tissues (Liu-Chen et al., 1993). Some modifications, such as palmitoylation, can be affected by external stimuli (Bonatti et al., 1989; Omary and Trowbridge, 1981; Alvarez et al., 1990). For 7TM receptors, palmitoylation may be particularly important because it reg-

ulates signal transduction both from receptors and the G-proteins with which they interact (Bouvier et al., 1995).

Clearly, a great deal of artifactual data can result from incomplete or incorrect expression of receptor protein from genetic material. This is an uncontrolled variable in receptor expression. For the purposes of this review, it will be assumed that the pharmacological effects observed are not the result of posttranslational modification differences, but, rather, that they reflect the behavior of natural receptors.

With the first cloning of a cDNA encoding a G-protein coupled receptor (retinal photon receptor and rhodopsin) (Nathans and Hogness, 1983) and the following description of the cDNA sequence for the β_2-adrenergic receptor (Dixon et al., 1986) has come a cornucopiea of expressed animal and human 7TM receptors. Table 2 shows a partial list of cloned 7TM receptors. This table is meant as a source of information regarding the behavior of different receptors in different expression systems. It should be noted that this table is not meant to be a complete listing of all cloned receptors, nor should it be used to ascribe temporal organization with respect to when the individual receptors were first expressed. Also, cloned genes are not included, only studies in which the gene is expressed in a cellular system and the binding and/or function of ligands on the expressed receptor is studied.

With this new technology has come the potential for new concerns in receptor pharmacology that stem from the unique nature of 7TM receptors as recognition and transduction units. These new concerns stem from the expectation that receptor activity will be immune to the removal of a receptor from its native environment and the expression of that receptor into a foreign one. Hopefully, this review will outline the limits for this expectation and some strategies for recognizing when observed effects reflect innate ligand receptor activity and when it might reflect activity modified by receptor environment. This involves the identification of system-dependent rather than solely receptor-type-dependent potency of ligands. Clearly, it would be advantageous to recognize the latter situation in terms of the subsequent expectation of drug activity in a therapeutic environment.

IV. Definitions

As a preface to the discussion, it is useful to define some terms to be used throughout this review. Drugs will be assumed to have two basic properties, *affinity* and *efficacy*. The first term relates to how well the drug binds to the receptor (as defined by the equilibrium dissociation constant of the drug-receptor complex). The second term relates to what happens to the receptor system as a result of the drug binding. The effects may promote physiological response, in which case the drug demonstrates *positive efficacy* and therefore is defined as an *agonist*. Conversely, the drug may do nothing to the receptor but bind to it and by its presence preclude activation of the receptor by an agonist. This would make it a *neutral antagonist* with *zero efficacy*. Recent data compels yet another scenario in which receptor systems produce measurable physiological response in the absence of agonist (vide infra). Such receptor systems are defined as being *constitutively active* and may be used to discover drugs that destabilize active receptor complexes. Such drugs are referred to as *inverse agonists* and have *negative efficacy*. It should be noted that in the absence of constitutive receptor activity, neutral antagonists and inverse agonists behave in an identical manner. However, it should not be assumed that they are pharmacologically the same because important differences in the receptor properties of neutral antagonists and inverse agonists may be very relevant to the therapeutic use of these drugs and the classification of drug receptors with them in heterologous expression systems. At this point, it is useful to suspend the common usage of the term efficacy as the property of a drug that promotes positive physiological response and consider efficacy to be the *property of a drug that modifies subsequent interaction of the 7TM receptor with other membrane proteins* (Kenakin, 1994).

V. 7TM Receptor Behavior

The behavior of 7TM receptors can be divided into two components namely, *intrinsic* and *interactive*. Intrinsic behavior refers to the basic properties of receptor proteins to exist in multiple conformational states and the effects of those states on observed drug activity. The interactive behavior relates to the result of receptor and G-protein interaction on the quality and quantity of drug response. It is worth discussing these separately.

A. Intrinsic Receptor Behavior

There is considerable circumstantial evidence to suggest that 7TM receptors can exist in 'active' and 'inactive' conformational states with respect to the fruitful interaction with G-proteins. It is useful at this point to consider the analogy with 'two-state' theory for ion channels (Katz and Thesleff, 1957) as applied to receptors (Colquhoun 1973; Karlin, 1967; Thron, 1973; Robertson et al., 1994). This hypothesis describes a population of receptors that can exist in a so-called 'inactive' state T (following the convention for ion channels) and an 'active' state R, the relative proportions of which are defined by an allosteric constant L (where L = [T]/[R]). A drug [A] binds to the two conformations T and R where the equilibrium dissociation constants of the resulting complexes are K_{AT} and K_{AR} respectively:

$$\begin{array}{ccc} & L & \\ T & \rightleftharpoons & R \\ K_{AT} \downarrow\uparrow & & \downarrow\uparrow K_{AR} \\ A\cdot T & & A\cdot R \end{array} \qquad [1]$$

TABLE 2
Cloned and expressed receptors

Receptor	Species	System	Reference(s)
Adenosine			
A_1	rat	A9-L + CHO	Mahan et al., 1991
	human	CHO	Libert et al., 1992
	canine	CHO	Libert et al., 1991
A_{2b}	rat	*Xenopus* oocytes	Yakel et al., 1993
	human	CHO-K1	Pierce et al., 1992
A_3	sheep	COS-1/CHO K1	Linden et al., 1993
A_3	human	CHO	Salvatore et al., 1993
Adrenergic			
α_1	hamster	COS-7	Cotecchia et al., 1988
α_1	bovine	COS-7	Schwinn et al., 1990
α_2	human	COS-7	Regan et al., 1988
		CHO	Fraser et al., 1989; Lomasney et al., 1990
	mouse	COS-7	Link et al., 1992; Chruscinski et al., 1992
		Xenopus oocytes	Kobilka et al., 1987
	fish	COS-7	Svensson et al., 1993
α_{2C}	mouse		Link et al., 1992
	rat	CHO/COS-7	Voigt et al., 1991a
α_{2A}	human	COS-7	Link et al., 1992
	human	COS 1	Lanier et al., 1991
α_{2B}, α_{2C}, α_{2D}	rat	NIH 3T3	Duzic et al., 1992
α_{2B}	rat	COS	Zeng et al., 1990
	human	Ltk cells	Weinshank et al., 1990
α_{2D}	rat	COS 1	Lanier et al., 1991
α_{2A}	human	*Xenopus* oocytes	Kobilka et al., 1987
	porcine	COS-M6	Guyer et al., 1990
α_{2B}	rat	COS	Zeng et al., 1990
α_{1D}	human	SK-N-MC	Esbenshade et al., 1995a
β_1	rat	L cells	Machida et al., 1990
	human	*Xenopus* oocytes	Frielle et al., 1987
	mouse	COS-7/L-cells	Cohen et al., 1993
		sf9	Ravet et al., 1993
β_1/β_2	human	CHO	Suzuki et al., 1991
β_2	mouse	Y-1	Allen et al., 1988
	human	*Escherichia coli*	Marullo et al., 1988
β_3	mouse	CHO	Nahmias et al., 1991
	rat	CHO	Granneman et al., 1991
	human	CHO cells	Emorine et al., 1989
Calcium	bovine	*Xenopus* oocytes	Racke et al., 1993
Dopamine			
D_1	human		
	rat	COS-7	Zhou et al., 1990
	human	COS-7	Sunahara et al., 1991a
	rhesus	C_6 cells	Machida et al., 1990
D_{1B}	rat	COS-7	Tiberi et al., 1991
D_2	human	COS-7	Stormann et al., 1990
	mouse	COS-7	Montmayeur et al., 1991
	rat	COS-7	Chio et al., 1990
		CHO-6, DUK 25	
	rat	mouse fibroblasts	Bunzow et al., 1988
D_2/D_3	human	CHO	Sokoloff et al., 1992
D_2/D_3	rat	LZR1, Ltk 59	Castro and Strange, 1993
D_3	rat	CHO	Sokoloff et al., 1990
D_4	human	COS-7	Van Tol et al., 1991
D_5	human	COS-7	Sunahara et al., 1991b
Histamine			
H_1	bovine	COS-7	Yamashita et al., 1991
H_2	rat	CHO	Traiffort et al., 1992
	canine	L-cells	Gantz et al., 1991
	human	Colo-320	Gantz et al., 1991

TABLE 2
Continued

Receptor	Species	System	Reference(s)
Muscarininc			
muscarinic	porcine	*Xenopus* oocytes	Kubo et al., 1986
			Akiba et al., 1988
muscarinic	porcine	CHO	Peralta et al., 1987
muscarinic	drosoph.	Y-1 cells	Shapiro et al., 1989
m1	mouse	Y-1, L-cells	Shapiro et al., 1988
	human	CHO-K1	Buckley et al., 1989
m1, m2	human	HEK	Peralta et al., 1987
m2	human	CHO-K1	Buckley et al., 1989
m3	human	CHO-K1	Buckley et al., 1989
		CHO	Tobin et al., 1992
m4	chicken	Y1/CHO	Tietje and Nathanson, 1991
	human	CHO-K1	Buckley et al., 1989
m5	human/rat	COS-7	Bonner et al., 1988
		CHO-K1	Buckley et al., 1989
Opioid			
κ	mouse	PC-12	Raynor et al., 1994a
		COS-1	Yasuda et al., 1993
	human	COS-1	Zhu et al., 1995
	rat	COS-7/*Xenopus* oocytes	Minami et al., 1993
		COS-7	Nishi et al., 1993
			Li et al., 1993
δ	mouse	CHO-DGH4	Raynor et al., 1994a
	human	CHO	Evans et al., 1992
		COS	Kieffer et al., 1992
μ	rat	COS-7	Raynor et al., 1994b
		COS-7	Chen et al., 1993
		COS-7	Bunzow et al., 1995
δ	human	COS-7	Knapp et al., 1994
	mouse	COS-1	Yasuda et al., 1993
μ	human	COS-7	Raynor et al., 1994b
Peptides			
Angiotensin type 2	human	COS-7	Tsuzuki et al., 1994
	mouse	COS-7	Nakajima et al., 1993
	rat	COS-7	Kambayashi et al., 1993
Bradykinin			
B_2	rat	*Xenopus* oocytes	McEachern et al., 1991
	human	COS-7	Hess et al., 1992
Calcitonin			
	human		Gorn et al., 1992; Moore et al., 1992
	rat		Sexton et al., 1993; Albrandt et al., 1993
	pig	COS	Lin et al., 1991
Cholecystokin			
A	human	COS	Ulrich et al., 1993
	rat	*Xenopus* oocytes	Wank et al., 1992
B	human	COS-7	Miyake et al., 1994
			Lee et al., 1993
Choriogonadotropin	porcine	COS-7	Loosfelt et al., 1989
Corticotropin releasing factor	rat	COS-7	Chang et al., 1993
$Endothelin_B$	human	COS-7	Webb et al., 1995
Gastrin	canine	COS-7	Kopin et al., 1992
Glucagon	rat	BHK	Jelinek et al., 1993
Gonadotropin releasing hormone	human	COS-7	Kakar et al., 1992
Growth hormone releasing hormone	rat	HEK 293	Mayo, 1992
Lutropin/luteinizing hormone	rat	HEK 292	McFarland et al., 1989
	mouse	L cells	Gudermann et al., 1993a
Neuropeptide Y	rat	293	Krause et al., 1992
$Neurotensin_A$	human	Baculovirus	Aharony et al., 1993
Parathyroid hormone	opossum	COS-7	Juppner et al., 1991
	rat	COS	Abou-Samra et al., 1992
Secretin	rat	COS	Ishihara et al., 1992
	human	HEK 293	Patel et al., 1995

TABLE 2
Continued

Receptor	Species	System	Reference(s)
Somatostatin	mouse/human	CHO	Rens-Domiano et al., 1992
R1	rat	COS-7	Li et al., 1992b
	human	CHO	Yamada et al., 1992a
R2	mouse	CHO	Yamada et al., 1992a
R3	rat	COS	Meyerhof et al., 1992
	human	COS-1	Yamada et al., 1992b
	mouse	CHO	Yasuda et al., 1992
R4	human	COS-7	Demchysyn et al., 1993
	human	COS-1	Rohrer et al., 1993
	mouse	COS-1DM	Bruno et al., 1992
R4, R5	human	CHO-K1/COS-1	Raynor et al., 1993
R5	human	CHO-K1	O'Carroll et al., 1994
	human	COS-7	Panetta et al., 1994
Substance P	murine	*Xenopus* oocytes	Sundelin et al., 1992
	rat	COS	Yokota et al., 1989
	human	COS-7	Takeda et al., 1991
TSH	human	COS-7	Misrahi et al., 1990
Thyrotropin	canine	COS	Parmentier et al., 1989
	rat	CHO-K1	Endo et al., 1995
VIP	rat	COP	Ishihara et al., 1992
	human	COS-6	Sreedham et al., 1991
5-HT			
1	rat	HEK 293	Voigt et al., 1991b
1A	rat	Ltk^-	Albert et al., 1990
	human	monkey kidney	Fargin et al., 1988
		NIH 3T3	Varrault et al., 1992
1B	rat	Y-1	Adham et al., 1993
	human	sf9	Ng et al., 1993
		HeLa	Hamblin et al., 1992
	mouse	NIH 3T3	Marotoeaux et al., 1992
1C	human	*Xenopus* oocytes	Julius et al., 1988
	mouse	*Xenopus* oocytes	Yu et al., 1991
1D	canine	COS-7	Maenhaut et al., 1991
	human	CHO-K1	Hamblin and Metcalf, 1991
1E	human	murine L cells	Guderman et al., 1993b
2	rat	(mammalian)	Pritchett et al., 1988
2B	human	AV12-664	Kursar et al., 1994
3	mouse	COS-1/*Xenopus* oocytes	Maricq et al., 1991
5A, 5B	mouse	COS-7	Matthes et al., 1993
5A	human	Cos M6	Rees et al., 1994
(S12)	human	Ltk	Levy et al., 1992
7	rat	COS-7, HEK 293	Shen et al., 1993
GP2-7	guinea p	CHO-K1	Tsou et al., 1994

The important thing to note from this scheme is that unless A has *identical* affinities for T and R, the presence of A will *alter* the relative proportions of T and R, i.e., drug A plays an active role in the equilibrium and is not a mere observer. Under these circumstances, the fraction of receptors in the activated form in the presence of any given concentration of ligand (normalized to $c = [A]/K_{AR}$) is given by:

$$\rho = \frac{1}{1 + L[(1 + Mc)/(1 + c)]} \qquad [2]$$

where M is the ratio of equilibrium dissociation constants of A for the two receptor states ($M = K_{AR}/K_{AT}$). Thus, a measure of the ability of A to alter the equilibrium is denoted by M, and a correlate to drug efficacy in this type of system can be given by (Colquhoun, 1973);

$$\epsilon = \frac{K_{AT}}{K_{AR}} - 1 = \frac{1}{M} - 1 \qquad [3]$$

There are two features of this type of system to note. The first relates to the drug constant M. There is no a priori reason to assume that a ligand will promote only receptor activation; in fact, it is equally possible that a ligand will destabilize activated receptor formation (i.e., have a selectively higher affinity for the inactivated receptor T). In a system where there are few activated receptors in the absence of ligand, drugs with selective affinities for the inactivated state will have little effect,

i.e., it is not possible to turn off a system that already is turned off. However, if there is appreciable R present because of a favorable L, then a ligand with selective affinity for T over R will *decrease* spontaneous receptor activity. Such a destabilizing property of the ligand would not be detectable in the absence of constitutive receptor activity.

The maximal receptor activation of receptors by a saturating concentration of ligand is given by:

$$\rho_{max} = \frac{1}{1 + L \cdot M} \quad [4]$$

and it can be seen from equation 4 that it is the product of L and M that determines the observed drug effect. Therefore, for a destabilizing ligand (also called an inverse agonist), (vide infra) where $M > 1$, no appreciable effect will be observed if the magnitude of the allosteric constant L is very small (i.e., $LM \rightarrow 0$).

The second idea relates to the allosteric constant L. The fraction of receptors in the activated state in the absence of ligand A is given by:

$$\rho = \frac{1}{1 + L} \quad [5]$$

Theoretically, it is possible to have a system where *all* of the receptors are in the active state (to produce a 'constitutively' fully activated receptor system). Under these circumstances, an agonist would produce no measurable response, as the system will already reside at the maximal asymptote for response. Also, a system in which the constant L is large, even an extremely efficacious ligand with powerfully selective affinity for only the activated state R, would not be able to effectively change the relative quantities of T and R. Thus, the *system* could impose the dominant regulation on drug activity.

The intrinsic behavior of 7TM receptors is germinal to the overall behavior of these receptors in expression systems. However, another property is equally important and sets these receptors apart from ion channels, namely the ability to translocate within the membrane and interact with other membrane bound proteins.

B. Interactive Behavior: Cellular Host Effects

There are tissue-specific effects on receptors that modify observed drug activity. These effects can be quite striking, as in the case of 5-HT receptor-mediated responses in brain regions. Whereas the agonist activities of serotonin and a range of other agonists is comparable in mouse hippocampus and cortical neurons, methysergide and metergoline are nearly full agonists in hippocampal neurons and complete antagonists in cortical neurons (Dumius et al., 1988).

Just as different regions of organs such as the brain may have different cellular hosts for 7TM receptors, the transfection of these receptors into different expression systems may cause differences in receptor behavior. Surrogate cell lines for the expression of receptors generally are chosen for technical reasons, i.e., robust expression levels, etc. There is a considerable body of evidence to show that many 7TM receptors appear to function normally when introduced into these systems. Antagonist profiles can be remarkably similar for a given cloned receptor in different cell lines. However, such positive evidence is less revealing than negative evidence, i.e., it never can be proven that a receptor behaves in a physiologically normal fashion in a surrogate cell line, only when it does not. Thus, the weight of confirming evidence may only belie the fact that drugs that would show the differences have not yet been tested.

Cellular hosts can have long-term effects on 7TM receptors. For example, while the human β_3-adrenergic receptor desensitizes to isoproterenol in SK-N-MC cells and 293 cells, it does not do so in Chinese hamster ovary (CHO) cells. Similarly, the rat β_3-adrenergic receptors that does not desensitize in rat adipocytes does so in 293 cells (Chaudry and Granneman, 1994). Similar differences for β_3-adrenergic receptor desensitization have been found in L and Chinese hamster fibroblasts cells (Nantel et al., 1995).

The most obvious cases of deficient host effects are for agonists because this activity requires the coupling of the activated receptor to a G-protein. If the appropriate G-protein is not present in the surrogate cell, or the stoichiometry of the receptors and G-proteins is aberrant, then different activity may result. The effects of this phenomenon on high affinity agonist binding will be dealt with separately. As well as host cell deficiencies, receptors may be expressed in cells containing components they do not normally encounter in native tissues, or that they do encounter but with different stoichiometries. A striking case of functional reversal was demonstrated by Duzic and Lanier (1992) who transfected the α_{2B}-adrenergic receptor into three cell lines. In DDT$_1$-MF2 and NIH 3T3 cells, the α_{2B}-adrenergic receptor agonist epinephrine produces a concentration-dependent inhibition of cyclic adenosine monophosphate (cAMP). Surprisingly, an *increase* in cAMP is mediated by this agonist in PC-12 cells (fig. 1).

Cellular hosts can affect parameters fundamentally thought to reflect receptor properties. For example, the potency ratios for porcine and human calcitonin, human calcitonin gene related peptide (CGRP), and rat amylin can be shown to vary dramatically for activation of porcine calcitonin receptors transfected in either CHO or COS cells (Christmanson et al., 1994). Whereas absolute potencies may vary between systems, relative potencies should not vary unless different G-proteins are activated selectively by the activated receptors in the two hosts. This effect of 'stimulus trafficking' by agonists is discussed more fully later.

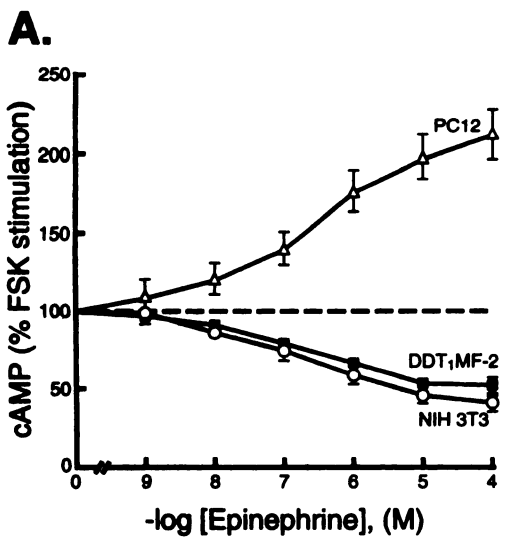


FIG. 1. (A) Effects of epinephrine on α_{2B}-adrenergic receptors transfected into three different cell lines. Ordinates: levels of cytosolic cAMP. Abscissae: Logarithms of molar concentrations of epinephrine. With permission from the American Society for Pharmacology and Experimental Therapeutics (ASPET) and from Duzic and Lanier (1992).

TABLE 3
Coupling of the same receptor to different cytosolic cascades in different systems

Receptor	Systems	Reference(s)
Dopamine D_2	Rat striatum Rat anterior pituitary Pituitary GH_4C_1 cells Ltk$^-$ fibroblasts	Meller et al., 1992 Vallar et al., 1990
α_{2B}-Adrenergic	NIH 3T3 cells PC12 cells DDT_1 MF-2 cells	Duciz and Lanier, 1992
Serotonin 5-HT_{1C}	Natural systems Syrian hamster tumor cells	Lucaites et al., 1992
α_{1B}-Adrenergic α_{1D}-Adrenergic	COS-1 cells, CHO cells	Perez et al., 1993
Muscarinic m2	Y-1, CHO	Tietje and Nathanson, 1991

Different host cells may present receptors with different arrays of G-proteins. For example, expression of α_{1B}- and α_{1D}-adrenergic receptors in COS-1 and CHO cells leads to two different couplings. In COS-1 cells, coupling is to a pertussis toxin-insensitive G-protein that leads to phosphosinositol (PI) hydrolysis and increased cAMP and a pertussis-insensitive G-protein to an L-type calcium channel to stimulate phospholipase A. In CHO cells, the coupling is via a pertussis-insensitive G-protein to increase PI hydrolysis (Perez et al., 1993). Another effect of the host cell system on responses of transfected receptors was reported for the chicken muscarinic m2 receptor which, when expressed in Y-1 cells, inhibited adenylate cyclase, but when expressed in CHO cells, inhibited adenylate cyclase and stimulated phosphoinositide metabolism (Tietje and Nathanson, 1991). A striking effect was obtained in cells transfected with high levels of 5-$HT_{1D\alpha}$ and 5-$HT_{1D\beta}$ receptors when antagonists such as yohimbine and dihydroergotamine produced agonist effects (Adham et al., 1993). Table 3 shows other examples of receptors in various cellular hosts that demonstrate differences in effector coupling.

In addition to effects on receptor/G-protein coupling, there are other tiers of interaction in cellular signaling. For example, there is evidence that 7TM receptors can interact with each other, as in the case of the abolition of natural 5-HT_1-like receptor effects in CHO cells by transfection and activation of 5-HT_{2C} receptors (Berg et al., 1994).

In general, it can be said that the automatic assumption of immutable receptor behavior irrespective of cellular host is not supported by data. The proclivity of receptors to interact with many G-proteins spontaneously (vide infra) raises the specter of the introduction of an uncontrolled variable in expression studies that may transfer to the observed activity of drugs.

C. Evidence for Spontaneous Receptor/G-Protein Coupling

One of the major characteristics of 7TM receptors is the fact that they have different recognition domains for ligands and G-proteins. This latter property confers the ability of the receptor, when in the active state, to couple to and activate G-proteins. Sequence similarity dendograms have shown that 7TM receptor evolution can be traced at two sites, namely the ligand *and* G-protein binding sites (Donnelly et al., 1994). There is now a large body of evidence to show that many receptors can spontaneously couple to G-proteins in the absence of agonists. For example, solubilized CGRP receptors from rat cerebellum were shown to bind ^{125}I-CGRP with high affinity. Treatment of the solubilized receptor supernatant with $G_{\alpha s}$ antiserum caused immunoprecipitation of the G_s with a concomitant loss in receptor binding upon centrifugation (Chatterjee et al., 1993). The most straightforward explanation for these data is that the loss in receptor binding represented G_s protein-receptor complexes that were present in the supernatant spontaneously in the absence of CGRP.

There are numerous systems in which receptors can be purified as complexes with G-proteins. For example, solubilized D_2 receptors from bovine striatum copurify with G_i and G_o (Elazar et al., 1989). The D_2-dopamine receptor of the bovine anterior pituitary copurifies with affinity chromatography with a pertussis toxin-sensitive G-protein (Senogles et al., 1987).

Another line of evidence to show receptor precoupling comes from receptor kinetic studies. For example, the biphasic kinetics of N-formyl peptide receptor binding are amenable to explanation by the proposal that the receptor population exists as a mixture of precoupled

and uncoupled states (Fay et al., 1991; Posner et al., 1994). Similar data have been obtained for the α_2-adrenergic receptor (Neubig et al., 1988).

In general, there are a great deal of data to suggest that most 7TM receptors spontaneously couple to G-proteins in the absence of agonist. Table 4 gives some examples of these systems. The assumption that the spontaneous association of receptor with G-protein involves the receptor in the 'activated' form is supported by evidence that shows spontaneous receptor/G-protein coupling is associated with the production of physiological response, i.e., elevation of cAMP (Samama et al., 1993; phosphoinositide turnover; Senogles et al., 1990) and guanine nucleotide G-protein exchange (Costa and Herz, 1989; Freissmuth et al., 1991). For example, figure 2 shows the activation of $G_{o,i}$-protein by adenosine A_1 receptors. This activity can be increased by the agonist N^6-(phenylisopropyl)-adenosine and reduced by the inverse agonist xanthine amine congener (XAC). Of relevance to this discussion is the fact that there is measurable spontaneous activation of the G-protein in the absence of agonists.

D. Receptor / G-Protein Promiscuity

It is now well known that many 7TM receptors are able to activate multiple biochemical cascades. This also can be shown in receptor expression systems: table 5 shows examples of multiple signaling from single receptors when they are expressed in surrogate cell lines. Multiple signaling can be the result of multiple receptor coupling at the membrane level or it can be the result of the activation of multiple internal biochemical cascades. For example, bradykinin receptors in cultured rat mesangial cells depress cAMP through a phospholipase C pathway (i.e., production of diacyglycerol from stimula-

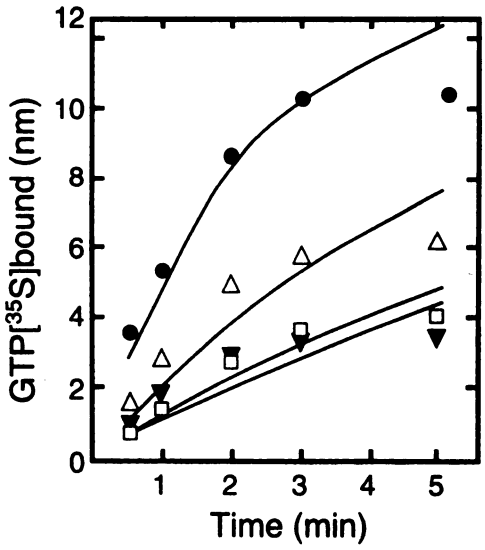


FIG. 2. Time course of GTP[^{35}S] binding to bovine brain $G_{o,i}$. Spontaneous G-protein activity in buffer alone (□), with adenosine A_1-receptor (△), adenosine receptor agonist R(−)-N^6-(2-phenylisopropyl)adenosine (●) and inverse agonist [^{3}H]xanthine amine congener (inverted filled triangles). With permission from the Biochemical Society and Portland Press and from Freissmuth et al., 1991.

TABLE 4
Evidence receptor precoupling to G-proteins

Receptor	System	Reference(s)
Opioid	NG108-15 cells	Costa et al., 1990
	Rat brain	Demoliou-Mason and Barnard, 1986
		Wong et al., 1989
		Georgoussi et al., 1995
		Li et al., 1992a
Dopamine D_1	Canine/bovine striatum	Niznik et al., 1986
Dopamine D_2	Bovine anterior pituitary	Wreggett and De Lean, 1984
		Senogles et al., 1987
		Senogles et al., 1990
	Reconstitution	Senogles et al., 1990
CGRP	Rat cerebellum	Chatterjee et al., 1993
Somatostatin	Rat brain	Law et al., 1991
Purinergic P_{2Y}	Turkey eryth.	Jeffs et al., 1991
Muscarinic	Rat cerebral cortex	Baron et al., 1985
		Matesic et al., 1989
β-Adrenergic	Cardiac membrane	Nerme et al., 1986
α_2-Adrenergic	Calf cerebral cortex	Sladeczek et al., 1984
	Rat brain	Matsui et al., 1985
	Human platelets	Neubig et al., 1988
	bovine aorta	Jagadeesh et al., 1990
Adenosine A_1	Bovine cerebral cortex	Leung and Green, 1989
Vasopressin		Fitzgerald et al., 1986

TABLE 5
Receptors coupled to multiple cytosolic cascades in cells

Receptor	System	Reference(s)
Muscarinic m3	IMR-32 cells	Pinkas-Kramarski et al., 1990
Muscarinic m1	CHO cells	Gurwitz et al., 1994
Muscarinic m2	CHO cells	Tietje and Nathanson, 1991
α_2-adrenergic	CHO cells	Fraser et al., 1989
		Eason et al., 1992
	CHO-K1 cells	Gerhardt and Neubig, 1991
PTH	COS cells	Abou-Samra et al., 1992
α_{1B}-Adrenergic	COS-1 cells	Perez et al., 1993
α_{1D}-Adrenergic		
Human 5-HT_{1D}	COS cells	Van Sande et al., 1993
Dog 5-HT_{1D}	Y1 Kin-8 cells	Van Sande et al., 1993
Human bradykinin B_2	CHO cells	Hess et al., 1994
Human calcitonin	BHK cells	Moore et al., 1992
Luteinizing H	Xenopus oocytes	Gudermann et al., 1993a
Rat endothelin A	Cardiac myocytes	Hilal-Danda et al., 1994
Human secretin	HEK-293 cells	Patel et al., 1995

tion of protein kinase C (PKC) inhibits stimulated cAMP production; Bascands et al., 1993). However, there are mechanisms whereby a single receptor can activate more than one biochemical pathway at the membrane level. For example, a single G-protein interaction can activate more than one biochemical cascade as in the activation of adenylate cyclase and phospholipase C by 5-HT_{1A} receptors (Fargin et al., 1991). Similarly, it has been shown that heterotrimeric G-proteins containing $G_{\alpha i3}$ can regulate multiple effector enzymes in the same cell (Hunt et al., 1994). As well as effector activation by α-subunits of G-proteins, it is now clear that the $\beta\gamma$ counterparts can directly activate effectors (Logothetis et al., 1987; Jelsma and Axelrod, 1987; Whiteway et al., 1989; Birnbaumer, 1992; Tang and Gilman, 1992; Camps et al., 1992; Katz et al., 1992; Blank et al., 1992; Iniguez-Lluhu et al., 1993; Boyer et al., 1994; Muller and Lohse, 1995). These effectors include adenylate cyclase, phospholipase A_2, K^+ channels, phospholipase C, calcium channels and receptor kinases (Clapham and Neer, 1993). This adds another level of complexity into 7TM receptor/G-protein signaling because the presence or absence of counterpart effectors for $\beta\gamma$-dimers of receptor linked G-proteins will affect the type and magnitude of agonist response. For example, a natural cellular system for a given receptor may contain a dedicated G-protein which, upon agonist-receptor activation, yields an α-subunit that interacts with one effector and a $\beta\gamma$ dimer that activates another. The summation of the effector cascades produce the cellular response. If this receptor is transfected into another cell type that has the appropriate G-protein, it still will not produce the same response unless *both* effectors for the α and $\beta\gamma$-dimer subunits are present in the membrane as well.

While receptor signaling can yield pleiotropic responses in the cytosol, there also is considerable evidence to show that receptors demonstrate G-protein specificity. For example, adenosine A_1 and dopamine D_2 receptors transfected into HEK 293 cells activate G_i (Wong et al., 1992) but do not interact with α_q to activate PLC, even when this subunit is overexpressed (Conklin et al., 1993). Structure-activity selectivity was shown in this latter study when a three-amino-acid substitution switched receptor selectivity of $G_{\alpha q}$ to that of $G_{\alpha i}$ (Conklin et al., 1993). Furthermore, although the adenosine A_1 receptor and dopamine D_2 receptors are indistinguishable in activation of G_i, they did discriminate chimeras of α_q to α_i (Conklin et al., 1993). Similarly, 5-HT_{1A} receptors expressed in *Escherichia coli* form high affinity agonist complexes with several G-protein α-subunits but neglect to do so with others (See fig. 3A).

It is well known from recombinant and natural systems that there is cross-reactivity of receptors between many different G-proteins. For example, Haga and coworkers (1989) have shown that the muscarinic receptor forms high affinity complexes with acetylcholine equally well when reconstituted with G_o, G_i and G_n. It has been found that cloned human 5-HT_{1D} receptors expressed in CHO cells and that dog 5-HT_{1D} receptors expressed in Y1 Kin-8 cells can both stimulate and inhibit adenylate cyclase by concomitantly interacting with G_s and G_i proteins (Van Sande et al., 1993). The α_{2B}-adrenergic receptor expressed in S115 mouse mammary tumor cells inhibits adenylate cyclase via G_i *and* increases cAMP upon treatment of the cells with pertussis toxin, presumably via G_s (Jansson et al., 1994). Kinetic studies have been used to delineate receptor/G-protein promiscuity. For example, the interaction of the thyrotropin-releasing hormone receptor with $G_{q/11}$ and another unidentified G-protein was inferred from the observation of biphasic kinetics of [^{35}S]GTPγS binding (Brady et al., 1994). Some other examples of this promiscuity at the biochemical level are given in table 6A. This promiscuity carries over to transfected receptors in cellular expression systems (table 6B).

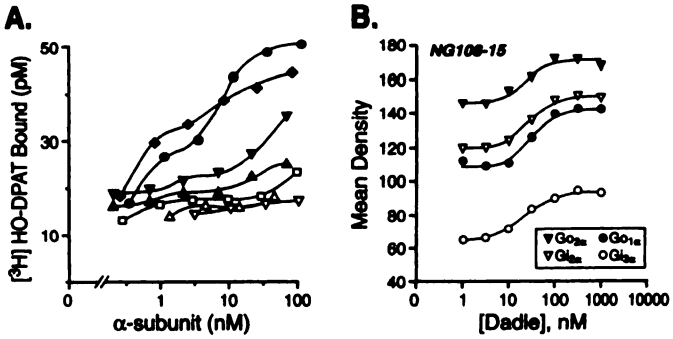


FIG. 3. Selectivity and promiscuity between receptors and G-proteins. (A) Interactions of 5-HT_1 receptors in *E. coli* membranes with various G-proteins. Formation of high affinity binding for [^{3}H]DPAT (ordinates) versus molar concentration of G-protein α-subunit (logarithmic scale). Data for $G_{\alpha i3}\beta\gamma$ (filled diamonds), bovine brain $G_{o,i}$ (filled circles), rat $G_{\alpha i2}\beta\gamma$ (inverted filled triangles), rat $G_{\alpha i1}\beta\gamma$ (filled triangles), and a lack of ternary complex formation with rat $G_{\alpha o}\beta\gamma$ (open squares), rat $G_{\alpha s1}\beta\gamma$ (open triangles) and rat $G_{\alpha s3}\beta\gamma$ (open inverted triangles). With permission from the Journal of Biological Chemistry and from Bertin et al. (1992). (B) Radioactive labeling of complexes between opioid receptors, the opioid agonist DADLE and various G-protein α-subunits in NG108-15 cells. With permission from ASPET and from Prather et al. (1994).

There also are numerous examples of natural systems in which a single receptor activates more than one G-protein (see table 7). In NG108-15, neuroblastoma × glioma cells the opioid receptor agonist [D-Ala^2, D-Leu^5]enkephalin (DADLE) has been shown to form three complexes with G_o, G_{i2}, and G_{i3} (Roerig et al., 1992). Adenosine A_1 receptors from bovine brain have been shown to copurify with G_{i1}, G_{i2}, and G_o (Munshi et al., 1991). Similarly, the muscarinic receptor in cerebellar and cardiac ventricular membranes was shown to form complexes with both G_i and G_o when activated by the agonists carbachol, pilocarpine and McN A343 (Matesic et al., 1991). Solubilized D_2 receptors from bovine striatum copurify with G_i and G_o (Elazar et al., 1989) and, as shown by Senogles et al. (1990), purified D_2 receptors activate GTPase of G_{i1}, G_{i2}, and G_{i3}. In patch

TABLE 6

Receptor	Coupler	Reference
Known Cross-Reactivity Between Receptors and G-proteins		
Muscarinic	G_i, G_o	Florio and Sternweis, 1985
	G_i, G_o	Haga et al., 1986; Kurose et al., 1986
	G_i, G_o, G_n	Haga et al., 1988, 1989
	G_i, G_p	Ashkenazi et al., 1987
	G_i, G_s	Dittman et al., 1994
β-Adrenergic	G_i, G_s	Asano et al., 1984
	G_i, G_s	Marbach et al., 1988
	G_s, G_{i1}, G_{i3}	Rubinstein et al., 1991
α_2-Adrenergic	G_i, G_s	Cerione et al., 1986
	G_i, G_o	Kim and Neubig, 1987
	G_{i2}, G_{i3}	Milligan et al., 1991
	G_{i2}, G_{i3}	Gerhardt and Neubig, 1991
	G_i, G_s	Eason et al., 1994
	G_i, G_s	Fraser et al., 1989
Serotonin	G_i, G_k, G_p, G_s	Roth and Chuang, 1987
	$G_{\alpha i1}$, $G_{\alpha i2}$, $G_{\alpha i3}$	Raymond et al., 1993
γ-Aminobutyric acid B	G_i, G_o	Asano et al., 1985
Dopamine D_2	G_i, G_o	Ohara et al., 1988
		Kimura et al., 1995
	G_{i1}, G_{i2}, G_{i3}	Senogles et al., 1990
Opioid	G_o, G_{i2}	Offermanns et al., 1991
Adenosine	G_{i2}, G_{i3}, G_o	Roerig et al., 1992
	G_{i1}, G_{i2}, G_o	Munshi et al., 1991
Neuropeptide Y	G_s, G_{i1}, G_{i2}	Ewald et al., 1989
Bradykinin	G_s, G_{i1}, G_{i2}	Ewald et al., 1989
Somatostatin	$G_{\alpha i1}$, $G_{\alpha i3}$	Law et al., 1991
	$G_{\alpha i2}$, $G_{o\alpha}$	Luthin et al., 1993
Calcitonin	G_s, G_i, (G_p)	Chakraborthy et al., 1991
5-HT_{1A}	$G_{\alpha i2}$, $G_{\alpha i3}$	Gettys et al., 1994
Evidence of Receptor/G-Protein Promiscuity in Transfected Cellular Systems		
α_{2B}-Adrenergic	S115 cells	G_i (G_s)	Jansson et al., 1994
α_{2A}-Adrenergic	Rat 1 fibroblasts	G_{i2}, G_{i3}	Milligan et al., 1991
		G_{i2}, G_{o1}	Grassie and Milligan, 1995
α_{2A}-Adrenergic	CHO cells	G_i, G_s	Eason et al., 1994
	LLC-PK1-O	G_{i1}, G_{i2} G_{i3}, G_o	Okuma and Reisine, 1992
Muscarinic m4	HEK 293 cells	G_i, G_s	Dittman et al., 1994
5-HT_{1A}	CHO cells	$G_{\alpha i2}$ $G_{\alpha i3}$	Gettys et al., 1994
5-HT_{1E}	BS-C-1 cells	G_i, G_s	Adham et al., 1994b
5-HT_{1C}, TRH	*Xenopus*	G_o, G_q	Quick et al., 1994

TABLE 7

Possible 'naturally promiscuous' receptor systems

Tissue	Receptor	Reference(s)
Chick heart	Muscarinic	Agnarsson et al., 1988
		Brown and Brown, 1984
		Brown and Goldstein, 1986
		Tajima et al., 1987
Rat atrium	Muscarinic	Eglen et al., 1988
		Imai and Ohta, 1988
		Kenakin and Boselli, 1990a, b; 1991
Guinea pig atrium	Muscarinic	Eglen et al., 1988
		Imai and Ohta, 1988
Rat striatum	Muscarinic	Kelly et al., 1985
Rat medulla pons	Muscarinic	Birdsall et al., 1980
Neuroblastoma cells	Muscarinic	Bruni et al., 1985
7315c cells	Angiotensin	Crawford et al., 1992
Rat anterior pituitary	Angiotensin	Enjalbert et al., 1986
3T3 fibroblasts	Thrombin	Murayama and Ui, 1985
Hippocampus (rat/ guinea pig)	Serotonin	De Vivo and Maayani, 1986
Rat hepatocytes	Glucagon	Wakelam et al., 1986
Rat phrenic nerve hemi-diaphragm	Adenosine	Silinsky et al., 1989
CHP212	CCK	Barrett et al., 1989
NG 108-15 cells	δ Opioid	Offermanns et al., 1991
Rat brain	Opioid	Wong et al., 1989
Bovine striatum	Dopamine D_2	Elazar et al., 1989
Rat anterior pituitary	Dopamine D_2	Lledo et al., 1992
αT3-1 cells	Gonadotrophin-releasing hormone	Shah and Milligan, 1994
LLC-PK_1 cells	Endothelin	Ozaki et al., 1994
Rat myometrium	Endothelin	Khac et al., 1994
Rat brain	Somatostatin	Murray-Whelan and Schlegel, 1992

clamp experiments, antisera to $G_{\alpha o}$ and $G_{\alpha i3}$ respectively reduced potassium currents caused by D_2 receptor activation (Lledo et al., 1992). When these types of interactions do occur, the concentrations of agonist producing the multiple ternary complex species usually are very similar, as in the formation of $G_{\alpha o2}$, $G_{\alpha o1}$, $G_{\alpha i2}$, and $G_{\alpha i3}$ complexes with DADLE and opioid receptors in NG108-15 cells (see fig. 3B) (Prather et al., 1994).

Receptor/G-protein cross-reactivity also can be seen with studies of signal down-regulation: for example, in the immortalized gonadotroph cell line αT3-1 cells that express gonadotropin-releasing hormone (GnRH) receptor. Exposure to a GnRH receptor agonist results in substantial down-regulation of the α-subunits of G-proteins G_q and G_{11} (Shah and Milligan, 1994). These and other data in this study suggest that this receptor interacts functionally with both $G_{q\alpha}$ and $G_{11\alpha}$.

Although receptors can be promiscuous with respect to the G-proteins with which they interact, they can also be promiscuous with respect to cell cycle. For example, calcitonin receptors in LLC-PK1 cells interact with two G-proteins to activate the cAMP and PKC pathways via cholera toxin G_s and pertussis toxin sensitive G_i protein, respectively, to produce opposite biological responses (Chakraborthy et al., 1991). Interestingly, the primary activation of one pathway over the other was cell cycle-dependent (i.e., G2 versus S phase).

These data in general lead to a model for 7TM receptor systems that must contain interactive receptors, G-proteins and ligands. As a preface to discussion of a current model for such systems, it is useful to trace the history of receptor models in pharmacology.

VI. Receptor Models

The discussion of these ideas is considerably easier with comparison of experimental data with a receptor model. The first mathematical application of a receptor theory to data was made by Clark (1933, 1937), and invaluable modifications were made by Gaddum (1937, 1957) and Schild (1947a, b; 1949; 1957) among others (see Parascandola, 1986 for review). With the introduction of the concept of efficacy into drug-protein interaction (Ariens, 1954, 1964; Stephenson, 1956), the ideas relating to allosteric states of enzymes and ion channels (Monod et al., 1965; Koshland, 1960; Karlin, 1967; Katz and Thesleff, 1957; Thron, 1973; Colquhoun, 1973) and the idea that receptors can translocate within the membrane and interact with other membrane proteins (Cuatrecasas, 1974) have come the basic ternary complex model (DeLean et al., 1980). Subsequent receptor studies and the availability of new receptor test systems have caused the modification of this model into the extended ternary complex model (Leeb-Lundberg and Mathis, 1990; Samama et al., 1993). It should be recognized that there are numerous other models available to describe drug-receptor interaction (for review see MacKay, 1977; Kenakin, 1984).

Alternative models such as the operational model of receptors (Black and Leff, 1983) are not bound by mechanistic constraints and can be used to quantify drug activity in general terms. This is a particular advantage in functional analysis of drug-receptor interaction in which null methods are used to negate systems effects. Because this review is specifically concerned with 7TM receptors, the known biochemical mechanisms of these systems will be used for modeling purposes. A recently described statistically complete model of 7TM receptor/G-protein interaction, termed the cubic ternary complex model (Weiss et al., 1996a, b), will be used to describe ligand effects.

A. The Cubic Ternary Complex Model

In general, there are three classes of interaction in 7TM receptor systems; these are shown schematically in figure 4. Part I shows receptor activation as a equilibrium between the active (R_a) and inactive (R_i) receptor forms and their interaction with the ligand A. The affinity constant of the ligand for the inactive receptor is denoted K_A, and it is modified by a factor α that quantifies the difference in affinity the ligand has for the activated over the inactivated receptor. The allosteric constant describing the equilibrium between R_i and R_a is denoted K_{act}. The concept of microscopic reversibility

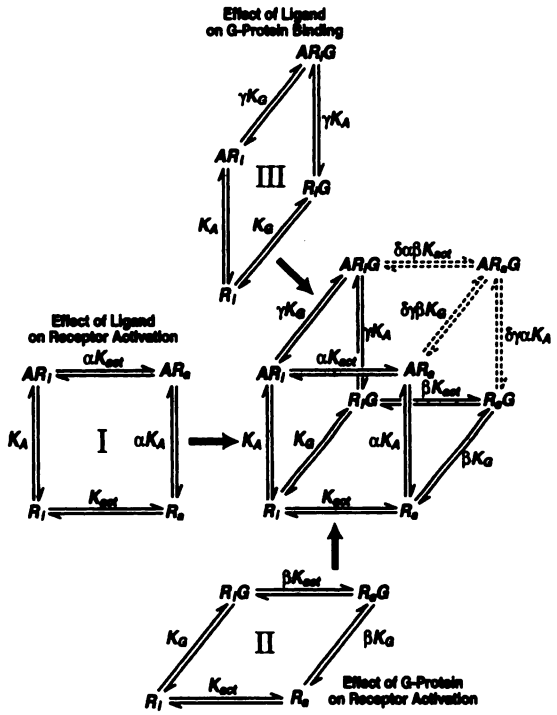


FIG. 4. Cubic ternary complex model for 7TM receptors. A. Three faces of the cube represent (I) the effect of ligand on receptor activation (A on R_i to R_a), (II) the interaction of receptors with G-protein (G on R_i and R_a) and (III) the added effect of ligand on receptor/G-protein interaction (A on R and G). (middle right) The completed cube with appropriate association constants mandatory by microreversibility.

(Wyman, 1975) sets the equilibrium association constants for the ligand bound receptor species to be αK_{act}.

The second class of interaction is the receptor behavior toward G- proteins (part II in fig. 4). Here it can be seen that, theoretically, both receptor forms can bind to G-protein; this is a deviation from the extended ternary complex model as described by Samama et al. (1993). Although there is no evidence that a stable complex between the inactivated receptor and G-proteins exists (species R_iG), all proteins have an unconditional association constant between them, albeit small; thermodynamically, a path must exist through this species for the system to be energetically correct. Thus, although K_G may be exceedingly small, the factor β can be large to favor coupling of the activated receptor over the inactivated receptor to G-protein. Again, microscopic reversibility sets the other equilibrium constant to βK_{act}.

Finally, the agonist effect on receptor/G-protein coupling is shown as part III in fig. 4. Here the presence of the agonist on the receptor produces a bias to receptor coupling by a factor γ. The full construction of the cube must interrelate these processes with the equilibrium constants shown in fig. 4 (described in detail in Weiss et al., 1996a, b). Clearly, the model is heuristic in that too many constants exist for useful modeling of data and ascription of chemical significance to ligand properties. However, the model is complete and is useful for describ-

ing and predicting receptor behaviors in different host systems. Moreover, the cubic ternary complex model subsumes many previous models of 7TM receptor systems; some of these are shown in table 8.

VII. Pharmacological Drug-Receptor Classification

As can be seen from the previous discussion, 7TM receptors can be thought of as societal proteins in membranes. They interact with other membrane proteins in promiscuous fashion and can carry on a signaling dialogue in the absence of agonists. When foreign ligands are thrust into this milieu, these systems are perturbed, and the manner in which the system adjusts to this perturbation yields measures of ligand-receptor activity that pharmacologists and medicinal chemists use to design drugs for therapeutic use. It is axiomatic that measures of drug activity must be independent of the systems from which they are obtained, and the usual methods to do this measure ligand *affinity* and ligand *efficacy* (Kenakin, 1984). These drug parameters should be unique for each receptor and thus transcend the measuring system to be predictive of activity in humans.

Theoretically, there are two approaches that can be taken to do this. One is the recreation of the physiological environment for the receptor of interest as the primary screening system for new drug entities. In view of the paucity of knowledge regarding the complete nature of these systems, this approach does not seem practical. A second approach, which strives to diminish the behavioral effects of receptors on drug activity and yield chemical constants of interaction, would appear to be more useful at present. The first step in this process is the recognition of when receptor behavior obscures and when it modifies observed drug activity.

The introduction of receptors into foreign host cells can produce artifacts with respect to the observed behavior of drugs. This can occur by mating the receptor with inappropriate membrane coupling proteins (heterologous match-making). Thus, the newly transfected receptor may be introduced to G-proteins not normally encountered. For example, the mouse 5-HT_{1C} receptor is known normally to couple to phospholipase C; however, when transfected into Syrian Hamster tumor cells, an unexpected inhibition of adenylate cyclase (blocked by pertussis toxin and thus related to receptor/G-protein interaction) was observed (Lucaites et al., 1992). Similar effects can be seen by varying the stoichiometry of 7TM receptors and G-proteins (vide infra). As a preface to the discussion of these issues, it is useful to consider the molecular nature of drug activity, namely the nature of affinity and efficacy. The two properties are native to molecules and therefore intimately related (i.e., see Colquhoun, 1987). The first step is to examine the conditions under which these properties can, if possible, be studied separately.

TABLE 8
7TM Receptor models

Model	Formulation	References
Classical	A + R ⇌ AR	Clark, 1933; 1937; Hill, 1909; Gaddum, 1937, 1957; Langley, 1878, 1909; Stephenson, 1956
Simple ternary complex	A + R ⇌ AR + G ⇅ ARG	Ross, 1989; Bourne et al., 1990; Birnbaumer et al., 1990; MacKay, 1988, 1990; Mayo et al., 1989
Ternary complex	R + A ⇌ AR + + G G ⇅ ⇅ RG + A ⇌ ARG	Wreggett and DeLean, 1984; DeLean et al., 1980; Costa et al., 1992; Ehlert, 1985; Cuatrecasas, 1974; Jacobs and Cuatrecasas, 1976; Abramson et al., 1987; Boeynaems and Dumont, 1977; Neubig et al., 1988; Minton and Sokolovsky, 1990; Lee et al., 1986
Simple two-state	R + A ⇌ AR ⇅ AR*	Karlin, 1967; Thron, 1973; Changeux et al., 1967; Katz and Thesleff, 1957; Kirschner and Stone, 1951; del Castillo and Katz, 1957
Full two-state	R + A ⇌ AR ⇅ ⇅ R* + A ⇌ AR*	Iyengar et al., 1980; Birnbaumer et al., 1980; Colquhoun, 1973; Karlin, 1967; Podleski and Changeux, 1970; Heidenreich et al., 1980; Ross et al., 1977
Extended ternary	R + A ⇌ AR ⇅ ⇅ R* + A ⇌ AR* + + G G R*G + A ⇌ AR*G	Leeb-Lundberg and Mathis, 1990; Samama et al., 1993; Lefkowitz et al., 1993

R*, activated receptor predisposed to G-protein coupling.

A. The Expectation of Zero Efficacy

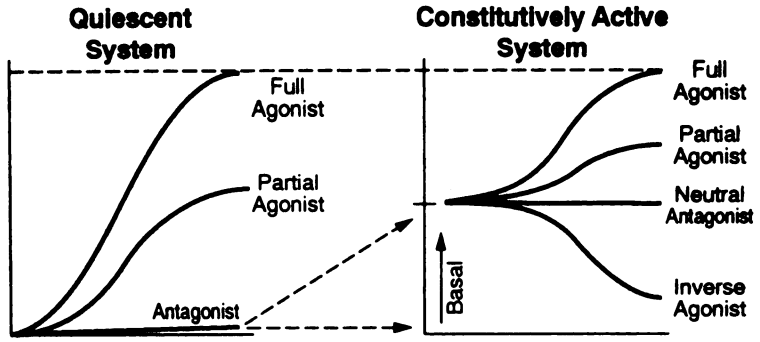
The efficacy of a ligand is usually observed as a change in the state of a receptor system in the presence of the ligand. By far, the predominance of experience with efficacious ligands has been in quiescent systems that demonstrate a ligand-induced physiological response. The first general idea to consider is the translation of ligand efficacy by the receptor system. Positive efficacy interacts with the intrinsic amplification stimulus- response mechanisms of the receptor system to yield an observable change of state. However, the sensitivity and power of these mechanisms can completely control what is observed. For example, the low β-adrenergic receptor efficacy of prenalterol can demonstrate full agonism in atria from thyroxine-treated guinea pig atria, partial

agonist activity in guinea pig left atria and *no* agonist activity in the extensor digitorum longus muscle of the guinea pig, where it acts as an antagonist (Kenakin and Beek, 1980; Kenakin, 1985a). Therefore, the lack of observation of an agonist response does not necessarily preclude the presence of ligand efficacy, only that the system was inadequate to make it observable (Kenakin, 1985a; Hoyer and Boddecke, 1993).

Considering efficacy as the property of a drug that, when it is bound to the receptor, modifies the interaction of that receptor with other membrane-bound proteins encompasses a larger potential than simply the production of cellular response. The cubic ternary complex model has a set of parameters that can be divided into those that are characteristic of the receptor system (K_G, K_{act}, β, [R], [G]) and those that are characteristic of the drug interacting with that system (K_A, α, γ, δ). If it is assumed that K_A is the chemical equilibrium dissociation constant of the complex between the inactive receptor and the ligand (i.e., a measure of true affinity), then the observed affinity of any ligand is given by (Weiss et al., 1996a):

$$K_{obs} = K_A \frac{1 + \alpha K_{act} + \gamma K_G[G] + \delta\alpha\gamma\beta K_G K_{act}[G]}{1 + K_{act} + K_G[G] + \beta K_G K_{act}[G]} \quad [6]$$

What can be seen from this equation is that, for K_{obs} to be equal to K_A, i.e., for simple affinity to be measured, then the condition that $\alpha = \gamma = \delta = 1$ must be true. If a drug has positive or negative efficacy (i.e., if either α, γ or δ are not equal to unity), then the observed affinity may be subject to systems conditions such as receptor/G-protein stoichiometry or level of spontaneous receptor activation. Figure 5 shows the observed affinity for a positive agonist (panel A), neutral antagonist (panel B), or inverse agonist (panel C) with changing level of receptor activation (K_{act}) and/or receptor expression level (G-protein level constant). As can be seen from this figure, the observed affinities of positive or negative agonists can vary with the system (i.e., cell type, receptor expression level). In general, positive agonism can increase the observed affinity of the ligand because the isomerization of the receptor to the active form (Colquhoun, 1985, 1987) and subsequent coupling to the G-protein creates a series of reactions that drives the binding of the agonist to the receptor beyond what would be dictated by the K_A (MacKay, 1987; 1988; 1990a, b; Leff and Harper, 1989; Kenakin et al., 1990). In contrast, the reverse is seen with inverse agonists. Because the higher affinity form of the receptor is the uncoupled



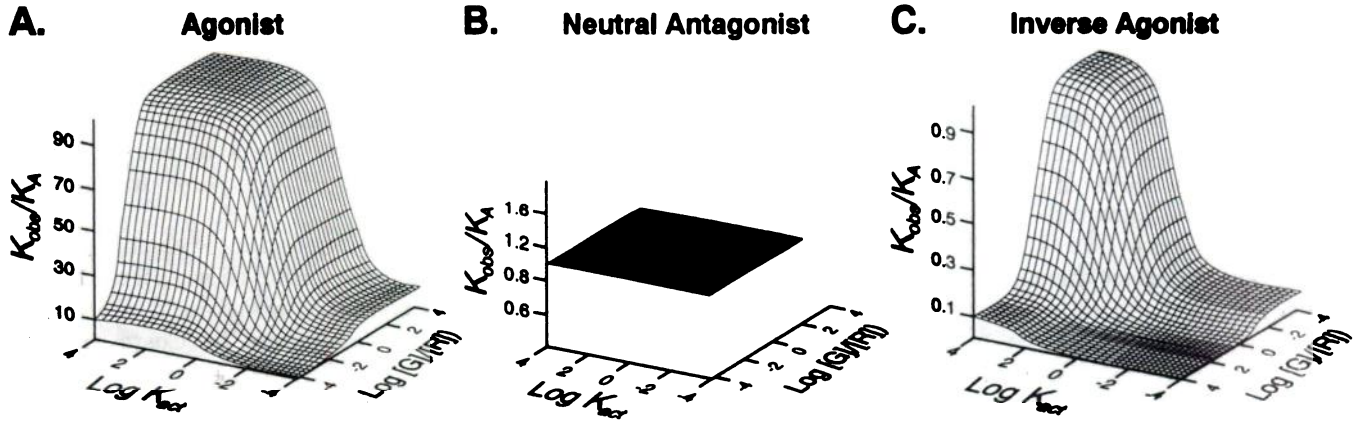


FIG. 5. Observed affinity of various types of ligands for 7TM receptors. K_{obs}/K_A calculated from equation 6 as a function of varying the ratio of receptors to G-proteins and varying setpoint levels of receptor activation (log K_{act}). Note how the systems parameters (K_{act} and [R]) have no effect on neutral antagonists (panel B) but increase the observed affinity for positive agonists (panel A) and decrease the observed affinity for inverse agonists (panel C).

free receptor, systems in which the receptor is precoupled to G-protein will demonstrate a lower observed affinity for the ligand. When uncoupling is complete, the observed affinity will equal K_A.

It can be seen from these simulations that the observed affinity of agonists and inverse agonists in different expression systems can be likened to traveling on a curvilinear surface and that the magnitude of the affinity is dependent on systems effects such as receptor level, availability of G-protein and/or the level of spontaneous receptor activation (i.e., ionic effects vide infra).

Neutral antagonists are special entities. For a drug to qualify as such means that it must in no way modify the relative proportions of active and inactive receptor (no effect on receptor activation). This condition ($\alpha = 1$) requires that the ligand recognize no difference between the active and inactive conformations of the receptor, i.e., that it see both conformations identically. Secondly, the presence of the ligand must in no way alter the interaction of the receptor with G-protein (i.e., $\gamma = \delta = 1$); therefore, the ligand-bound receptor must adopt a conformation formally identical to the unbound receptor with respect to the binding of the G- protein. The ligand must also block the effects of an agonist. Only under these circumstances would the ligand qualify as a true neutral antagonist and would its affinity not be subject to systems effects (i.e., host cell type, receptor/G-protein stoichiometry, relative levels of receptor activation).

Neutral antagonists are of great value in receptor classification because they can be relied upon to chemically classify receptors in any host cell. It might also be supposed that true neutral antagonists might be less prevalent than previously thought and that the only reason that they appear to be so common in the literature is the fact that the existing test systems severely bias the observation toward neutral antagonism and not low levels of positive or negative efficacy. With the advent of constitutively active receptor systems (vide infra), many ligands thought to be neutral antagonists can be seen, in fact, to be inverse agonists. At this point, it should be stressed that the constitutively active receptor systems discussed here refer to those that demonstrate a truly spontaneously activated receptor and are not simply a system with an elevated baseline response. While all constitutively active receptor systems show elevated basal responses, there are other ways in which basal response can be elevated (i.e., release of endogenous agonist, vide infra).

In view of the strict thermodynamic requirements for neutral antagonism ($\alpha = \gamma = \delta = 1$), positive and negative efficacy may be thought to be a knife edge with ligands either stabilizing or destabilizing receptor/G-protein complexes. To what extent these nuances in coupling are pharmacologically relevant is as yet unknown. It is useful to differentiate constitutive activity and inverse agonism as a physiologically relevant phenomenon from its utility as a pharmacological looking glass into the properties of drugs. It is not at all clear that constitutive activity is prevalent in natural systems and that, therefore, inverse agonists will be therapeutically special. However, the availability of constitutively active receptor systems has allowed the reclassification of antagonists and has given new insights into 7TM receptor mechanisms. From this standpoint, this area of pharmacological research has proven to be useful.

The expectation of zero efficacy introduces the concept of the 'antagonist assumption' in receptor pharmacology (Kenakin et al., 1995). If a ligand is prematurely classified as a neutral antagonist on the basis of experiments in quiescent nonconstitutively active systems, then it automatically assumes an identity equal to that of other neutral antagonists. When such ligands are used to classify receptors and expression systems, then the tacit assumption is made that the ligands are all equal. Judgments as to the similarity of expression systems to natural systems of physiological interest are made on the basis of correlations of affinity of antagonists in the genetically created versus the natural system. If the ligands used for such correlations are *not* uniform (i.e., some are in fact inverse agonists and thus possibly subject to systems effects), then erroneous conclusions can be made. In these cases, it may be more warranted to reclassify the ligand rather than the receptor.

The previous discussions have defined a theoretical class of ligand, namely the inverse agonist. This entity destabilizes receptor/G-protein complexes, a property that is obvious only when receptor/G-protein complexes are present in such quantities as to be observed.

B. Detection of Inverse Agonism

Before the advent of drug testing in constitutively active receptor systems, drugs that blocked the effects of agonists but produced no positive response were classified as neutral antagonists. As in the case of ion channel two-state theory, if a system does not have spontaneous activity, then the effects of a drug that suppresses spontaneous activity will not be evident. Over the past few years, various methods of detecting such negative effects of drugs have been reported. In general, the main tenet of these approaches is that conditions are met for the increased prevalence of the spontaneously active receptor state, and/or the effects of the spontaneously active receptor state are amplified to the point at which they are observed.

One potential method of detecting inverse agonism is by increasing the basal activation of receptors. The first instance of biochemical detection of an inverse agonist for a 7TM receptor was reported by Costa and Herz (1989). They showed that the inverse agonist for opioid receptors ICI 174864 produced little negative effect on GTPase activity in NG108-15 cells until the constitutive GTPase activity of these membranes was elevated by substitution of NaCl by KCl (see fig. 6A). Spontaneous precoupling of opioid receptors and G-proteins is

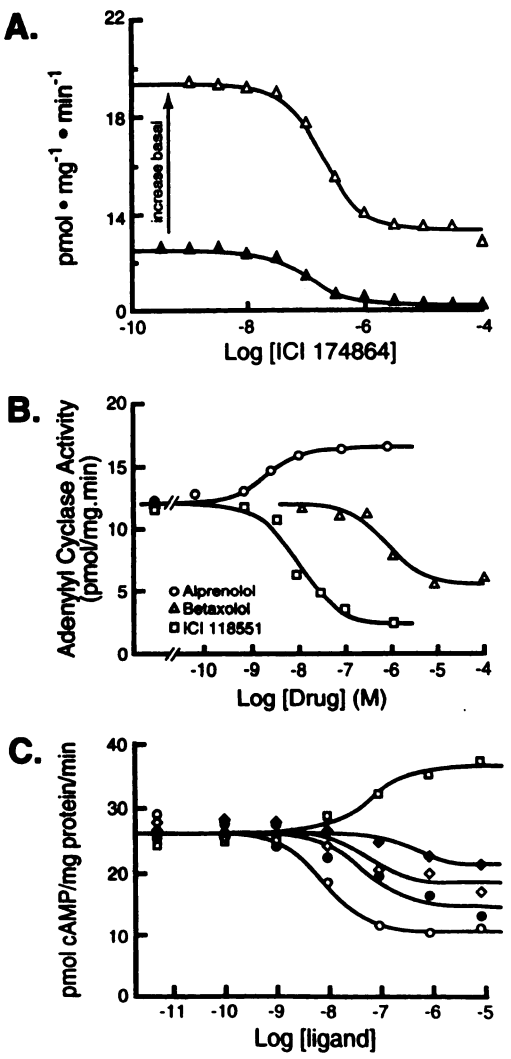


FIG. 6. Detection of inverse agonism. (A) Increased basal GTPase activity in membranes from NG108-15 cells by substitution of Na^+ with K^+ and the effects of the opioid inverse agonist ICI 174864 ([N,N'-diallyl-Tyr^1, Aib^2, 3]Leu^5-enkephalin). With permission from the National Academy of Sciences and from Costa and Herz (1989). (B) Effect of β-adrenergic receptor ligands on basal adenylate cyclase activity mediated by a constitutively-active mutant of the β_2-adrenergic receptor. With permission from ASPET and from Samama et al. (1994). (C) Effects of β-adrenergic blockers on basal adenylate cyclase activity in membranes from sf9 cells overexpressed with wild type β_2-adrenergic receptor. Responses to isoproterenol (open squares), dichloroisoproterenol (filled diamonds), labetolol (open diamonds), pindolol (filled circles), and timolol (open circles). With permission from ASPET and from Chidiac et al. (1994).

destablized by Na^+ (Costa et al., 1992). In addition, it is known that Na^+ modulates receptor affinity for G-proteins (Jagadeesh et al., 1990; Costa et al., 1990), and it is known that Na^+ produces dextral displacement of concentration-response curves to agonists for α_2-adrenergic receptors (Limbird et al., 1982), dopamine D_2 receptors (Hamblin and Creese, 1982) and δ-opioid receptors (Pert et al., 1973).

The original method reported by Costa and Herz (1989) has been extended to other receptors. Thus, removal of Na^+, shown to stimulate spontaneous association of G-proteins and opioid receptors (Costa et al., 1990, 1992), also produces constitutive activity for α_2-adrenergic receptors (Tian et al., 1994). Similarly, the binding of the α_2-adrenergic receptor inverse agonist [^{3}H]rauwolscine is increased 75% in PC-12 membranes with added Na^+ (Shi and Deth, 1994). There is evidence to suggest that, like GTP-induced cancellation of receptor/G-protein complexation, biochemical factors such as Na^+ may be important in the modulation of constitutive activity; although inverse agonism can be detected for some drugs in membrane systems in which the ionic milieu can be controlled, the same is not true in whole cell systems. Thus, the inverse agonism detected for some δ-opioid antagonists in membrane systems (Costa and Herz, 1989) was not observed in whole cellular systems (Costa et al., 1990). It may be that biochemical systems can be optimized for the detection of inverse agonism more easily than can functional systems.

Inverse agonists also have been discovered in binding studies by observing effects of guanine nucleotides. Thus, unlike the effects of GTP analogues on positive agonist binding (affinity is reduced), the binding of inverse agonists is *increased* by GTP. For example, studies on the reverse effects of GTPγS on binding have been used to detect negative efficacy in spiroperidol (De Lean et al., 1982). The adenosine receptor antagonist [^{3}H]xanthine amine congener preferentially binds to free adenosine receptors in bovine cerebral cortex (Freissmuth et al., 1991; Schutz and Freissmuth, 1992), where the receptors are spontaneously coupled to G-proteins (Leung and Green, 1989). This approach, although useful in some receptor systems, requires a kinetically favorable system for rapid exchange of guanosine diphosphate (GDP) to GTP. There are known G-protein systems that do not temporally respond adequately for this reaction to take place on an appropriate time scale, making this approach unreliable. This is discussed later in relation to agonist receptor coupling. A variant approach is by the cancellation of G-protein effects with toxins. Thus, pertussis toxin has been shown to *increase* the affinity of the opioid receptor inverse agonist ICI 174864 (Costa and Herz, 1989) and the α_2-adrenergic receptor inverse agonist rauwolscine (Jagadeesh et al., 1990; Shi and Deth, 1994).

Another approach is to study ligand effects on constitutively active mutant receptors. This has been used for the study of inverse agonism of β_2-blockers ICI 118,551 and betaxolol in CHO cells transfected with mutant constitutively active β-adrenergic receptors (See fig. 6B; Samama et al., 1994). Constitutively active mutants have been made also of α_{1B}-adrenergic receptors (Milano et al., 1994a) and α_2-adrenergic receptors (Ren et al., 1993). In general, the technical demands could limit the applicability of this technique. Also, the potential for differences between ligand activity on mutant versus wild type receptors always is open to question.

To date, the most fruitful approach is the testing of ligands in overexpressed receptor systems. There is evidence to suggest that the relative stoichiometry of receptors and G-proteins can be important in the production of constitutive activity. The concept here is that increasing the concentration of the reactants for spontaneous receptor/G-protein coupling will increase the quantity of activated receptor and G-protein and that this, in turn, will be observed either directly as GTPase activation or by subsequent activation of response elements (i.e., adenylate cyclase, etc.). Constitutive cellular activity has been shown to be related directly to β-adrenergic receptor expression levels in CHO cells (Samama et al., 1993), NG108-15 cells (Kim et al., 1995) and membranes from transfected sf9 cells (Chidiac et al., 1994). In CHO cells transfected for β-adrenergic receptor expression, the receptor density can be correlated with cellular basal adenylate cyclase activity. Thus, in clones expressing 2500 fmol/mg protein receptor, the basal level of cAMP approaches the maximally isoproterenol-stimulated levels in cells expressing 170 fmol/mg protein receptor (George et al., 1988). Figure 6C shows the positive and inverse effects of β-adrenergic receptor ligands on basal adenylate cyclase activity from membranes of sf9 cells transfected with high levels of β_2-adrenergic receptor. The stoichiometry also can be altered by increasing G-protein levels. For example, the reconstitution of dopamine D_2 receptors and G_{i2} proteins leads to spontaneous GTPase activity in the absence of agonist (Senogles et al., 1990).

Yet another approach is the testing of drugs in tissues from transgenic animals that produce constitutive activity by receptor overexpression. Transgenic mice (TG-4, TG-33) have been shown to demonstrate cardiac-specific overexpression of the wild type β_2-adrenergic receptor (Milano et al., 1994b). This receptor overexpression resulted in a three-fold increase in the baseline twitch tension of left atria that was selectively decreased by the β_2-adrenergic receptor inverse agonist ICI-118,551 (fig. 7A); this inverse agonism was blocked by the β_2-adrenergic receptor antagonist alprenolol (Bond et al., 1995). In vivo, this inverse agonism could be shown as well as decreases in left ventricular developed pressure (dp/dt); this effect also was blocked by alprenolol (fig. 7B). Identical responses could be seen with adenylate cyclase activity in membranes from TG-4 mouse hearts (fig. 7B).

There are practical and theoretical caveats to be made to the experimental support of inverse agonism. For example, the possibility of residual agonist present in bathing media of experimental preparations may demonstrate apparent inverse agonism for a neutral antagonist under partial agonist activation (i.e., Maenhaut et al., 1990). Also, the restriction of movement of agonist among free receptors in membrane systems may produce reduced G-protein activation (Mahama and Linderman, 1994). On balance, however, there are specific criteria for the demonstration of the data with

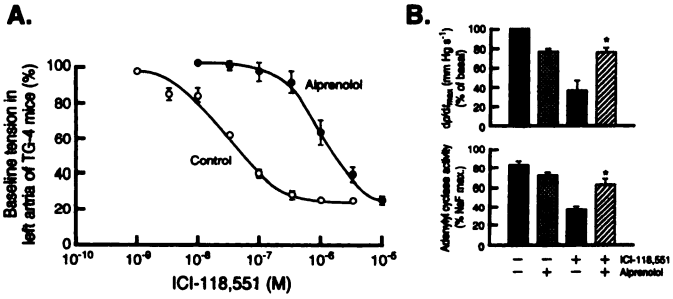


FIG. 7. Effects of inverse agonists in tissues from transgenic TG-4 mice with overexpressed cardiac β_2-adrenergic receptors. (A) Effect of the inverse agonist ICI-118,551 on baseline stimulated left atrial tension from TG-4 mice. Data from atria obtained in the absence (open circles) and presence of alprenolol (0.1 μM; filled circles). (B) Effects of ICI-118,551 on left ventricular dp/dt_{max} (upper bar graph) and adenylate cyclase activity (lower bar graph) in membranes from TG-4 mouse hearts. Although a minimal effect is seen with the neutral antagonist alprenolol, significant depression of constitutive activity is observed with ICI-118,551. This inverse agonism is reversed by alprenolol (1 μm). With permission from MacMillan Magazines and from Bond et al. (1995). (B) Effect of ICI 118,551 (5 μg i.v.) or alprenolol (10 μg i.v.) to mice (n = 7) (upper bars) and effect of 100 nM ICI 118,551 and alprenolol (1 μM) on adenylate cyclase activity. From Bond et al. (1995).

constitutively active receptor systems that have been met in many studies. They are as follows:

- Inverse effects have been observed in receptor systems with added G-protein (Schutz and Freissmuth, 1992).
- Reverse effects of GTP analogs and G-protein toxins have been noted on binding curves.
- Inverse agonism has been observed in transgenic animals.
- Inverse agonist responses can be blocked selectively by neutral antagonists (i.e., see fig. 7A).

This latter point is perhaps the most important. When this can be shown, it indicates that the phenomenon is a receptor mechanism.

With the development of sensitive test systems for the detection of inverse agonism will come a reclassification of many drugs. If it is accepted that efficacy is based on the differential affinity of a ligand for receptor states, then an expectation of 'zero' efficacy sometimes may be unrealistic because this would require identical affinities for two different tertiary forms of the receptor protein. Given this, it might be observed that numerous previously classified neutral antagonists may be inverse agonists. There are suggestions that this is true for β-blockers. For example four β-blockers tested in sf9 cells expressing β_2-adrenergic receptors produced inverse agonist effects (Chidiac et al., 1994) (fig. 6C). Similarly, Samama et al. (1994) found negative efficacy in two of three β-blockers tested (fig. 6B). In PC-12 cells expressing α_2-adrenergic receptors, five commonly used α_2-antagonists depressed [^{35}S]GTPγS binding indicative of inverse agonism (Tian et al., 1994). Table 9 shows a

TABLE 9
Putative inverse agonists: wild type receptors

Receptor	System	Drug	
β_2-Adrenergic	Sf9 membranes	DCl[a] pindolol labetolol timolol	Chidiac et al., 1994
	CHW membranes	labetolol pindolol alprenolol propranolol timolol	
	turkey erythrocytes	propranolol pindolol	Gotze and Jakobs, 1994
	TG-4 murine atria	ICI 118,551	Bond et al., 1995
β_1-Adrenergic	cardiomyocytes	atenolol propranolol	Mewes et al., 1993
α_2-Adrenergic	PC-12 cells	rauwolscine yohimbine WB 4101 idazoxan phentolamine yohimbine	Tian et al., 1994
	bovine aorta	rauwolscine	Jagadeesh et al., 1990
	PC-12 cells	rauwolscine[b]	Shi and Deth, 1994 Jagadeesh and Deth, 1992
Muscarinic Acetylcholine	frog/rat atrial myocytes	atropine[c]	Hanf et al., 1993
	frog heart	QNB[d]	Burgisser et al., 1982
Bradykinin	Rat myometria	HOE140 NPC17731 NPC567	Leeb-Lundberg et al., 1994
5-HT_{2C}	NIH 3T3 cells	mianserin mesulergine ketanserin	Barker et al., 1994
	NIH 3T3 cells	clozapine cyproheptadine ketanserin mesulergine	Westphal and Sanders-Bush, 1994
	Sf9 cells	metergoline methysergide ritanserin mianserin mesulergine ketanserin clozapine spiperone	Lebrecque et al., 1995
δ-Opioid	NG108-15 cells	ICI 174864	Costa and Herz, 1989
Dopamine*	P. anterior pituitary	spiroperidol	De Lean et al., 1982
Adenosine*	Bovine brain	XAC	Freissmuth et al., 1991
		Constitutively active mutants	
β_2-Adrenergic	CHO membranes	betaxolol ICI 118,551	Samama et al., 1994
α_{1B}-Adrenergic	Rat-1 Fibrob.	prazosin WB 4101 phentolamine	Cotecchia et al., 1995

[a] Dichloroisoproterenol.
[b] Increased binding in the presence of added Na^+.
[c] At 1 μM.
[d] [^{3}H]Quinuclidinyl benzoate.
* Suggested by the increased binding observed with inclusion of GTP in medium.

list of possible inverse agonists, previously thought to be neutral antagonists from data in quiescent systems. It should be noted that in many of the cases cited, blockade of the inverse effect with a neutral antagonist was not shown; therefore, the data are consistent with but not necessarily proof of true inverse agonism.

Presently, it is unclear to what extent, if any, inverse agonists will affect therapeutic approaches to 7TM re-

ceptors. Clearly, an inverse agonist will have a somewhat different profile of antagonism across various tissues in the body than a neutral antagonist in that they will block endogenous agonist *and* any constitutive receptor activity, whereas the latter will only block the effects of endogenous agonists. Insofar as receptor overexpression may lead to constitutive basal activity in tissues, an inverse agonist would be a unique drug. For example, dopamine D_4 receptors are elevated six-fold over control in patients with schizophrenia (Lee et al., 1978; Seeman et al., 1993). If this increase in receptor density leads to constitutively active foci of activity, these would be resistant to standard dopamine antagonists such as haloperidol but sensitive to negative antagonists. Also, there is evidence that 7TM receptor mutation sometimes may lead to a pathologically relevant, constitutively active receptor mutants (vide infra). Under these circumstances, an inverse agonist would be needed. At present, it is premature to speculate on the potential merits of inverse agonists (Milligan et al., 1995a); however, in view of the fact that they are a newly discovered drug type, it will be extremely interesting to see what place they find in therapy.

It may be advantageous to detect inverse agonism in antagonists for other reasons. For example, a theoretical case could be made for a greater risk of receptor upregulation (and therefore, of tolerance to blockade) for inverse agonists versus neutral antagonists (Milligan et al., 1995b). If normal levels of receptor on the membrane are controlled by phosphorylation of spontaneously activated receptors, then an inverse agonist could prevent this normal process and thus produce an imbalance in the receptor synthesis/destruction cycle.

C. Receptor Expression Levels and Relative Stoichiometry

1. Agonist coupling. Heterologous expression of 7TM receptors particularly tests the assumption that high agonist affinity binding will be observed in surrogate cell systems. In some expression systems, there is reasonable correspondence between the type and quantity of high affinity binding observed in natural systems and heterologous expression systems. For example, ^{125}I-VIP demonstrates high (K_d = 0.3 nM) and low (K_d = 23 nm) binding in rat lung, with a relative proportion of 13% high affinity sites (Leroux et al., 1984). In COS cells transfected for expression of rat VIP receptors, similar complex ^{125}I-VIP binding could be observed (high affinity K_d = 0.16 nM, low affinity K_d = 20 nM, 7% high affinity sites: Ishihara et al., 1992). One novel approach has been the study of radioligand binding to the effector to detect ternary complex formation. For example, increased binding of [^{3}H]forskolin to adenylate cyclase in response to $G_{\alpha s}$ and transfected receptor (prostanoid, β-adrenergic) interaction in NG108-15 cells provided insight into the stoichiometry of receptor-effector relationships (Kim et al., 1995).

The definition of a 'high-affinity' site for a ligand often is subjective when agonist radioligands are used. Commonly, a single population of binding sites is observed because accurate binding data at the high concentrations of radioligand required to define low affinity binding (i.e., the uncoupled form of the receptor) is unobtainable. Thus, if a single homogeneous population of receptors is observed with an agonist radioligand, it is not possible to determine whether this is a completely coupled ternary complex binding product or an uncoupled receptor unless an antagonist radioligand is used to determine the receptor population size. For example, saturation binding of transfected rat 5-HT_7 receptors in COS-7 cells yields a population size of 5 to 15 pmol/mg protein when measured with the antagonist [^{3}H]lysergic acid diethylamide and 2 to 10 pmol/mg protein when measured with the agonist [^{3}H]5-HT (Shen et al., 1993). These data suggest the labeling of a coupled receptor subset with the agonist.

The determination of G-protein binding by elimination of high affinity sites with analogues of GTP (i.e., Gpp(NH)p) is one potential method of determining whether observed high affinity binding is because of a two-stage process of receptor/G-protein coupling. Some of these effects can be striking, as in the 1480-fold change in affinity of carbachol in rat heart membranes produced by GTP (Matesic et al., 1989). Such studies have also been done in membranes from surrogate cells transfected with receptor genes. For example, the high affinity binding of [^{3}H]5-HT to human 5-HT_{1B} receptors in HeLa cells (Hamblin et al., 1992) and rat 5-HT_1 receptors in HEK 293 cells (Voigt et al., 1991b) is greatly reduced by inclusion of analogues of GTP. Similar sensitivity of complex agonist binding to GTP analogues has been observed for rat α_2-adrenergic receptor subtypes α_{2B} and α_{2D} (but not α_{2C}) in NIH 3T3 fibroblasts (Duzic et al., 1992), rat dopamine D_2 and D_3 receptors expressed in various cell lines (Sokoloff et al., 1990; Castro and Strange, 1993), rat adenosine A_1 receptors in A-9 cells (Mahan et al., 1991), human adenosine A_1 receptors in CHO cells (Libert et al., 1992), human 5-HT_{1D} receptors (Hamblin and Metcalf, 1991), human bradykinin B_2 receptors (Hess et al., 1992), human somatostatin receptors (Demchyshyn et al., 1993), mouse β_2-adrenergic receptors (Allen et al., 1988), human 5-HT S12 receptor (Levy et al., 1992), human dopamine D_4 receptor (Van Tol et al., 1991), human cholecystokinin (CCK)-B receptor (Miyake et al., 1994) and the guinea pig leukotriene B_4 receptor (Falcone and Aharony, 1991).

On the other hand, the cancellation of receptor coupling by GTP is a one-way experiment in that, if high affinity binding is eliminated, G-protein binding is implied, but if high affinity binding is not eliminated, other factors, such as kinetics of GDP/GTP exchange, may have confounded the experiment. Under these circumstances, it cannot be assumed that G-protein coupling is not present. For example, in Y-1 cells transfected with

genes encoding the mouse m1 muscarinic acetylcholine receptor, carbachol produces a clearly seen population of high affinity binding sites (26% pK_H = 5.4, 74% pK_L = 3.9) that are resistant to treatment of the membranes with Gpp(NH)p (Shapiro et al., 1988). The same results were obtained for the chick muscarinic acetylcholine receptor expressed in CHO cells, namely a lack of effect of Gpp(NH)p on complex inhibition of [^{3}H]QNB binding by carbachol (Tietje et al., 1990). Similarly, the binding of the agonist [^{3}H]5-HT to rat 5-HT_7 receptors transfected into COS-7 cells is unaffected by GTP (Shen et al., 1993). An interesting contrast was observed in CHO cells transfected with genes for human D_2 and D_3 receptors, respectively. Although high affinity dopamine binding was sensitive to Gpp(NH)p in D_2 receptor transfected cells, high affinity binding of dopamine to D_3 receptors in the same cell line was resistant to this procedure (Sokoloff et al., 1992). Similar heterogeneity of GTP-effects was observed for 5-HT_2 receptors transfected into HEK 293 and NIH 3T3 cells. Whereas agonist coupling was insensitive to GTP analogues in HEK 293 cells, it was partially sensitive in NIH 3T3 cells (Szele and Pritchett, 1993).

Variability in the 'GTP-shift' has been analyzed in a model proposed by Onaran and coworkers (1993), who extended the ternary complex model to include the dissociable subunits of the G-protein and the effects of nucleotides. In general, this model predicts large differences in the observed effects of nucleotides on ligand binding that depend on system characteristics, namely the coupling factor modifications of unconditional equilibrium constants among the G-protein subunits, the receptor and the α-subunit. As shown in figure 8A and B, the concentration of $\beta\gamma$ subunits affect the observed potency of agonists differentially under conditions of high to low GTP. The difference between these curves (at a fixed quantity of $\beta\gamma$ subunit in the system) is the 'GTP shift,' and it can be seen that the magnitude of this shift is determined by the availability of $\beta\gamma$ subunit. Interestingly, this model also predicts that the availability of $\beta\gamma$ subunits also affects spontaneous constitutive and agonist-induced receptor activation. As seen in figure 8C, constitutive activity is more sensitive to the concentration of $\beta\gamma$ subunits than is agonist activation. This has been observed experimentally as well (Cerione et al., 1985; Okabe et al., 1990; Hildebrandt and Kohnken, 1990). As seen in figure 8D, where the basal effect is subtracted, the concentration of $\beta\gamma$ subunits has a biphasic effect on agonist activation, increasing it at lower concentrations and then decreasing it at higher concentrations. Thus, the $\beta\gamma$ subunits buffer the system for both spontaneous activity and agonist effect. The importance of $\beta\gamma$ subunits (a cellular host effect) has been shown in antisense oligonucleotide experiments where certain β-subunits were found to be involved in signal transduction cascades in GH3 cells (Kleuss et al., 1992). Recent provocative data with $\alpha_{2A/D}$-adrenergic receptors

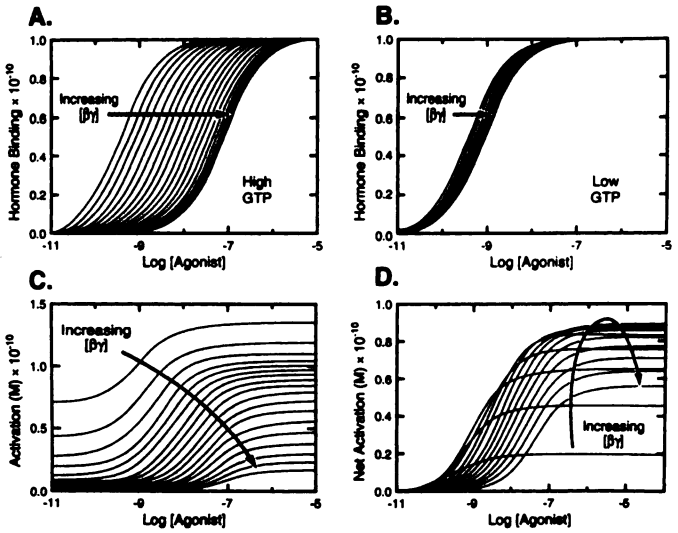


FIG. 8. The effects of $\beta\gamma$ subunits on high affinity ligand binding and receptor activation. (A) Simulation of high affinity binding with various concentrations of $\beta\gamma$ subunits in the presence of high concentrations of GTP and in the presence of low GTP (panel B). The relative locations of the dose-response curves for any given concentration of GTP reflects the GTP-shift induced by GTP, indicating agonist efficacy and receptor/G-protein coupling. (C) Effects of $\beta\gamma$ subunits on receptor activation. Note how increasing $\beta\gamma$ concentration depresses constitutive activity, changes maximal responses to an agonist and decreases sensitivity of the system to the agonist, but that each of these systems characteristics is not equally sensitive to $\beta\gamma$ concentration. (D) Simulation shown in C with the agonist-independent (constitutive) activity subtracted. This shows the biphasic effect of $\beta\gamma$ concentration on the maximal effect of an agonist with an intial increase then decrease with increasing $\beta\gamma$ concentration. Data from Onaran (1993).

in NIH 3T3 and PC-12 cells provide evidence for the involvement of a specific membrane-associated protein in the interaction of agonist-induced activation of G-proteins (Sato et al., 1995). A similar protein has been found for adenosine receptors. This factor causes tight receptor/G-protein coupling that is refractory to GTPγS (Nanoff et al., 1995). The implications of another membrane interactant in the receptor cascade are extremely important in terms of defining and quantifying agonist efficacy for drug therapy.

The expectation of observing a high affinity agonist binding in an expression system presupposes that the appropriate G-protein is present in the expression cell and also that it is there in sufficient quantities to produce observable ternary complexation. This can vary with different expression systems as was illustrated in studies of the expressed rat 5-HT_{5B} receptor. Accordingly, a high and low affinity state for this receptor for a 5-HT agonist that was greatly reduced by the presence of the nonhydrolyzable GTP analogue Gpp(NH)p (showing receptor/G-protein coupling) could be demonstrated when expressed in COS-1 cells; no corresponding G-protein activation could be demonstrated when this receptor was transiently expressed in COS-7, COS-293 or CHO cells (Wisden et al., 1993). Clearly, if the appropri-

ate G-protein is not available in the receptor compartment, then the uncoupled (and presumably low affinity) state of the receptor will be present, and technical considerations may preclude the observation of agonist binding with radioligands. In cellular expression systems in which the stoichiometry of receptor and G-proteins becomes a variable, the 'overexpression' of receptors can cause conditions whereby the ratio of uncoupled to coupled receptor is large, giving the appearance of a failure to G-protein couple the expressed receptors. For example, rat 5-HT_2 receptors expressed in mammalian cells show high affinity binding for the antagonist [^{3}H]spiperone but no detectable binding of [^{3}H]5-HT (Pritchett et al., 1988). A list of receptors demonstrating a range in proportions of receptor coupling efficiencies in expression systems is shown in table 10. It can be seen from this list that as long as an adequately strong signal can be obtained from the bound radioligand, exceedingly small percentages of coupled receptor can be detected.

The observation of G-protein coupled receptor events also may differ in binding and functional systems. There are cases where the biochemical amplification of minute G-protein signals allow agonist activation to be observed (i.e., cellular response), but where the amount of G-protein isomerization of the receptor is insufficient to be observed with ligand binding. For example, the adenosine receptor agonist 5'-N-ethylcarboxamidoadenosine (NECA) produces a clear increased cAMP response in CHO-K1 cells transfected with a cDNA for adenosine receptors from human brain, but no [^{3}H]NECA binding was observed (Pierce et al., 1992). Presumably, a low level of G-protein activation was sufficiently amplified by the biochemical cascade mechanisms in the cell to produce a measurable response, but there was an insufficient quantity of receptor distribution into a ternary complex to allow for the observance of high affinity agonist binding.

2. Relative expression level and promiscuity of coupling. In natural systems, the stoichiometry of receptors and G-proteins is fixed by nature. With receptor expression comes two new potential phenomena, namely the induction of constitutive receptor activation and also increased receptor promiscuity with respect to activation of numerous G-proteins. Increased complex receptor coupling with increased levels of receptor expression is becoming a commonly observed experimental phenomenon.

TABLE 10
Two affinity states for agonists in expression systems

Receptor	Cell	Agonist	pK_H	pK_L	%H/%L	Reference
Agonist Saturation Binding in Expression Systems						
r 5-HT_1	HEK 293	[^{3}H]5-HT	8.4	7.2	NG	Voigt et al., 1991b
h 5-$HT1_B$	sf9	[^{3}H]5-HT	11	10	50/50	Ng et al., 1993
r 5-HT_{1A}	Ltk$^-$	[^{3}H]DPAT	8.8	8.0	50/50	Albert et al., 1990
r α_{2D}	NIH 3T3	[^{3}H]UK 14,304	8.15	5.85	37.5/62.5	Duzic et al., 1992
m 5-HT_{1B}	sf9	[^{3}H]5-HT	11	9.9	36/64	Ng et al., 1993
r α_{2B}	NIH 3T3	[^{3}H]UK 14,304	8.3	6.0	34/66	Duzic et al., 1992
h 5-HT_{1B}	HeLa	[^{3}H]5-HT	9.27	8.1	30/70	Hamblin et al., 1992
r secretin	COS + G_s[a]	^{125}I-secretin	9.2	7.7	15/85	Ishihara et al., 1991
h 5-HT_{1D}	LS12/6.2	[^{3}H]5-HT	7.7	6.7	11.8/88.2	Levy et al., 1992
r VIP	COS	^{125}I-VIP	9.8	7.7	7/93	Ishihara et al., 1992
h 5-HT_A	COS-7	[^{3}H]DPAT	10.2	7.65	2.5/97.5	Fargin et al., 1988
r secretin	COS	^{125}I-Secretin	9.2	7.7	1.8/98.2	Ishihara et al., 1991
Agonist Inhibition Binding of Antagonist Radiolabels						
m musc m1	CHO	carbachol	5.26	3.9	85/15	Shapiro et al., 1988
c musc m4	CHO	carbachol	5.27	3.9	85/15	Tietje et al., 1990
r A1	A-9	r-PIA	9.15	7	72/28	Mahan et al., 1991
m musc m1	Y-1	carbachol	5.5	4.2	70/30	Shapiro et al., 1988
r D_{2short}	LZRI	dopamine	8.1	6.1	58/42	Castro and Strange, 1993
r D_{2long}	CHO	dopamine	7.5	5.45	48/52	Castro and Strange, 1993
h D_3	CHO	dopamine	8.4	7.1	47/53	Sokoloff et al., 1992
r D_3	CHO	dopamine	8.6	7.2	40/60	Castro and Strange, 1993
h D_2	CHO	dopamine	7.66	5.8	34/66	Sokoloff et al., 1992
rh D_1	C6 cells	dopamine	8	6	32/68	Machida et al., 1990
h m1	HEK	carbachol	4.1	3.3	30/70	Peralta et al., 1987
h m2	HEK	carbachol	7	3.3	28/72	Peralta et al., 1987
r D_{2long}	Ltk59	dopamine	8.1	6	25/75	Castro and Strange, 1993
h m2	HEK	oxotremorine	8.1	5.3	20/80	Peralta et al., 1987
h m4	HEK	carbachol	5.1	3.25	6.5/93.5	Peralta et al., 1987
h m3	HEK	oxotremorine	7.66	5.4	5/95	Peralta et al., 1987

[a] Cotransfected with G_s.

r, rat; h, human; m, mouse; c, chick; rh; rhesus monkey; VIP, vasoactive intestinal peptide; r-PIA, r-phenylisopropyladenosine.

There is an intrinsic association constant between receptors and all G-proteins (i.e., K_G in the cubic ternary complex model), and selectivity of receptor/G-protein coupling can be achieved by this K_G and the relative 'concentrations' of the receptor and G-protein (i.e., very little receptor/G-protein complex will be formed by a receptor with an association constant K_G of 10^4 at a concentration of 10 μM). However, if the concentration of receptor were increased 100-fold, then an appreciable amount of even this unfavored complex will be formed.

If it is accepted that receptors can be promiscuous with respect to the G-proteins that they encounter in the membrane, then there is a potential for dissimulation of effect with receptor overexpression. One method of achieving signaling selectivity in nature is to control the stoichiometry of receptors and G-proteins; if this is overridden in a heterologously expressed system, then system-dependent data may result that may not reflect the physiology of the receptor. For example, receptor expression level has been shown to determine the cellular responses mediated by transfected α_2-adrenergic receptors in CHO cells (Fraser et al., 1989). Thus, in cells containing 50 fmol/mg protein, primarily inhibition of cAMP levels was observed with epinephrine, whereas in cells containing 1200 fmol/mg protein, a biphasic inhibition and stimulation of cAMP level was seen. The inhibition phase was sensitive to treatment of cells with pertussis toxin, suggesting that this receptor activated two separate G-proteins in CHO cells (Fraser et al., 1989). Similarly, muscarinic receptors expressed in JEG-3 cells can either inhibit or stimulate adenylate cyclase, the nature of the response being dependent upon the receptor expression level (Migeon and Nathanson, 1994). Another example of receptor density controlling the effect in a cellular system is with the expression of α_{2A}-adrenergic receptors in COS cells (Eason et al., 1992). In this study, an α_2-adrenergic receptor level of 1 pmol/mg protein yielded a system that mediated inhibition of adenylate cyclase (via G_i protein), whereas higher receptor expression levels (5 and 10 pmol/mg protein) produced biphasic interaction of the receptor with both G_i and G_s to inhibit and then subsequently stimulate adenylate cyclase. The α_{2A}-adrenergic receptor expressed in Rat-1 fibroblasts has been shown to activate several cellular elements including phospholipase D (MacNulty et al., 1992). Similarly, expression of muscarinic m4 receptors in HEK 293 demonstrated a biphasic response with respect to adenylate cyclase activity that depended upon receptor number. Thus, in cells with low levels of receptor expression, activation of m4 receptors resulted in inhibition of cAMP, whereas in cell lines exhibiting the highest levels of receptor expression, a biphasic response of inhibition and stimulation was observed. This was consistent with receptor competition with a pertussis-sensitive and -insensitive G-protein in the cell membrane (Dittman et al., 1994). In CHO cells transfected with muscarinic m3 receptors, carbachol produced both increased accumulation of inositol 1,4,5-triphosphate and cAMP (Burford et al., 1995). However, a 50% reduction in the receptor transfection level resulted in loss of cAMP response.

An interesting difference in signaling was shown for the human calcitonin receptor. Expression of the cDNA, obtained from T47D cells, in BHK cells led to a receptor profile similar to that found in T47D cells. However, the function of the expressed system differed. Although calcitonin increased intracellular calcium, inositol phosphate production and cAMP in BHK cells, only the cAMP response was observed in T47D cells (Moore et al., 1992). This difference in coupling may have been related to the difference in receptor densities in the two cell types (BHK cells 800,000 receptors/cell; T47D 40,000 receptors/cell).

Another case of receptor-coupling promiscuity tied to receptor expression level was observed in African green monkey cells (BS-C-1) transfected with human 5-HT_{1E} receptors. While low expression levels (2 pmol/mg protein) produced 5-HT-mediated inhibition of cAMP production, higher levels (5 pmol/mg protein) produced cells showing a biphasic decrease and increased cAMP response to this agonist (Adham et al., 1994b). Each respective response could be eliminated by treatment of cells with pertussis and cholera toxins(s), indicating promiscuous coupling of this expressed receptor to separate G-proteins.

There are theoretical reasons for concern over receptor density versus availability and type of G-protein coupler present in the membrane. Figure 9 shows the results of modeling using the cubic ternary complex model with one receptor binding to two G-proteins (Kenakin and Morgan, 1989). The simulation is for the maximal ternary complex produced by an agonist that activates a single receptor toward interaction with two G-proteins. G-protein G_1 is favored and is the primary physiological coupler for the receptor-agonist pair, but there is a weak interaction between the agonist-activated receptor and the second G-protein G_2. As can be seen from figure 9, at receptor levels from 0.01 to 10, essentially all of the ternary complex is formed with G_1. However, as the quantity of receptor increases beyond this level, appreciable levels of ternary complex with G_2 can be observed. The resulting cellular response resulting from the biochemical cascades initiated by both G_1 and G_2 might be expected to be quite different when compared with the result emanating from only G_1; thus, a qualitative and quantitative difference in agonist response probably would be seen as a function of receptor expression level (Kenakin, 1995a).

D. The Nature of Efficacy: Receptor Activation

There has been much effort placed into the study of how receptors can activate G-proteins and effectors. As discussed previously, theoretical and practical data suggest that an active conformation of the receptor can bind

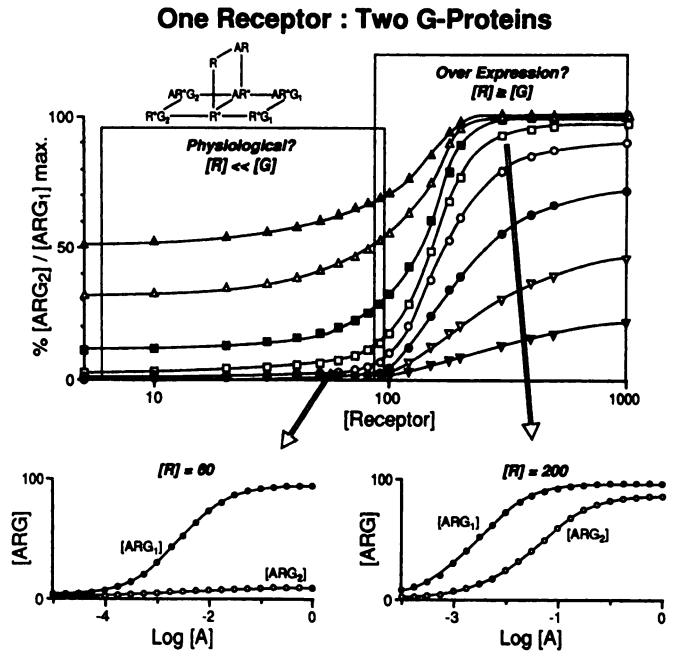


FIG. 9. Simulation of a single receptor plus two-G-protein membrane system. Ordinate axes show the maximum quantity of ternary complex formed between agonist/receptor and primary G-protein G_1 (right-hand axis) and agonist/receptor and secondary G-protein G_2 as a function of receptor density (abscissae). The relative concentration of G_1/G_2 is 10 to 20. Other system-dependent parameters are: [R] = variable, J = 0.1, β = 1000: Agonist dependent parameters = M_1 = 0.01, α_1 = 1000, M_2 = 0.01, α_2 = 10 (see schematic inset). The agonist promotes a preferential ternary complex with G_1. As can be seen from this figure, at receptor concentrations from 3 to 50, essentially all of the ternary complex is formed with G_1 (binding curve inset for [R] = 60). However, as the receptor concentration is increased beyond 50, a second complex of agonist/receptor/G_2 appears with increasing prevalence (binding curve inset for [R] = 200).

to G-proteins and begin the process of effector activation. There is evidence that short synthetic peptides from the third and fourth intracellular loops of G-protein receptors in close apposition to the membrane are known to stimulate G-proteins in vitro (Okamoto et al., 1991; Cheung et al., 1991; Ikezu et al., 1992). This would suggest that inactive receptors have these binding domains inaccessible to G-proteins and that agonists 'relax' the receptor to expose these domains and thus initiate activation (Lefkowitz et al., 1993). Under these conditions, inactive receptors could be thought to be under tonic constraint with respect to these intracellular domains (Lefkowitz et al., 1993). There is evidence that mutations of some receptors in specific regions produce constitutively active receptors and that the inactive receptor is the exception, not the rule. For example, substitution at position 293 of the α_{1B}-adrenergic receptor with any one of 19 other amino acids (different from the wild type) produces a receptor that spontaneously produces inositol phosphate production (Kjelsberg et al., 1992). In the bacterial chemoreceptor Trg, 20 mutations led to nine constitutively active receptors and 11 quiescent ones (Yaghmai and Hazelbaurer, 1992). Thus, in general terms, it may be that the inactive conformation of the receptor is the special one, designed to keep inaccessible the G-protein-activating amino acid sequences, and that deviation from such conformation(s) leads to a partially or completely activated receptor.

A central question in pharmacology is the nature of agonist efficacy, i.e., what makes agonists enrich the membrane population of activated receptors? A useful early delineation of ideas was proposed by Burgen (1966), who suggested that receptors could impart signals either by 'conformational selection' or 'conformational induction.' The first idea describes a condition whereby the receptor pre-exists in at least two states, one of which elicits cellular signaling (the activated receptor state). Agonists selectively bind to this activated state and enrich the population and thus produce a drug-induced response. The second idea describes an active receptor conformation created by the agonist and thus not present in the absence of the agonist.

Although it necessitates at least two receptor states, the most parsimonious hypothesis is conformational selection because it does not require additional receptor conformations other than those that exist naturally. There is evidence that naturally activated receptors exist and can activate a variety of effector systems. In fact, there is evidence to show that receptors can form different conformations and display complex binding kinetics in receptor systems stripped of G-proteins. Thus, muscarinic receptors, solubilized with digitonin-cholate and further processed to remove G-proteins displayed biphasic binding curves for the agonist oxotremorine (Wreggett and Wells, 1995). These data can be described with a tetravalent oligomeric receptor model that involves different receptor conformations not dependent upon G-proteins (Wreggett and Wells, 1995).

In terms of the concept of receptors existing in different conformations, selective binding to the activated form of the receptor by a ligand will enrich the relative proportion of that activated species and produce response (i.e., fig. 2). There are data becoming available to probe the nature of the receptor species responsible for physiological response. The most valuable systems to explore this area are those in which a single receptor species is capable of interacting with two or more G-proteins. The relative activation of the G-proteins involved may provide an insight into the activated receptor species formed by agonists.

1. Receptor trafficking of stimulus. There is evidence that some agonists specifically direct receptor signaling traffic toward specific G-proteins making agonist responses selective for receptor/G-protein combinations (shown schematically in fig. 10A). For example, in CHO cells transfected with 5-HT_{1A} receptors, a range of full and partial agonists differentially produced activation of $G_{\alpha i2}$ and $G_{\alpha i3}$. Whereas 5-HT appeared to be equiactive for G-proteins, the agonist ipsapirone showed a distinction (Gettys et al., 1994). Agonists for m1 acetylcholine

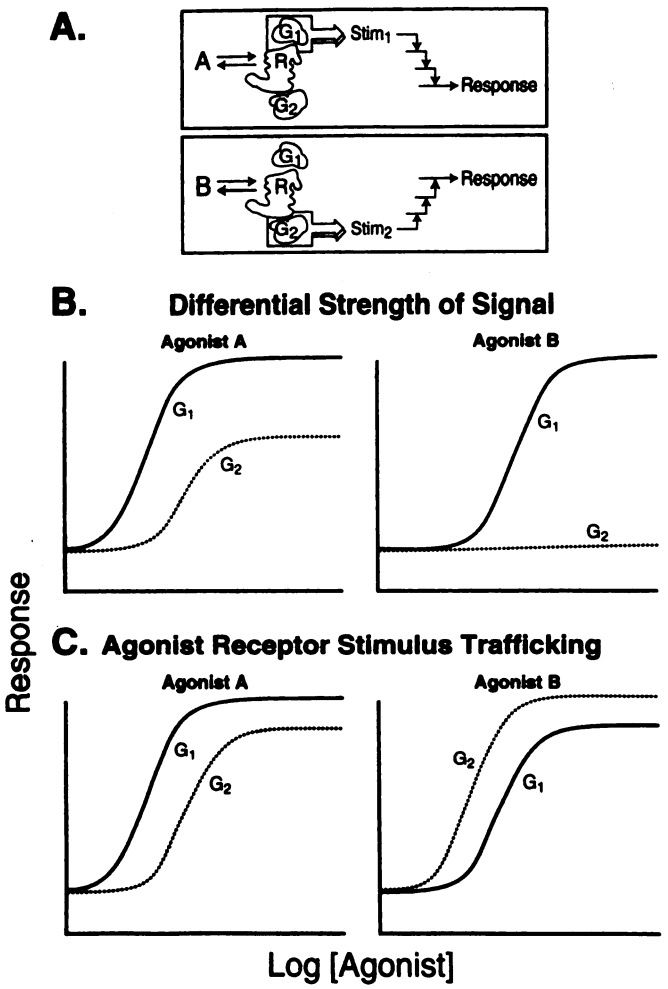


FIG. 10. Agonist trafficking of receptor stimulus to different G-proteins. (A) Schematic diagram of the concept of receptor stimulus trafficking by agonists. Agonist A produces response by directing receptor activation to G-protein 1 while agonist B uses another G-protein. Presumably, this is caused by differences in the conformation of the activated state either stabilized by or created by the different agonists. From Kenakin (1995b). (B) Simulated data consistent with differences in G-protein activation by differential strength of signal. Agonist A is highly efficacious and activates G_1 and the less sensitive G_2, whereas agonist B is weaker and can only activate the most sensitive process, namely G_1. (C) Data simulating true agonist directed trafficking of receptor stimulus. Agonist A preferentially activates G_1, whereas agonist B preferentially activates G_2.

(m1AChR) receptors also may direct trafficking to selective biochemical cellular pathways. In CHO cells transfected with m1AChR, quantitative differences in the potencies of carbachol, pilocarpine and AF102B for activation of phosphoinositide hydrolysis, arachidonic acid release and cAMP accumulation indicate selective activation associated with receptor recognition of ligands (Gurwitz et al., 1994). A clear distinction between G-protein activation of G_s and G_i by α_2-adrenergic receptors was shown by oxymetazoline and epinephrine in CHO cells (Eason et al., 1994). Whereas epinephrine activated both G-proteins in a similar concentration range, oxymetazoline could be shown only to activate G_i.

Another possible indication of agonist directed trafficking comes from the observation of high and low affinity binding states with different agonists in various expression systems. For example, carbachol and oxotremorine are known agonists for human muscarinic acetylcholine receptors. Transfection of subtypes 1 to 4 into HEK cells leads to interesting differences in high and low affinity inhibition of [^{3}H]QNB binding. Specifically, whereas the m2 subtype shows comparable high and low affinity states with both agonists, oxotremorine exclusively produces two states in cells transfected with subtype m3, and carbachol exclusively produces two states for transfections with subtype m1 and m4 (Peralta et al., 1987). Although the ability to produce a demonstrable high affinity state is dependent on the intrinsic efficacy of the agonist, these data suggest that whatever G-proteins are available in the HEK cell for complexation with the receptors are differentially used by the agonists in producing coupling states.

One possible explanation for these data is that the various agonists produce different activated receptor conformations that have different relative affinities for G-proteins, i.e., these agonists 'traffic' the receptor stimulus toward different G-proteins. At this point, the nature of the receptor species that activates the G-protein should be defined. At present, there is an abundance of evidence that different regions of 7TM receptors activate different G-proteins (i.e., the same sequences do not universally activate all G-proteins) and that selectivity for G-protein coupling can result (Wong et al., 1990; Kosugi et al., 1992; Okamoto and Nishimoto, 1992; Yamada et al., 1994; Nussenveig et al., 1994; Wu et al., 1995). However, it is not clear whether the activated receptor exposes all or just some of these upon conformational change to the active state. There is suggestive biochemical evidence to indicate that agonist-bound receptor complexes differ from those not containing agonist with respect to G-protein binding. For example, whereas some antisera for α-subunits of G-proteins do not differentiate spontaneously receptor-bound G-proteins and those produced by agonists, the amount of spontaneously coupled α_2-adrenergic receptor to $G_{\alpha i}$ is reduced by the α_2-adrenergic receptor agonist p-aminoclonidine (Okuma and Reisine, 1992). This indicates that the receptor complex spontaneously coupled to this G-protein and the activated receptor formed by p-aminoclonidine were seen to be different by the G-protein (as indicated by immunoprecipitation with the antiserum).

Although there is suggestive evidence, there is still a paucity of definitive data to indicate that agonist-selective activated receptor complexes exist. Also, before this complex hypothesis can be considered, the more simple scenario of selective G-protein activation graded by strength of stimulus must be eliminated. This idea states that the spectrum of G-protein activation is produced by the actual strength of stimulus, in the case of

receptor conformational selection, by the actual quantity of activated receptor state. Thus, a powerful agonist that produces a great deal of activated receptor will activate many G-proteins, whereas a weaker agonist will only produce enough activated receptor to activate the most efficiently coupled G-protein (i.e., highest K_G). For example, although carbachol, pilocarpine and McN A343 are all muscarinic receptor agonists, it can be shown that they produce a spectrum of maximal amounts of G-protein activation by immunoprecipitation (Matesic et al., 1991).

In general, cellular cascades consisting of sequential saturable biochemical reactions lead to progressive amplification of receptor stimuli. Under these conditions, it is possible that a given receptor stimulus will be of sufficient strength to trigger another signal in the cytosol. The relevance to receptor classification is the possibility that the strength of signal may determine the pleiotropy or lack of it, i.e., whereas a strong efficacious agonist may trigger many biochemical cascades in the cell, a weaker one (partial agonist) may induce only the most sensitive and highly amplified one. For example, the opioid agonist DADLE stimulates high affinity GTPase and also inhibits basal adenylate cyclase in NG108-115 cells. However, upon reduction of receptor stimulus through alkylation, the less sensitive response (GTPase) is eliminated, and the more sensitive one remains (Costa et al., 1988). Similar effects were observed by Saussy et al. (1989) who showed that the partial LTD4 receptor agonist LTE4 activated only a portion of the signaling system available to the receptor when it was activated by LTD4 in U-937 cells. In liver membranes, glucagon has been shown to activate adenylate cyclase as well as elevate IP_3, whereas the partial agonist des-His^1[Glu^9] glucagon amide only elevated IP_3 (Unson et al., 1989). In general, the simple demonstration of multiple versus single activation of biochemical pathways cannot be used as definitive evidence of differences of agonist effect at the receptor (see fig. 10B).

The possibility of selective G-protein activation by strength of stimulus always exists in systems where some agonists activate numerous G-proteins and others only a few. However, if the relative potency of selective G-protein activation could be shown to be different for different agonists, this would truly imply that the agonists concerned produced selective receptor activation states (see fig. 10C). For example, the PACAP receptor PACAP-R transfected into LLC PK1 cells mediates stimulation of cAMP levels and inositol phosphate production (Spengler et al., 1993). However, the relative potency of the agonists PACAP-38 and PACAP-27 is reversed for these two responses, indicating that some preferential ternary complex was formed for each agonist. Similar data have been reported for octopamine/tyramine receptors in *Drosophila*, where a clear reversal of potency for cAMP attenuation and Ca^{2+} transients is seen for octopamine and tyramine (Robb et al., 1994).

In addition to the reversal of agonist potency suggesting that stimulus trafficking can be produced by some agonists, study of the interactions of receptors with different G-proteins can be useful. For example, Molero and Miller (1991) found that two radioactive agonists for cholecystokinin receptors, cholecystokinin and the partial agonist OPE (D-Tyr-Gly-[($Nle^{26,31}$)CCK-26-32]-phenyl ester) demonstrated high affinity binding in bovine gall bladder. However, whereas ^{125}I-OPE binding was sensitive to Gpp(NH)p, high affinity binding of ^{125}I-CCK was not. These data can be interpreted as suggesting that the two agonists activate a different array of G-proteins in this preparation, the binding of some of which are sensitive to Gpp(NH)p (Molero and Miller, 1991). Similar results were obtained by Lallement et al. (1995), who showed that gastrin activation of CCK B receptors in Jurkat cells was sensitive to stable guanyl nucleotides, whereas CCK binding was only slightly affected (Lallement et al., 1995). Another related approach to this technique is the study of selective G-protein activation by agonists with specific antisera for different G-proteins (i.e., Izenwasser and Cote, 1995).

Agonist trafficking of stimulus can be measured as selective efficacy of agonist for receptor/G-protein pairs. An interesting example of this was shown by Meller et al. (1992), who showed that reversed relative efficacy of the dopamine agonists quinpirole and 3-(3-hydroxyphenyl)-N-n-propylpiperidine in the rat anterior pituitary and striatum. These data suggest that these agonists produce differences in coupling to different G-proteins in these two tissues; the reversal of relative potency (i.e., reversed intrinsic efficacy) cannot be accommodated by a strength of signal hypothesis.

There are two important implications of agonist trafficking of receptor stimulus. The first relates to the testing of new drug entities for therapeutic potential. If certain agonists preferentially couple receptors to select G-proteins, then ligands that interfere with receptors and G-protein coupling (i.e., have either positive or negative intrinsic efficacy) may be selective on the basis of the system used to test for drug activity. In this sense, the screen would be for a selected receptor/G-protein pair and not just the receptor. Parenthetically, this has relevance to site-directed mutagenesis studies aimed at defining the binding locus of molecules within receptors. One of the major methods used in these types of studies is the selective antagonism of certain agonists over other agonists. For example, the antagonism of responses to substance-P and septide by RP 67580 differs considerably in COS cells transfected with neurokinin-1 receptors (Pradier et al., 1994). These data can be interpreted as evidence that the two agonists, substance-P and septide, bind to different regions of the neurokinin receptor and that the antagonist does not access both regions equally well. However, an alternative explanation might be that the two agonists use different G- proteins to produce response and that the antagonist discerns the

receptor/G-protein pairs. A second implication of agonist trafficking of receptor stimulus relates to the potential for more selective agonists. It is possible that some agonists produce therapeutic and toxic effects by activation of many G-proteins and that the toxic effects could be reduced by eliminating some of these activations (Gettys et al., 1994).

2. Ligands with protean efficacy. The production of response from a 7TM receptor system consists of two theoretical steps, namely the activation of the receptor (production of activated receptor) and the subsequent coupling of that receptor to the G-protein. In theoretical terms, there is no reason to suppose that all ligands will produce the same effects on receptor activation and receptor/G-protein coupling. In terms of the cubic ternary complex model, the relative magnitudes of the thermodynamic multipliers α and γ may differ. This could occur if the ligand froze the receptor into a unique conformation that had different activating characteristics from the natural spontaneous active conformation. If this were to occur, then the receptor would take on new coupling characteristics with respect to coupling to G-proteins. Under these circumstances, a potentially very interesting experimental condition could exist, because the observed effects of the system would be because of the summation of the spontaneously activated and coupled receptor *and* the agonist-activated G-protein coupled receptor. Unless the two species were identical in terms of their catalytic properties, differences between constitutive activity and agonist-induced activity should be seen. Therefore, depending upon the set point of the receptor system, these compounds may function as positive agonists or inverse agonists, i.e., their efficacy would be protean (Kenakin, 1995c, 1996a). Specifically, the inverse agonism would be observed when the system was predisposed to constitutive activity, and positive agonists when the constitutive activity was low and the response emanates from agonist activation.

Such a ligand may have unique characteristics which, if detected, could in fact offer indirect evidence of a unique agonist-selective active receptor conformation. This type of behavior is modeled in figure 11A. Such simulations suggest that the change from positive to negative efficacy (it should be noted that efficacy is used to describe the combined effect of ligand and receptor as defined by Stephenson (1956) and does not describe the ligand constant intrinsic efficacy) can occur with differences in recetor/G-protein stoichiometric ratios or differences in K_{act} (as in fig. 11A). This latter factor may be effected by changes in ionic environment (Na^+ effect). Another possibility would be changes in K_G that may be approximated by changes in the coupling of the G-protein with activated receptor, which in turn may be effected by GTP availability. Therefore, there may be differences in observed agonist profile in the presence and absence of GTP (fig. 11B).

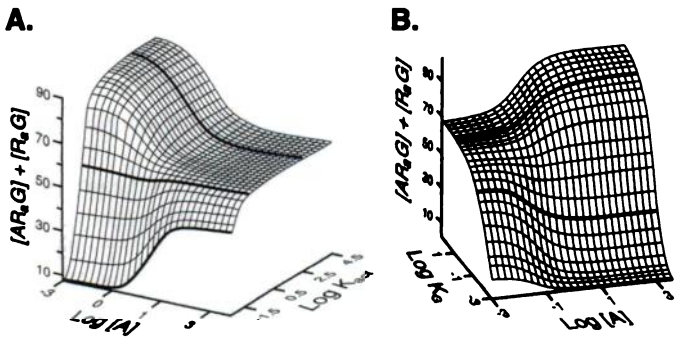


FIG. 11. Protean efficacy. Simulations for a ligand that creates a unique receptor active state which promotes response but is less efficacious than the natural activated state. Ordinates: Basal and ligand-induced response as measured by the quantity of spontaneously coupled receptor [R_aG] plus the ternary species [ARaG]. Data for the observed effects of the ligand in a range of systems. X-axis; Logarithms of molar concentration of ligand. (A) Effect of spontaneous receptor activation (Y-axis = logarithm of Log K_{act}). Under conditions of very low to undetectable spontaneous receptor activation, the ligand is a positive partial agonist. As the amount of highly active spontaneous species ([RaG]) increases (increasing K_{act}), formation of the less efficacious agonist species blocks constitutive activity, and the ligand is an inverse agonist. Systems parameters: β = 10, K_G = 0.1, [R] = [G] = 100. Ligand parameters: α = 100, γ = 0.03, δ = 1. B. Effects of cancellation of the accumulation of ternary complex (i.e., simulated effects of GTP). Y-axis: logarithm of magnitude of K_G. Systems parameters: β = 10, [R] = [G] = 100. Ligand parameters; α = 300, γ = 0.01, δ = 1.

In this type of situation, the agonist could be considered an allosteric effector of the receptor with respect to its coupling to the G-protein. Allosteric effectors for the binding of other ligands such as γ-aminobutyric acid, muscarinic agonists, dopamine and adenosine have been described (i.e., see Birdsall et al., 1995). This idea could be extended to allosteric modification of receptors toward G-proteins to describe changes in the conditional constant γ in the cubic ternary complex model for agonism. There is experimental evidence that this occurs for the adenosine receptor allosteric effector PD 81,723. Specifically, this ligand can be shown to potentiate adenosine agonism by stabilizing receptor/G-protein interaction (Kolias-Baker et al., 1994; Bhattacharya and Linden, 1995).

There are compounds that appear to have complex actions on receptors, being positive agonists in some systems and inverse agonists in others. For example, dichloroisoproterenol is a well known β-adrenergic receptor partial agonist (i.e., see Fleming and Hawkins, 1960); however, in membranes from Sf9 cells overexpressed with β_2-adrenergic receptors, dichloroisoproterenol produced inverse agonism (Chidiac et al., 1994). It is premature to postulate that protean ligands are true pharmacological entities, but if they are found to be so, they may offer a window into agonist-activated receptor states.

3. The molecular nature of efficacy. Efficacy is a molecular property that, under ideal conditions, can be

quantified for the characterization of drug activity (Besse and Furchgott, 1976; Kenakin, 1984, 1985b). What can be seen from the current models of 7TM receptor mechanisms is that efficacy can be thought of as being receptor-related and drug-related. Thus, receptors have efficacy for G-proteins as measured by the equilibrium dissociation constants of the resulting receptor/G-protein complexes (i.e., K_G, fig. 4). The effect of receptor activation on receptor efficacy is given by the conditional term β. These constants quantify the interaction of the receptor protein and G-protein in the membrane. Added to this is the influence of ligands characterized by the effect they have on receptor activation (α term), on G-protein binding (γ term) and the synergy between these two effects (δ term). Theoretically, the delineation of these molecular terms to characterize drugs and receptors would be ideal because the influence of each of these effects on receptor function is different, i.e., there may be different types of efficacy that will result in different types of response in physiological systems.

The previous points have highlighted certain ideas regarding the molecular nature of receptor efficacy. In general, it is reasonable to consider a model of a receptor conformation that exposes various portions of the intracellular loops to G-proteins and that these amino acid sequences serve to activate the G-proteins. In terms of the design of agonist ligands, it is not clear how many active receptor conformations exist in nature. It is well known that proteins are dynamic and that, while at any given instant the protein molecule may be in a distinct conformation, it most likely (at least at physiological temperatures) does not stay there and in fact goes on to explore an 'energy landscape' of different conformations. These fluctuations in conformations may be relaxations toward an equilibrium form or equilibrium fluctuations brought on by ligands (Frauenfelder et al., 1991). An analogy could be made to myoglobin that is thought to exist in two globally distinct macrostates (models of R_i and R_a) and a spectrum of conformational microstates (Frauenfelder et al., 1988). Thus, an energy landscape such as the schematic shown in figure 12A might exist for a given 7TM receptor. Thermal and other energy would control the exploration of the receptor over this energy landscape. In terms of relative populations within this scheme, figure 12B shows the relative population of the quiescent receptor system, most of the species existing in the 'inactive' (R_i) macrostate. A spectrum of potential active microstates exist. It would theoretically be possible for ligands to differentially enrich the relative populations of these various active microstates. Figure 12C shows the relative populations for a hypothetical agonist A1 and figure 12D for another agonist A2. The point would be that these microstates could have different affinities for different G-proteins; thus, agonist selective trafficking of stimulus could occur. At present, these ideas are speculative, but they offer a framework upon which to design experiments to detect agonists that are stimulus-selective.

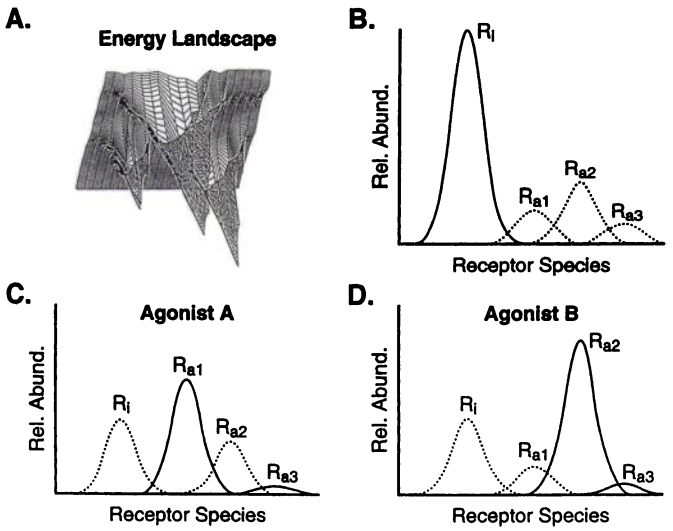


FIG. 12. Energy states of 7TM receptors. A. Simulated 'energy landscape' for 7TM receptors with differing levels of resting energy. The receptor explores this landscape as a function of thermal energy. (B) At any given instant, the relative proportions of receptor states can be illustrated with Boltzman distributions. This shows most of the receptors in the inactive [R_i] state with three putative activated states R_{a1}, R_{a2}, and R_{a3}. C. Theoretical effects of an agonist that enriches active states R_{a1} and R_{a3}. (D) Another agonist enriches active states R_{a2} and R_{a3}. It is postulated that these two different agonists present different arrays of activated receptors to the G-protein complement of the host system. From Kenakin (1996b) with permission.

Parenthetically, the energy landscape idea reconciles Burgen's initial (apparently opposing) views of conformational selection and induction (Burgen, 1966). If a particular receptor conformation was exceedingly rare and a given agonist had a high selective affinity for that conformation, then the agonist would enrich a unique conformation not found in nature in appreciable amounts. Under these circumstances, this would, for all intents and purposes, be conformational induction occurring with a selection mechanism. From this standpoint, conformational selection and induction can be seen to be extremes of the same molecular mechanism of efficacy (Kenakin, 1996b).

VIII. Quantitative Measurements on 7TM Receptors

In general, a large part of pharmacology is the measurement of drug affinity and efficacy; because these are inherently chemical terms specific to the drug and the receptor, they, in turn, can be used to classify receptors. Correct measurement of these drug properties can lead to predictions of therapeutic activity in humans. However, the delineation of systems effects on observed potency first must be achieved.

A. System-Dependent Observed Affinity

The ideal situation is to characterize receptors with neutral antagonists. If a ligand possesses efficacy, then its potency may be *system-dependent* as opposed to only *receptor-dependent*. This is because the components of the system (i.e., receptor level, G-protein composition, level of receptor activation) will affect the ligand receptor binding distribution (i.e., see equation 6). In practical terms, it may be important to detect system-dependent drug activity. This is because, although drugs theoretically are screened from the most simple and stable systems available, they eventually are used in the most complex of systems imaginable, namely the human body under pathological control. Drug discovery screens are designed for robustness and consistency and often will not detect low levels of positive or negative efficacy. However, the resulting discovered compounds are then used under in vivo conditions in which they encounter a spectrum of organs containing different densities of receptor, varying efficiencies of receptor coupling, possibly constitutive receptor foci, and different levels of endogenous agonist tone. In addition, the different membrane milieu for the receptors may contain different mixtures of G-proteins in varying ratios reacting to external hormonal input that possibly would interact (i.e., modulate or potentiate) the receptor signal. For these reasons, it is important to detect system dependence of ligand potency. If such effects are not detected, then the observed potency of a ligand will be assumed to reflect the chemical binding constant, and all differences in potency will be assumed to reflect differences in receptors. This could be dissimulating when receptors are expressed in various host systems, i.e., the potential for artifacts, because of systems effects increases. Moreover, the lack of recognition of system-dependent potency could lead to unexpected differences in activity between screening systems and therapeutic applications in humans.

One way to detect possible system dependence (i.e., efficacy of a ligand) is to measure the variation of repeated estimates of potency. For a true neutral antagonist, the only error associated with measurement would be random measurement error at a given level. If, however, systems effects bias the magnitude of the observed potency, then an added measure of error (that associated with changing relative quantities of components) might be expected in the observed measurement of ligand activity. Figure 13A shows the observed potency of a ligand with positive efficacy in 2000 simulated cell lines, i.e., computer-generated random combinations of [R], [G], and K_{act} for a given receptor/G-protein pair (K_G constant) for a ligand with a constant molecular efficacy ($\alpha = 100$, $\gamma = 1$, $\delta = 1$). As can be seen from this simulation, the observed potency is never less than the equilibrium dissociation constant of the ligand-receptor complex for the inactive receptor (K_A) but often is increased by G-protein coupling, as is commonly observed

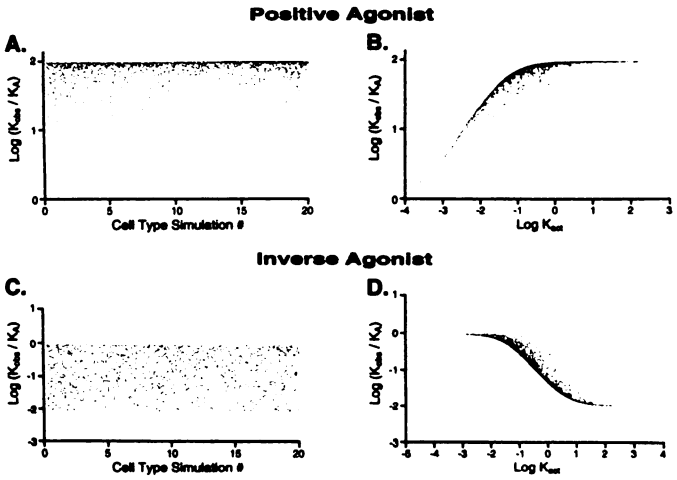


FIG. 13. Simulations of observed affinity for ligands with positive or negative efficacy in 2000 hypothetical cell lines. (A) Observed affinity (as calculated with equation 5) for a positive agonist ($\alpha = 100$, $\gamma = 1$, $\delta = 1$) in a system with varying [R], [G] and K_{act}. It was assumed that the activated receptor/G-protein coupling is favorable ($\beta K_G = 10$). (A) Ordinates: Log (K_{obs}/K_A). Abscissae: Computer simulation number, also a unique randomized configuration of levels of [R], [G] and K_{act} which might correspond to host cell type. (B) Data shown in A grouped as a function of log K_{act}. (C) Same analysis as in A but for an inverse agonist ($\alpha = 0.01$, $\gamma = 1$, $\delta = 1$). (D) Data grouped as a function of log K_{act}.

with agonists. Figure 13B shows the correlation of the observed potency with the working constant for receptor activation (i.e., K_{act}).

An identical analysis was done for an inverse agonist of $\alpha = 0.01$, $\gamma = 1$, $\delta = 1$ (fig. 13C and D), where it can be seen that the observed potency is *decreased* by receptor/G-protein coupling effects. However, the observed potency is never above K_A. These simulations show the expected variation of observed potency of ligands with efficacy (either positive or negative). What should be stressed is that the estimations in each particular cell system are correct for that particular milieu of receptor and G-protein. Moreover, it can be shown that the magnitude of the efficacy is directly proportional to the variation in observed potency (i.e., weak agonists will vary less than strong agonists). This can readily be inferred from equation 6 and the placement of ligand constants α, γ, and δ. Therefore, one practical test of system dependence is the presence of an inordinately high error for the mean measurement of affinity. If the measured affinity of a given ligand is found to be variable with repeated testing (i.e., in a series of transient expression systems or on the receptor when it is expressed in different cellular hosts), then this might imply that the ligand possesses efficacy that causes different interactions of the receptor with G-proteins in different systems.

B. The Manipulation of Receptor Systems

Just as it is possible to experimentally manipulate some aspects of signal strength from natural receptors

(Kenakin, 1984), there are techniques becoming available to modify the strength of recombinant receptor expression signals; i.e., receptor gene induction, increased expression (Collins et al., 1991; Charness et al., 1983, 1986, 1993; Gianoulakis, 1989; Hu and Hoffman, 1993). For example, steroid hormones are well known to affect gene transcription for some receptors (Collins et al., 1989; Davies and Lefkowitz, 1984; Emorine et al., 1987). In contrast, estrogen reduces the number of α_2-adrenergic receptors in the high affinity state in rat hypothalamus (Karkanias and Etgen, 1994). Short-term exposure to β-adrenergic receptor agonists or cAMP analogues can produce elevations of β_2-adrenergic receptor mRNA (Collins et al., 1988). Chronic treatment of SH-SY5Y cells with low efficacy μ-opioid agonists increases the abundance of G-proteins (Ammer and Schulz, 1993). Cotransfection of vectors containing cDNA for the human β_2-adrenergic receptor and for dihydrofolate reductase led to a control of expression levels for the receptor by stepwise increases in methotrexate concentration in the culture medium of CHO cells (Lohse, 1992). This technique, however, was cell-specific, as identical conditions in HeLa cells led to cell death. In cyclosporin A-induced hypertensive rats, increased gene expression for angiotensin type II receptors has been reported (Iwai et al., 1993). Another means of affecting receptor transcription has been shown for 5-HT type 2 receptors, where chronic treatment with antagonists led to reductions in both receptor and mRNA (Toth and Shenk, 1994)

In general, there is an increasing body of experimental evidence to indicate that, by the use of various promoters and plasmids, the control of receptor stoichiometry in host cell systems can be achieved. A strategy using adenovirus-mediated gene transfer has been used with several mammalian genes (Mulligan, 1993), including the expression of thyrotropin-releasing hormone receptors in several cell lines (Falck-Pedersen et al., 1994). Receptor expression can be induced, as was the case for α_1-adrenergic receptor transfected with isopropyl-β-D-thiogalactoside-inducible vectors in SK-N-MC cells (Esbenshade et al., 1995a, b). One of the most promising areas in this technology is the co-expression of receptors and G-proteins to create 7TM receptor systems. For example, cotransfection of Rat-1 fibroblasts with cDNA for α_{2A}-adrenergic receptors and cDNA for G_{o1} produces activation of this foreign G-protein in the transfected cell (Grassie and Milligan, 1995). Similarly, the cotransfection of $G_{\alpha s}$-subunit with secretin receptors greatly increased the amount of high affinity ternary complex coupling seen with ^{125}I-secretin (Ishihara et al., 1991). Co-expression of G-protein with receptors also has been carried out successfully with 5-HT_{1c} receptors (Quick et al., 1994), 5-HT_{1A} receptors (Butkerait et al., 1995), somatostatin-3 receptors (Law et al., 1994), α_2-adrenergic receptors (Coupry et al., 1992) and opioid receptors (Tsu et al., 1995).

The measurement of drug affinity and efficacy uses techniques unique to two broad disciplines, radioligand binding and those specific for functional systems. It is worth considering these separately.

C. Radioligand Binding

Binding studies offer a unique perspective on drug receptor interaction, in that theoretically the complex between the ligand and the receptor can be studied directly. The most simple model upon which all analyses initially are based is the Langmuir adsorption isotherm (Langmuir, 1916). The basic premise of this model is that molecules bind to an inert surface and that the equilibrium dissociation constant of the molecule/surface complex is a chemical term dependent only upon the nature of the two entities bound to each other. In fact, Langmuir derived his equation in terms of the actual area bound and not bound by an adsorbent material onto a surface. If this can be shown to be the case for a drug and receptor, then this chemical term assumes great importance, as it can be used to characterize that receptor in any tissue in which it resides. There are two major areas for error in the classification of receptors with ligand binding. One is the introduction of systems effects because of undetected ligand efficacy, and the second is heterogeneity of receptor populations.

Tests for Langmurian kinetics generally ask the question, "Is the observed binding consistent with the interaction of a ligand with a single stable receptor population?" The two windows into ligand binding behavior are by saturation binding of a radioligand and by inhibition binding of a fixed amount of radioligand by a nonradioactive ligand. The first expectation of Langmurian kinetics is that the binding curves for both types of experiment be monophasic and have a Hill coefficient not significantly different from unity. There have been numerous publications on mechanisms and nuances that produce complex behavior of binding curves (i.e., see Limbird, 1996; Swillens et al., 1995) that need not be reiterated here. Interestingly, the very nature of transfected cellular expression systems in which the receptor levels may be high can lead to artifacts with the use of standard binding models that assume that the concentration of radioligand is not altered by receptor binding (Swillens, 1995).

In general, two conditions can lead to complex binding curves or nonadherence to Langmurian kinetics: the presence of efficacy in a ligand that interacts with the receptor and one or more G-proteins, and/or the presence of a mixture of binding sites, either multiple stable binding sites (as in splice variants of a receptor) or multiple pre-existing coupling states. The latter condition (multiple coupling states) requires that the ligand have efficacy before differences in observable binding can be seen.

1. Saturation binding experiments. The study of expressed receptors in surrogate cell lines theoretically

The cell type used for expression could be very important for the functional study of receptors. For example, whereas transfection of the isoform for the somatostatin receptor mSSTR2A into CHO-K1 cells showed somatostatin-mediated inhibition of adenylate cyclase (Strnad et al., 1993; Vanetti et al., 1993b), stable transfection of the same receptor in CHO-DG44 cells or transient transfection into COS-1 or HEK 293 cells failed to show this same functional effect (Rens-Domiano et al., 1992; Law et al., 1993). These data may partially be explained by the fact that $G_{\alpha i1}$ protein (Tallent and Reisine, 1992), found to be necessary for somatostatin receptor function in AtT-20 cells, is present in CHO-K1 cells (Gerhardt and Neubig, 1991), but not in CHO-DG44 cells (Rens-Domiano et al., 1992).

1. New technologies for cellular systems. One obvious advantage of functional experiments is the increased sensitivity obtained by using the biochemical cascades in cells to amplify low levels of stimulus. The sequential relationship between saturable biochemical reactions in cells leads to amplification of minute membrane signals. For example, carbachol in CHO cells shows a 2.6-fold amplification between receptor occupancy and phosphoinositide hydrolysis and a further 88-fold amplification from PI hydrolysis to nitric oxide release (Wang et al., 1994).

Exquisite selectivity can be gained with functional preparations because many of them yield strong signals (maximal responses) with occupation of very small fractions of the receptor population. Figure 16 shows a schematic diagram depicting a typical 7TM receptor biochemical cytosolic cascade. Technology now has allowed viewing of the consequences of a drug-receptor interaction at various stages along this pathway. Denoting the interaction of the agonist and the 7TM receptor as reaction 1, the first consequence is the activation of G-proteins in the cell membrane (step 2). This can be viewed several ways, including as activity of the intrinsic GTPase activity of the G-protein and binding of radioactive GTP analogues to G-proteins (i.e., Freissmuth et al., 1991; Traynor and Nahorski, 1995; Odagaki and Fuxe, 1995; Thomas et al., 1995). Another first step in this cascade is the direct activation of ion channels with $\beta\gamma$ subunits of G-proteins resulting from G-protein activation (Jelsma and Axelrod, 1987; Okabe et al., 1990).

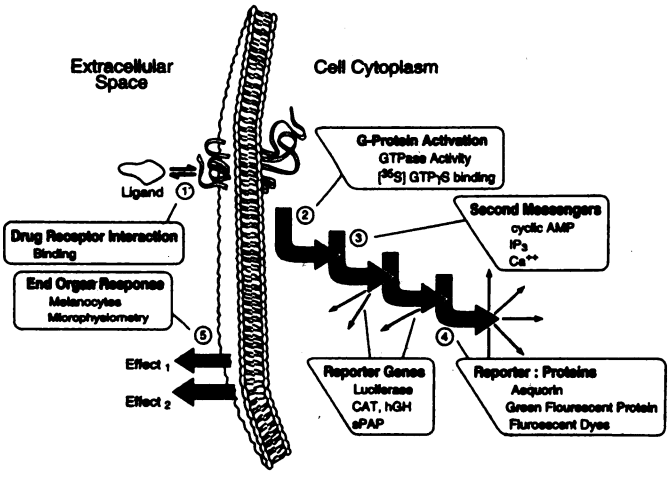


FIG. 16. Stimulus-response cascades in the cytosol for a 7TM receptor. Steps refer to the binding of a ligand to the receptor (1), the activation of a G-protein (2), the production of a second-messenger (3), the interaction of the second-messenger with cytosolic mechanisms (and detection with a reporter system (4)), and the complex observable end-organ response (5).

Further on in the sequence is the production of second-messengers such as cAMP or IP_3 (step 3). Electrical readouts of response from receptors expressed in oocytes is a well established technology (for typical examples see Wank et al., 1992; Racke et al., 1993; Kubo et al., 1986; Yu et al., 1991; Sundelin et al., 1992; Yakel et al., 1993; Maricq et al., 1991; McEachern et al., 1991; Minami et al., 1993). Interesting variants on the use of oocytes for response measurements is the co-injection of antisera to identify which G-proteins are activated by agonists (McFadzean et al., 1989; Harris-Warrick et al., 1988; Jones et al., 1994). New approaches coupled with molecular biology have extended these approaches to yield a new collection of functional assays for the study of efficacy in transfected cells. A rapidly expanding technology is in the field of reporters for cytosolic second-messengers. Thus, the next step in the cascade can be viewed with reporters for receptor-active cytosolic products. Basically, there are two types of reporters; reporter genes and reporter proteins. The former produce a readout of receptor activation by introducing a gene that is affected by the second-messenger. Under these circumstances, receptor activation leads to increased transcription and expression, and the magnitude of this secondary response is quantified some hours later. A second type of reporter system is the introduction (now by genetic means) of reporter proteins that signal the elevation of second-messengers directly in the cytosol.

The use of fusion genes is increasing as a means of assessing gene expression and thus, indirectly, assessing increases in cytosolic second-messengers. With this method, a promoter activator binding site or enhancer sequence is attached to a gene directing synthesis of a reporter molecule. The quantity of the reporter molecule in the cytosol thus becomes an indicator of the avidity of gene expression which, in turn, indicates the level of second-messenger present in the cytosol during expression. For example, elevation of intracellular cAMP or calcium (by calmodulin kinase) results in phosphorylation, and subsequent activation of the transcription factor cAMP response element binding protein (Gonzalez and Montiminy, 1989; Yamamoto et al., 1988). The level of the reporter can be assayed either from the cell lysate or culture medium (for secreted reporter proteins). This latter factor is relevant in terms of whether the response is monitored in real-time or stop-time techniques.

the concentration of radioligand is a straight line. Therefore, even if the K_d and/or the K_i for the drugs are not known, deviation from the model can be detected by observing the relationship between the IC_{50} and [A*]. This then becomes a very simple test for receptor and/or binding-site heterogeneity along various regions of the saturation binding curve. If the relationship between radioligand concentration and IC_{50} is not linear, this would imply that something other than, or in addition to, binding to a single static population of sites was occurring.

3. Binding and receptor biochemistry. The ability to label and track receptor protein has led to many techniques for the study of receptor structure, state and, relevant to the discussion of receptor classification, coupling to G-protein. For examples, receptors can be solubilized and immunoprecipitated with antisera for G-proteins (i.e., Law et al., 1991; Chatterjee et al., 1993; Gurdal et al., 1995) or receptors (Matesic et al., 1989, 1991). Similarly, antisera directed the C-terminal region of G-proteins have been used to disrupt agonist activation of receptors and/or high affinity binding (McKenzie and Milligan, 1990; Milligan et al., 1995b). Antisera directed to the N-terminal end of G-proteins have been used to co-immunoprecipitate receptors with G-proteins (Okuma and Reisine, 1992; Law et al., 1991). The cross-linking of receptor proteins to G-proteins also has enabled the study of receptor/G-protein interaction (i.e., Kermode et al., 1992). Another approach is to observe agonist-induced incorporation of [α-^{32}P]AA-GTP (a GTP analogue azidoanilidido[α^{32}P]GTP) into various G-proteins (Prather et al., 1994; Palmer et al., 1995). A novel method to study the activation of G-proteins by agonist-stimulated receptors is the observation of the half-time for degradation of G-protein in the presence and absence of agonist (Wise et al., 1995). Agonist affinity columns have been used to purify receptor/G-protein complexes as well (Munshi and Linden, 1989; Munshi et al., 1991). Another method of purification is with a biotinylated radioactive agonist for receptor binding followed by separation over a strepavidin affinity column (Eppler et al., 1992; Luthin et al., 1993).

Other methods use pertussis toxin (PTX)- or cholera toxin (CTX)-catalyzed ribosylation of G_α subunits. Receptor and agonist activation of PTX-sensitive G-proteins can be detected by taking advantage of the fact that PTX preferentially catalyzes ADP ribosylation of the G-protein heterotrimer (Milligan, 1987). Thus, agonist activation of a particular G-protein, if PTX-sensitive, will be diminished (Brass et al., 1988). In contrast, CTX interacts preferentially with the free α-subunits (Milligan and McKenzie, 1988); thus, agonist and receptor activation of a CTX-sensitive G-protein would increase adenosine diphosphate ribosylation (i.e., Milligan and McKenzie, 1988; Klinz and Costa, 1989). Theoretically, these methods may furnish a direct way to detect agonist trafficking of receptor stimulus if it could be shown that different agonists produce different patterns of ternary complexation.

D. Functional Studies in Receptor Classification

Early receptor classification relied completely on functional experimentation, and a great deal of pharmacology was concerned with the cancellation of tissue effects (usually through the null method). The introduction of receptor binding technologies has added another dimension to receptor pharmacology, and new insights into drug-receptor interaction were obtained. Now, the availability of new technologies and the advancement of biochemical techniques for receptor study have expanded the realm of receptor research back into functional receptor experiments beyond isolated tissues. It is now possible, by a variety of mechanisms, to study agonist efficacy in membrane receptor preparations and in cell culture (vide infra). This allows the considerable theoretical advantages of functional systems to be exploited. Many of these advantages (and disadvantages) are common to all methods of functional experimentation.

Before these are discussed specifically, there are several tacit assumptions that should be considered when transfected receptors and host cells are assembled for functional assays. The fact that the correct signal transduction apparatus may exist in the membrane of some cells still does not ensure that the biochemical mechanisms for transforming the stimulus to a cellular response are present as well. For example, whereas transfection of human dopamine D_3 receptor cDNA into CHO-K1, SK-N-MC, or CCL1.3 cells produces high affinity radioligand binding (with sensitivity to GTP analogues, indicating G-protein coupling), no effects on cAMP accumulation, inositol phosphate production or arachidonic acid release was observed with dopamine agonists (MacKenzie et al., 1994). Similarly, whereas β_1-, β_2- and β_3-adrenergic receptors co-exist in hamster brown fat cells, it appears that only the β_3-adrenergic receptors participate in thermogenesis (Zhao et al., 1994). It cannot be assumed that the activation of a receptor system to elevate the cytosolic level of a second-messenger automatically will make the intracellular second-messenger accessible to all parts of the cell machinery. For example. both dopamine and isoproterenol elevate intracellular cAMP in transfected kidney CV_1 cells. However, although the dopaminergic elevation of cAMP produces a translocation of transfected progesterone receptors from the cytoplasm to the nucleus, isoproterenol was *not* able to produce this same effect (Power et al., 1991). The need for other systems for the production of a functional response also can be very important. For example, transfection of the rat neurotensin receptor into 293 cells was insufficient to produce a functional system for producing cyclic guanosine monophosphate synthesis until nitric oxide synthetase cDNA was subcloned into the expression vector (Slusher et al., 1994).

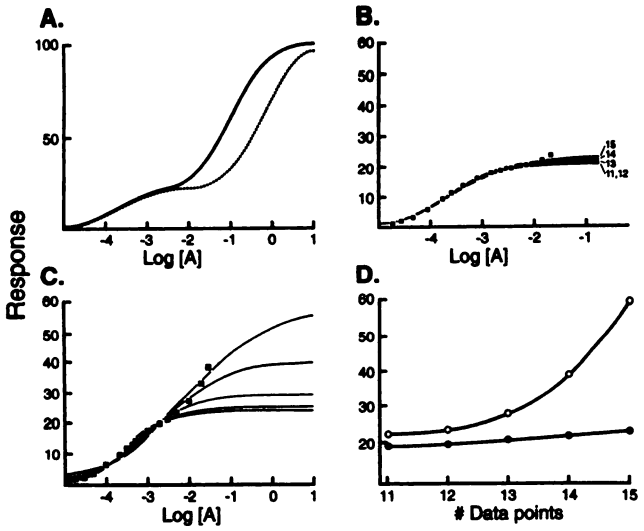


FIG. 15. Total saturation binding curves for two agonists with varying intrinsic efficacy to promote formation of the ternary complex (i.e., observed as high affinity binding). Ordinates; Production of [ARaG] and [AR] by the agonist. Abscissae; Logarithms of molar concentration of agonists. (A) Complete saturation curves for two agonists of varying efficacy; Solid line $\alpha = 100$, $\gamma = 100$, $\delta = 1$ and dotted line $\alpha = 10$, $\gamma = 1000$, $\delta = 1$ in a G-protein limited expression system ($\beta = 10$, [R]=100, [G]= 20, $K_G = 0.1$). (B) Statistically fit saturation curves for datasets of varying size for dotted line agonist in A. Numbers next to curves represent the number of datapoints used for the fit. (C) As for B but for solid line agonist in A. For this ligand, the size of the dataset severely affects the estimated B_{max}. (D) The estimated B_{max} values for the two agonists (dotted line agonist represented by filled circles) as a function of the number of datapoints used for the fit.

coupling to at least two different G-proteins. Both of these conclusions greatly affect how the receptor data from such a system is interpreted; therefore, the veracity of different B_{max} values becomes paramount.

In view of data clearly showing that receptors can be promiscuous with respect to the G-proteins with which they interact and also, that the expression level of receptors can dramatically alter G-protein coupling, the possibility of aberrant receptor/G-protein coupling must be considered in all transfection experiments. It is difficult to gauge the 'correct' physiological receptor/G-protein interaction because mass stoichiometry has no meaning within the constraints of the membrane. As discussed by Neubig (1994), cytoskeletal elements in the membrane and other factors can severely limit access of receptors to G-proteins. Therefore, a 100-fold greater bulk amount of G-protein over receptor is meaningless in terms of the actual G-protein accessed by the receptor in a membrane. There may, in fact, exist microdomains of receptors and G-proteins in cell membranes with assymetrically distributed receptors or G-proteins (Van Zastrow et al., 1993; Keefer et al., 1994; Neer, 1994). There are examples of situations in which the relative quantity of high affinity binding sites is limited by the availability of G-protein in the surrogate cell system. For example, as discussed earlier, in COS cells transfected with cDNA encoding the rat secretin receptor, only 1.7% of the sites binding ^{125}I-secretin were of high affinity. However, cotransfection of G_s protein increased the relative quantity of high affinity binding to 15% of the total (Ishihara et al., 1991). It should not be assumed that receptors have the same access to G-proteins in a membrane and will universally couple to those G-proteins (i.e., converse to receptor promiscuity). For example, α_{2B} and α_{2A} adrenergic receptors expressed transiently in human choriocarcinoma (JEG-3) cells have access to the same complement of G-proteins yet selectively couple to different effectors (Pepperl and Regan, 1993). Similar results were obtained by Hughes et al. (1986), who showed that, although muscarinic receptors normally interact with G_i, they do not do so in 1321N1 astrocytoma cells, even though a functionally active G_i can be shown with adenosine receptors.

2. Inhibition experiments. Another standard binding assay used to classify ligands and receptors is the quantitative inhibition of the binding of a fixed amount of radioligand to a receptor by a nonradioactive ligand. This procedure is essentially based on the mathematical model for simple competitive antagonism first presented by Gaddum (1937):

$$\frac{[A \cdot R]}{[R]} = \frac{[A]/K_A}{[A]/K_A + [B]/K_B + 1} \qquad [7]$$

in which the concentration of the ligand being blocked (i.e., radioligand in binding studies) is denoted by A, the equilibrium dissociation constant of the complex between A and the receptor denoted K_A, the blocking ligand denoted B, and the equilibrium dissociation constant of the complex between the blocking drug and receptor denoted by K_B. From this model comes Schild analysis for functional studies (Arunlakshana and Schild, 1959; vide infra) and the models for calculating the equilibrium dissociation constants for ligand/receptor complexes in binding studies (denoted K_i). Rearrangement of equation 7 can give a very useful, experimentally accessible relationship between the concentration of radioligand and the concentration of nonradioactive ligand required to block it. Thus, it can be shown that the concentration of ligand that reduces the binding of a given concentration of radioligand by 50% (denoted as the IC_{50}) is related to the concentration of radioligand by the following relationship:

$$\frac{IC_{50}}{K_i} = \frac{[A^*]}{K_d} + 1 \qquad [8]$$

in which K_d denotes the equilibrium dissociation constant of the radioligand-receptor complex obtained from saturation binding studies. The equation often is referred to as the Cheng-Prusoff relationship (Cheng and Prusoff, 1973). The important point to note about this equation is that the relationship between the IC_{50} and

should have the advantage that a single protein is expressed; therefore, a subtle presence of a mixture of receptor subtypes will not complicate the analysis. Under these circumstances, a monophasic saturation curve representing ligand binding to a single population of receptors is expected (in the absence of ligand efficacy). However, the transfection of cells with genomic receptor clones may not always lead to the expression of pure populations of receptors. For example, a genomic clone for the bradykinin receptor isolated from a mouse cosmid library leads to the expression of an apparently mixed population of B_1 (30%) and B_2 (70%) bradykinin receptors in COS-7 cells (McIntyre et al., 1993). While this appears not to be due to RNA splicing, it is not clear whether the mixed population is the result of incomplete post-translational modification or RNA editing. An alternative possibility would be the promiscuous coupling of the expressed receptor with different G-proteins.

This raises a practical point in receptor classification, namely the differentiation between heterogeneous receptors, receptor binding states (with G-proteins) and heterogeneous binding *sites* on the same receptor molecule. The technique of site-directed mutagenesis has opened a new era for the study of structure-activity relationships. Thus, the effects of genetically induced single-point mutations in receptors on the affinity of different ligands can be used to delineate separate loci of binding of different molecules on the same receptor. Notable examples of this approach are the different binding sites for peptides and nonpeptides on peptide receptors (i.e., see Perlman et al., 1995; Gether et al., 1993, 1994, 1995; Xue et al., 1994; Schwartz, 1994). An important tool in this endeavor is the saturation binding curve of different ligands because, in theory, these allow the ability to *count* binding sites as well as determine affinity. Therefore, a simple test for the assumption of different binding loci on the same receptor is the criterion that the *number* of sites for both selective ligands be the same (fig. 14A). Failure to demonstrate this leads to conclusions of different ligand-induced receptor conformations interacting with different G-proteins to produce different binding species in the membrane (fig. 14B). Especially in expression systems in which the stoichiometry of receptor to G-proteins may encourage promiscuous receptor coupling, the potential for multiple agonist activated ternary complexes with different G-proteins should be considered. If these agonist-selective species are formed, then separate structure-activity relationships could be observed for them, i.e., agonist selective functional antagonism, or binding affinities, could be observed. This possibility should be eliminated before consideration of specific receptor regions for binding.

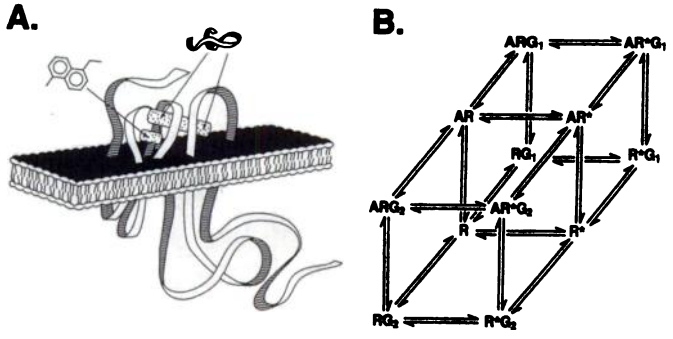


FIG. 14. Two potential views of selective agonist activation of 7TM receptors. (A) Two ligands bind to two separate allotopic sites on the receptor. (B) The receptor can form two separate complexes with two different G-proteins. The agonists differentially direct the receptor toward each G-protein, respectively.

The saturation maximal asymptote for radioligand binding can be useful to differentiate selective sites from selective confirmational species. However, care must be taken in the use of B_{max} values because of their inherent inaccuracy (Klotz, 1982; Klotz and Hunston, 1984). Usually, the maximal asymptote of a saturation binding curve is difficult to define with real data, and the shape of the saturation curve is used to estimate its value. However, in the case of agonists being tested in a possibly overexpressed receptor systems, high affinity binding measures the G-protein coupled receptor and the B_{max} value depends upon the ratio of receptor to G-protein available for coupling. In highly expressed systems, the receptor densities may outstrip the G-protein coupling capability, leading to a relatively small population of high affinity binding sites when compared with total receptor binding. Under these circumstances, the magnitude of the ratios between the high and low affinity binding sites could greatly affect the estimated B_{max} by saturation analysis. For example, figure 15A shows the saturation binding curve for a hypothetical agonist with efficacy values $\alpha = 10$, $\gamma = 1000$, $\delta = 1$ in a system of limited G-protein ([R]/[G] = 3.3). The delineation between the apparent high and low affinity sites for this agonist are clear, and the B_{max} estimate from data points is relatively immune to the size of the dataset. Figure 15A also shows the saturation curve for another agonist with a less clear delineation between the apparent high and low affinity sites ($\alpha = 100$, $\gamma = 100$, $\delta = 1$). For this agonist, the estimated B_{max} value depends very much on the size of the dataset in that, the higher the concentrations of radioligand that are used, the further up the secondary phase of the curve goes the binding (see fig. 15C). This is not a relevant issue for the agonist with the more clearly delineated biphasic binding (fig. 15B); however, the dependence of the size of the dataset for the two agonists can cause an increase in the apparent B_{max} (fig. 15D). It can be seen from these simulations that complex saturation curves should be analyzed carefully before conclusions regarding the relative size of receptor populations are made. The conclusion drawn from different B_{max} values is far reaching in an expression system because it implies the production of either two different expressed species, or that the receptor is

Reporter assays can be radioactive or nonradioactive. The firefly luciferase reporter system uses a bioluminescence reaction catalyzed by luciferase and luciferin. The intensity of the light observed is an indirect estimate of the efficiency of transcription of the luciferase gene. This can be particularly useful if a reporter cell line is established into which receptors could be transfected. For example, a reporter cell line containing the reporter *Photinus pyralis* luciferase gene (De Wet et al., 1987) under the transcriptional control of either a regulatory sequence responsive to cAMP (Himmler et al., 1993) or IP_3/diacylglycerol (Weyer et al., 1993) has been developed and used to study the function of transfected 5-HT_2 receptors (Weyer et al., 1993), NK1, NK2, NK3 receptors (Weyer et al., 1993; Stratowa et al., 1995), dopamine D_1 and D_5 receptors (Himmler et al., 1993), and muscarinic m1 and m4 receptors (Migeon and Nathanson, 1994).

Another nonradioactive reporter assay uses β-galactosidase (coded from the *E. coli* lac Z gene fused to a cAMP responsive element); the level of this reporter is assayed colorimetrically or fluorometrically from photoactive substrates. This method has been used to assay the function of receptors linked to G_s and G_q (Chen et al., 1995). Similarly, α_{1B}-adrenergic, m4 muscarinic, NK1 neurokinin and trkA neutrotrophin receptors transfected into NIH 3T3 cells yielded functional responses with this reporter (Messier et al., 1995). Some reporter assays use radioisotopes as in the chloramphenicol acetyltransferase assay. In this method, radioactive chloramphenicol is incubated with cells for a period of time after which the acetylated and nonacetylated forms of the substrate are measured. This approach has been used to study α_2-adrenergic receptor function in JEG-3 cells (Pepperl and Regan, 1993). A similar radioactive reporter assay uses human growth hormone (Selden et al., 1986).

Some reporter assays use secreted products and thus can be used for real-time assays. For example, a gene that encodes a truncated secreted human placental alkaline phosphatase (SPAP) can be used for colorimetric or bioluminescent assays (Berger et al., 1988). The levels of the secreted SPAP are directly proportional to SPAP mRNA and protein (Cullen and Malim, 1992). The secretion aspect of this assay is advantageous in that the cells are not disturbed during the assay; therefore, results can be obtained in real time, the background signal is nearly absent and the assays can be automated.

Reporter assays also have been developed for in vivo use. Under these conditions, reporter proteins can be encoded in transfected reporter genes and the cells made to express them in situ. Thus, green fluoresent protein (Chalfie et al., 1994) can be expressed in cells and used to monitor gene expression. The availability of luciferase substrates capable of crossing the cell membrane (i.e., caged luciferin) theoretically allows the use of the luciferase reporter assay in real time (Bronstein et al., 1994; Yang and Thomason, 1993).

As well as reporter genes for the measurement of cellular response, reporter proteins have been used. These molecules directly signal the level of cytosolic messengers such as Ca^{2+} (i.e., aequorin, Fura dyes) or other ions (Tsien, 1989). These can be introduced into the cells (i.e., microinjection of aequorin into oocytes, Giladi and Spindel, 1991) or co-expressed in the cell. The use of green fluorescent protein may be extremely versatile. This bioluminescent protein, when excited with light at 396 nm, will emit light at 508 nm. No preparation is required in that the cells need only to be illuminated with light and the resulting luminescence measured. The level of luminescence is directly proportional to the level of green fluorescent protein; thus, it can be used as a reporter for any inducible promoter (i.e., cAMP or diacylglycerol reporter genes).

There also are functional assays recording more complex responses from cells. For example, the measurement of cellular metabolism by microphysiometry allows a broad range of cellular responses to be measured. The concept relates to the fact that the rate of cellular metabolism is directly linked to the hydrogen ion extrusion by the cell, and this can be measured as the pH in the medium surrounding the cell. This is an extremely valuable technique because it can be used for virtually any cell type that can be sustained in culture (McConnell et al., 1992; Hafeman et al., 1988). Figure 17A shows the effects of human calcitonin on type 2 human calcitonin

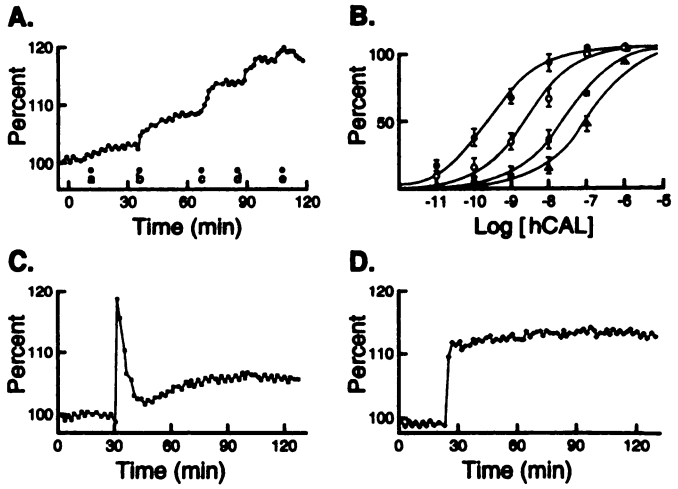


FIG. 17. Cytosensor microphysiometer responses for HEK 293 cells transfected with human calcitonin receptor type 2. (A) Cumulative dose-response curve for human calcitonin. Ordinates: percentage increase of basal cellular hydrogen ion secretion. Abscissae: Time in min. a = 10 pM, b = 100 pm, c = 1 nM, d = 10 nM human calcitonin, and e = 100 nM salmon calcitonin. (B) Schild analysis for human calcitonin responses. Responses in the absence (filled circles, n = 12) and presence of various concentrations of peptide calcitonin antagonist AC512 (Watson et al., 1995) 10 nM (circles, n = 12), 100 nM (filled squares, n = 14), and 300 nM (open triangles (n = 12). (C) Cytosensor response to 1 nM human calcitonin in a high expression HEK cell line (28,000 fmol/mg protein receptors). (D) Cytosensor response to 1 nM human calcitonin in a low expression HEK cell line (65 fmol/mg protein).

receptors expressed in HEK 293 cells. As can be seen from this figure, a cumulative concentration-response curve can be obtained from this system. Figure 17B shows the dextral displacement of human calcitonin dose-response curves by the peptide calcitonin antagonist AC512 (Watson et al., 1995). The resulting Schild analysis yields a linear Schild regression with a slope not different from unity. Figure 17 C and D show the effects of receptor expression level on steady-state response and underscore the value of real-time data and the potential problems with high expression levels. Although a low receptor density yields a monotonic response pattern with a sustained steady-state response (Clone 134–4-7: fig. 17D, 65 fmol/mg protein), the high receptor expression clone (Clone 134–2-23, fig. 17C, 28,000 fmol/mg protein) shows a triphasic response. This is consistent with promiscuous coupling of the calcitonin receptor to different G-proteins (Horne et al., 1994) to produce conflicting signals and complex responses. In general, high receptor expression levels may not be desirable for functional experiments.

There are alternative methods to detect ligand intrinsic efficacy biochemically or in cell lines. For example, *Xenopus laevis* melanocyte cell lines can be used to study the recombinant activity of G-protein receptors that modulate either cAMP or phosphoinositide production. Specifically, the dispersion or aggregation of pigment-containing melanosomes is affected by second-messengers and thus can be traced by observing light transmission at 620 nm (McClintock et al., 1993; Karne et al., 1993; Graminski et al., 1993; Potenze et al., 1992, 1994; Lerner, 1994).

The central dogma regarding functional receptor pharmacology is the idea that, if the end organ response is the result of a succession of saturable biochemical functions, then an amplification of the original signal is produced. In terms of drug development, this may be an advantage because an extremely weak initial signal may become measurable if viewed further on down the series of reactions. An example of this is the extremely weak activity of the β-adrenergic receptor partial agonist prenalterol on adenylate cyclase and the powerful end-organ cardiac response (Hedberg et al., 1982). Another example is the rate of myocardial relaxation of isolated cardiac preparations. In the guinea pig atrium, the dose-response curve for increased rates of myocardial relaxation can be detected at concentrations of externally applied dibutryl cAMP, which do not produce any other physiological effect on the preparation (fig. 18; Kenakin et al., 1991). This carries over into the study of weak partial agonists as well. Thus, while the β-adrenergic receptor partial agonist prenalterol produces nearly negligible inotropic activity in guinea pig left atria, a powerful myocardial relaxant effect can be observed. This illustrates the idea that, by selection of the biochemical readout from the cytosolic cascade, measures of efficacy can be obtained (Kenakin et al., 1991).

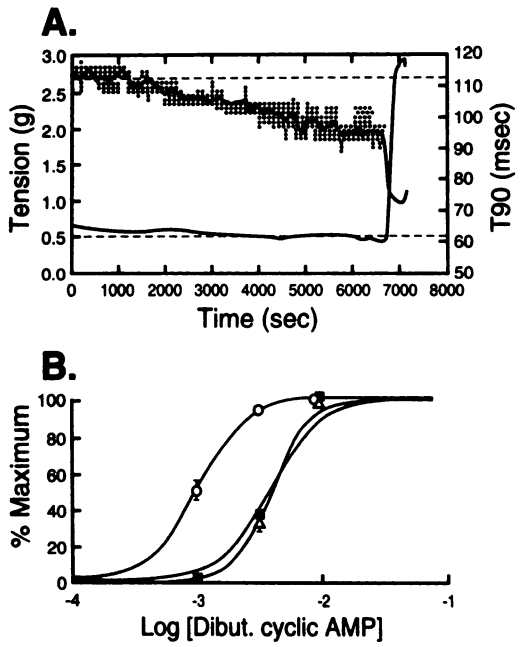


FIG. 18. Effects of dibutryl cAMP on guinea pig atrial function. (A) Temporal response to 1 mM dibutryl cAMP. Upper tracing quantifies the T_{90} (time to 90% relaxation after twitch contraction in msec); lower tracing shows lack of inotropic response (in g tension) in the same preparation. (B) Dose-response curves for myocardial relaxation (open circles) and inotropy measured at peak response (filled circles) and at 90 min (open triangles). Data expressed as the percentage of the maximal response to 10 μm forskolin in the same preparations. From Kenakin et al. (1991).

Reporter systems can be particularly sensitive for the detection of agonism because the second-messenger produced, in the case of the luciferase assay, goes on to initiate a series of reactions, including transcription, that result in the expression of luciferase. The fact that sequential biochemical reactions are involved leads to considerable amplification.

On the other end of the spectrum, increased sensitivity also can bring increased complexity, leading to complicating cross-reactions that may obscure the signal. With the measurement of end organ responses, or even responses that are processed by cytosolic reactions, can come a loss in fidelity of the signal.

Another major advantage of functional studies is that many of the techniques yield data in real time as opposed to the use of arbitrary windows of reaction time (although there are binding techniques such as fluorescence cytometry that allow binding studies in real time as well, i.e., Fay et al., 1991; Heithier et al., 1994; Neubig and Sklar, 1993). Both of these strengths can be especially important in functional studies of cell lines transfected with receptors because of the phenomenon of overexpression. If there is altered stoichiometry between the receptors and G-proteins, then aberrant coupling may produce conflicting physiological responses. For example, different levels of α_{2A}-adrenergic receptor expression levels in CHO cells produce different re-

sponses to the α_2-adrenergic receptor agonist UK-14304. At a receptor level of 1 pmol/mg protein, a dose-dependent inhibition of adenylate cyclase activity is obtained, whereas at 5 pmol/mg protein receptor, a biphasic inhibition followed by stimulation is observed (Eason et al., 1992). In this case, further analysis indicated that the receptor coupled to G_i at low levels and both G_i and G_s at higher levels. When these complex responses occur in expressed systems, unless the time course for activation of the pleiotropic pathways is identical, a corresponding complex temporal pattern of response will be obtained as well. Thus, responses may vary both with concentration and time. The additional complication of temporal organization of response makes the observance of response in real time essential. Responses may be temporally complex under these circumstances and, without visualization of the time course of response, steady states may be impossible to measure. Stop-time experiments with no knowledge of the kinetics of response clearly could be very dissimulating.

One of the theoretical disadvantages of stop-time techniques such as reporter genes is the fact that the kinetics of response production and the reporting of that response production may affect the magnitude of the response. Specifically, the time course of a typical first-order response is given by:

$$\rho_t = \rho_e(1 - e^{-(k([A]+K_A)t)}) \quad [9]$$

in which ρ_e is the response at equilibrium, [A] is the concentration of agonist, K_A is the equilibrium dissociation constant of the agonist-receptor complex, and k is the rate of onset. For stop-time reporter assays, the reaction is allowed to progress for a specified amount of time, and then it is stopped. The amount of product formed (which corresponds to the area under the curve describing ρ_t as a function of time), corresponds to the magnitude of the receptor stimulus (i.e., cAMP). The area under the first order rate of onset curve is given by the integral of equation 9 (Kenakin, 1993b):

$$R_t = R_e\, t + \frac{e^{-k([A]/K_A+1)t}}{k([A]/K_A + 1)} - \frac{1}{k([A]/K_A + 1)} \quad [10]$$

If the relative amount of product formed by two drugs of radically different rates of onset are measured in this manner, it is possible that the agonist of slower onset may appear to be of artificially low potency if the reaction is stopped too quickly. This suggests that longer time points for reporter assays done in stop-time format may reduce artifacts attributable to kinetics.

The issue of real time versus stop time becomes important when agonists produce complex transient responses. Whereas waveforms can be visualized in real time and appropriate decisions made as to where response will be measured, no such luxury always is afforded to stop-time experiments. In these, the reaction between agonist and receptor is terminated at some point and the product of the reaction assayed. If the response is transient, then differences between the total amount of product formed by low concentrations of agonist (which may produce sustained responses) may bear little relationship to the product formed by higher concentrations. Such behavior should be detectable by multiphase dose-response curves. Similarly, the study of constitutive receptor activity with stop-time assays must show that constitutive activity is still actively present at the time of exposure to inverse agonist. Because stop time assays measure a historical response (as in the case of the luciferase reporter gene assay), it may be that the observed response was a burst of constitutive activity that had desensitized back to zero by the time the inverse agonist was added. Under these circumstances, no inverse agonism would be seen. Similarly, if the cell line is very constitutively active, then the diminution in reaction product produced by a short exposure time to inverse agonist may be insignificantly small when compared with the historical reporter response produced by the assay before addition of drug.

2. Quantitative techniques for functional classification. The functional classification of drugs and receptors is achieved by the measurement of equilibrium dissociation constants of agonists and antagonists at receptors. There are theoretical and practical reasons to be cautious about considering classification of receptors with agonists.

One of the most common measures is the relative potency of agonists, because this is thought to reflect their affinity and efficacy, both chemical terms unique to the drug and the receptor. However, efficacy is a term specifically related to the agonist, receptor and G-protein (i.e., the γ and δ terms in the cubic ternary complex model); therefore, if receptors couple to different G-proteins in different cellular hosts, then the efficacy of the agonists will differ as well (Kenakin, 1989; fig. 19).

Another common means of classification with agonists is the measurement of agonist affinity by receptor alky-

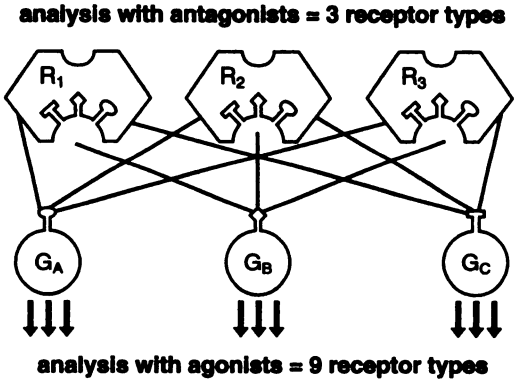


FIG. 19. Schematic diagram of three receptors interacting with three G-proteins. Analysis with antagonists would delineate three receptors, whereas analysis with agonists could define as many as nine 'receptor types.' From Kenakin (1989).

lation (method of Furchgott, 1966). As with potency ratios, the influence of G-protein can be confounding with this technique as well. Specifically, this method may yield an artifactually high measure of agonist potency that is increased by the 'distribution' of the receptor between the G-protein coupled and uncoupled states (Black and Shankley, 1990; MacKay, 1988; 1990a, b). Because the augmentation factor depends upon the degree of distribution which, in turn, depends upon the relative stoichiometry of the receptors and G-proteins, the cellular host system may impose an overriding influence on the resulting estimates of affinity.

The measurement of true neutral antagonist affinity is the most straightforward means of determining chemical terms with which drugs and receptors can be classified. For functional studies, the determination of affinity values by Schild analysis is by far the most powerful method to do this (Kenakin, 1992). This technique is most widely used in isolated tissue studies but also can be applied easily to binding and biochemical studies. Thus, the equilibrium dissociation constant of the antagonist (K_B) should be related to the equiactive dose-ratio of the agonist (determined in the presence and absence of antagonist) by the relationship (Arunlakshana and Schild, 1959):

$$\log (dr\text{-}1) = \log [B] - \log K_B \qquad [11]$$

Thus, various dose-ratios (dr) are plotted as a regression as log (dr-1) upon the logarithms of the molar concentrations of antagonist to yield a straight line of slope unity and intercept pK_B ($-\log K_B$). In general, the Schild method inspects the interaction of an agonist and antagonist over a concentration range and tests the assumption that they have singular properties (i.e., receptor activation for the agonist and receptor occupancy for the antagonist) in a single homogeneous receptor population. These stringent criteria can be a useful tool in themselves as deviation of Schild regression from ideal behavior can be used to detect nonequilibrium steady states such as agonist degradation (Furchgott, 1972), antagonist degradation (Kenakin and Beek, 1987a), temporal inequilibrium (Kenakin, 1980) and the presence of heterogeneous receptor populations (Furchgott, 1972; Kenakin, 1982, 1992). This latter application of Schild analysis can be extremely useful operationally, as general methods to detect mixtures of receptor populations can be derived from Schild analysis in functional studies (Kenakin, 1992). It is well established that Schild analysis is amenable to functional studies in isolated tissues and also can be used in similar approaches in cell cultures (i.e., Gudermann et al., 1993b; Poyner et al., 1992; De Vivo and Maayani, 1986).

An extension of Schild analysis and the additive dose-ratio technique (Paton and Rang, 1965) is a powerful approach to the cancellation of secondary drug properties and the resulting measurement of receptor parameters. Termed *resultant analysis* (Black et al., 1986), this method essentially applies the Schild technique to allow the measurement of dose-ratios under conditions in which the antagonist with suspected secondary actions (termed the test antagonist, $[B_{test}]$) is used in conjunction with a reference antagonist ($[B_{ref}]$). The resulting summated antagonism can be analyzed to yield the contribution of each antagonist (with resulting pK_B estimates). Because the antagonist with the secondary properties is present throughout the analysis, the effects of the secondary properties cancel under the null conditions of the experiment. Thus, Schild regressions for the reference antagonist in the absence and presence of various concentrations of test antagonist are obtained, and the dextral displacement of these regressions can be related to the equilibrium dissociation constant of the test antagonist (Black et al., 1986; for examples see Leff and Morse, 1987; Kenakin and Beek, 1987b; Kenakin and Boselli, 1989)

IX. Mutation of 7TM Receptors

There is increasing evidence that within the field of 7TM receptor research exists a subpopulation of potentially very useful drug targets, namely mutant 7TM receptors. There is also evidence to suggest that the inactive form of 7TM receptors may be a special conformation aimed at keeping inaccessible amino acid sequences that automatically activate G-proteins (Lefkowitz et al., 1993). Existing studies suggest that many man-made mutants of 7TM receptors are spontaneously active (vide infra). In addition, there is increasing evidence that spontaneous 7TM receptors in nature can lead to constitutively active basal responses and thus be associated with human pathology. Some examples of where 7TM receptor mutation can lead to constitutive activity: melanocyte stimulating hormone (Robbins et al., 1993), rhodopsin (Robinson et al., 1992), β_2-adrenergic (Samama et al., 1993), α_2-adrenergic (Ren et al., 1993), leutinizing hormone (Shenker et al., 1993), and thyrotropin (Parma et al., 1993). There are certain diseases that are associated with 7TM receptor mutation; some of these are given in table 11.

There are selected instances in which constitutively active mutant receptors may be associated with disease states: for example, the severe ligand-independent hypercalcemia and hypophosphatemia associated with Jansen-Type metaphyseal chondrodysplasia may relate to a mutant constitutively active PTH receptor (Schipani et al., 1995). Still, it is not clear to what extent the constitutive 7TM receptor activity in general is relevant to human disease (Clapham, 1993; Milligan et al., 1995b). However, the chronic elevation of second-messengers in cells (for example, by constitutive G-protein activity, Lyons et al., 1990; Weinstein et al., 1990; see review by Spiegel et al., 1993) has been shown to lead to cell transformation. In accordance with these findings is the fact that receptor genetic material can function as an

TABLE 11
7TM Receptor mutants in disease

Color blindness	Nathans et al., 1989
Night blindness	Dryja et al., 1993
Retinal degeneration	Robinson et al., 1992
Familial male precocious puberty	Shenker et al., 1993
Familial glucocorticoid deficiency	Clarke et al., 1993
Thyroid adenoma	Parma et al., 1993
Nephrogenic diabetes insipidus	Rosenthal et al., 1993
Neonatal hyperparathyroidism	Pollak et al., 1993
Hypocalcemia	Pollack et al., 1994
Genetic obesity	LaNoue and Martin, 1994
Multigenic Hirschsprung's disease	Puffenberger et al., 1994
Jansen-type metaphyseal chondrodysplasia	Schipani et al., 1995
Nocturnal asthma	Turki et al., 1995
Schizophrenia	Van Tol et al., 1992

agonist-dependent pro-oncogene (Julius et al., 1989; Gutkind et al., 1991; Allen et al., 1991). For example, transfection of functional 5-HT_{1C} receptors into NIH 3T3 cells leads to cell transformation, and injection of the transformed loci into nude mice leads to generation of tumors (Julius et al., 1989). Similarly, fibroblasts transfected with α_{1B}-adrenergic receptors have been shown to be tumorogenic when injected into nude mice (Kim et al., 1994). Agonist *independent* proto-oncogene activity has been observed with transfection of mutant α_1-adrenergic receptors (Allen et al., 1991).

Receptor mutation may play a larger role in the pathology of disease states than thought previously (Pearce and Trump, 1995). Molecular biology techniques such as 'mutational analysis' now are being used to link sequence variations in genes to pathological conditions (Gejman and Gelernter, 1993). For example, there is considerable evidence of polymorphism in human dopamine receptors (Inoue et al., 1993). A link in receptor mutation and disease has been proposed for the dopamine receptor in view of the observed functional polymorphism within the dopamine D_4 receptor gene (Van Tol et al., 1992) and D_3 receptor gene (Nimgaonkar et al., 1993). Significant polymorphic variation in the human population has been detected for dopamine D_4 receptors; this variation may be related to responsiveness to antipsychotic treatment (Van Tol et al., 1992). Similarly, George et al. (1993) found that the dopamine D_4 receptor genotype in 72 patients with chronic alcoholism was heterogeneous, with individuals being homozygous and others heterozygous for the various D_4 receptor alleles. Mutations of dopamine D_4 receptors also appear to be relevant to patients with psychosis, in whom it was found that variation of the gene encoding this receptor conferred susceptibility to delusional disorders (Catalano et al., 1993). Although evidence that some central nervous system diseases may be related to such polymorphism (i.e., psychosis, Catalano et al., 1993; alcoholism, George et al., 1993), there is still debate as to the relevance of this finding to diseases such as schizophrenia (Catalano et al., 1993; Barr et al., 1993).

An interesting polymorphic loci within the coding of the β_2-adrenergic receptor has been discovered that produces mutant receptors (Green et al., 1994). One of these (GLY16) demonstrates accelerated receptor down-regulation and is overrepresented in patients with nocturnal asthma, suggesting a possible correlation and mutant receptor drug target (Turki et al., 1995).

X. Conclusions

This review cites many papers describing the effects of drugs on 7TM receptors that have been expressed in surrogate cell systems. In many of these papers, the receptor antagonist profiles are uniform, high affinity agonist binding is observed, and a functional readout for receptor-mediated response can be obtained. There are also instances in which abnormal stoichiometry and/or the presence or absence of an essential component of the receptor system produces an aberrant behavior that does not reflect therapeutically relevant drug activity. It may be that the expression of 7TM receptors into foreign cells may not always faithfully reflect wild type receptor activity because of the intrinsic organization of cell systems (Neer, 1995). Cells may have optimal ratios of receptors and G-proteins to function (Moghe and Tranquillo, 1995), and the disturbance of these ratios may introduce confounding variables into experiments aimed at measuring drug activity. What is clear is that molecular biology has spawned a renaissance in receptor research, because now these systems can be manipulated in ways never before possible. The use of these new technologies no doubt will aid both in the understanding of drug-receptor mechanisms and the finding of new drug entities.

XI. References

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