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Drug Efficacy at Guanine Nucleotide-Binding Regulatory Protein-Linked Receptors: Thermodynamic Interpretation of Negative Antagonism and of Receptor Activity in the Absence of Ligand

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SUMMARY

The mutual effects that a hormonal ligand (H) and a guanine nucleotide regulatory protein (G protein) exert on each other when simultaneously occupying distinct sites of the receptor molecule (R) can be viewed as the molecular mechanism of drug efficacy. These effects are predictable on the basis of a model assuming that the ternary complex between the three partners (HRG) reaches equilibrium in the membrane [*J. Biol. Chem.* **255**:7108–7117 (1980)]. Ligands can be classified as agonists, neutral antagonists, or negative antagonists, depending on whether they enhance, leave unchanged, or reduce, respectively, the spontaneous tendency of R to interact with G. Using this model and the assumption that the G protein response observed in membranes reflects the sum of ligand-independent (RG) and ligand-dependent (HRG) receptor-G protein complexes, we can explain virtually all the phenomenology reported earlier for opioid receptor-mediated stimulation of GTPase, i.e., 1) existence of ligands with both "positive" and "negative" intrinsic activity (the latter termed negative antagonists), 2) equipotency of neutral antagonists for the competitive blockade of the responses elicited both by agonists and by negative antagonists, and 3) apparent heterogeneity of binding sites for the binding isotherms of negative antagonists. The ternary complex model can also explain the differential effects of sodium on ligand binding and ligand-dependent GTPase activity, if we assume that this ion reduces the stability constant between receptor and G protein in membranes. Computer simulations predict that a negative antagonist exhibits a discrepancy between "biological" K_i (obtained by Schild plots) and true dissociation constant for the receptor, which increases as the fraction of "precoupled" receptors in the membrane increases. The demonstration of negative antagonism is definitive evidence for the existence of receptor coupling (hence activity) in the absence of ligand. Using this experimental paradigm, we show here that spontaneous receptor activity occurs in isolated membranes but not in intact NG108-15 cells.

Affinity and efficacy are the two key factors that determine the initial interaction between a receptor protein and its ligand. From a molecular point of view, drug efficacy indicates the strength of the microscopic change that ligands impart to the receptor molecule and that propagates to the generation of a biological signal. Pharmacologists have developed techniques to define and measure intrinsic efficacy as a dimensionless parameter based on the stimulus-response relationship of the receptor, rather than on its biochemical properties. This concept is universally applicable to any receptor for which a cellular response can be measured (1–3). However, because our ability to manipulate the molecular structure of receptors has been enhanced by the use of site-directed mutagenesis techniques and the assessment of such manipulations relies on the biochemical response of recombinant receptors transfected into cells, there is a clear need for understanding and quantifying efficacy in molecular terms.

For some types of receptors, ligand-binding subunits and those involved in the generation of the intracellular signal are all assembled in the same macromolecular domain and, therefore, the distinction of ligand-induced stimulus from ligand-generated biological signal is not easy. In contrast, G protein-linked receptors are single polypeptides that lack the ability to generate second messengers (4). The stimulus generated by the ligand is transmitted to transducer coupling proteins (G proteins) (5), which, in turn, translate and distribute it to a host of effector molecules endowed with the ability to generate intracellular signals. Thus, for these receptors the degree of activation of G proteins is a biochemical measure of both type

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ABBREVIATIONS: G proteins, guanine nucleotide-binding regulatory proteins; G_α and $G_{\beta\gamma}$, α and $\beta\gamma$ subunits of the guanine nucleotide-binding protein heterotrimer; EC_{50}, effective concentration inducing 50% of response; DADLE, [D-Ala^2,D-Leu^5]enkephalin; ICI174, [*N*,*N*′-diallyl-Tyr^1,Aib^{2,3}] Leu-enkephalin; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

and intensity of ligand-induced stimuli before their amplification and conversion into intracellular signals.

From a thermodynamic standpoint, the interaction between hormone, receptor, and G protein can be viewed as a case of simultaneous binding of multiple ligands (hormone and G protein) to distinct sites of a single monomeric protein (the receptor). The energetics of ligand binding at multiple sites of proteins were reviewed by Weber (6). Based on Weber's formalism, DeLean *et al.* (7) proposed a model of receptor action known as the ternary complex model, which assumes that the stability constant that governs the association between receptor and G protein can be altered in an allosteric fashion by their respective ligands (i.e., agonist for the receptor and guanine nucleotide for the G protein).

An implication of the model is that "efficacy" can be defined in thermodynamic terms as the mutual effects hormone (H) and G protein (G) exert on the binding of each other to receptor (R) (6, 7). If there is a strong positive interaction, the ligand H facilitates binding of G to R. In classical pharmacological terms, this ligand is an agonist; the greater its efficacy, the greater its ability to stabilize the ternary complex. If there is no interaction, the ligand H does not alter the spontaneous tendency of R to associate to G and, therefore, is an antagonist that binds to the receptor site but induces no stimulus. However, the model also predicts an additional type of antagonist, the binding of which opposes that of the G protein (and vice versa) and, thus, promotes destabilization of the ternary complex. Indirect evidence for the existence of this type of antagonist comes from the observation that guanine nucleotides can enhance the apparent binding affinity of some antagonists (8–10). More direct experimental evidence for the existence of antagonists that destabilize receptor-G protein complexes formed in the absence of ligand has been obtained in both reconstituted systems (11, 12) and native membranes (13–16). In this study we have examined in detail the theoretical aspects of negative antagonism predicted by the ternary complex model.

Materials and Methods

The Model and Its Assumptions

The model consists of three components, hormone H, which interacts at a specific recognition site on the receptor R, and transducer protein G, which interacts with R at a site different from that occupied by H. It is assumed that the interaction reaches equilibrium in the membrane, so that it can be described as multiple simultaneous binding equilibria between two ligands and a protein (6–8, 17). The following scheme applies:

$$\begin{array}{ccc} & \overset{K}{\rightleftharpoons} & \\ H + R + G & & HR + G \\ \quad M \uparrow\downarrow & & \uparrow\downarrow \alpha M \\ H + R\,G & \underset{\alpha K}{\rightleftharpoons} & HRG \end{array}$$

where K and M are the unconditional equilibrium affinity constants describing the formation of the binary species HR and RG, respectively; αK and αM are conditional equilibrium affinity constants for the formation of the ternary species HRG.[1] As pointed out previously (6, 7, 17), thermodynamic balance implies that the ratios between each pair of conditional and unconditional constants are equal. Thus, the model is sufficiently described by the following equilibria:

$$K = \frac{[HR]}{[H][R]} \quad (1)$$

$$M = \frac{[RG]}{[R][G]} \quad (2)$$

$$\alpha = \frac{[HRG][R]}{[HR][RG]} \quad (3)$$

and equations for conservation of mass:

$$R_t = [R] + [HR] + [RG] + [HRG]$$

$$G_t = [G] + [RG] + [HRG]$$

$$H_t = [H] + [HR] + [HRG]$$

The "coupling" constant α, which is both G protein- and ligand-related, indicates at what extent and to which direction the spontaneous interaction between R and G is perturbed after the introduction of H. α is related to the free-energy coupling between H and G, i.e., the difference between the sum of free energy changes associated with the bimolecular reactions (H + R → HR and G + R → RG) and the standard free energy of formation of the biliganded species (G + HR → HRG and H + RG → HRG) (6). Depending on whether this energy of interaction is equal to, larger than, or smaller than zero, there is "neutrality," "antagonism," or "cooperation" between the binding of H and G, which means that the binding of H may leave unchanged, decrease, or enhance, respectively, the probability of R to interact with G (and vice versa). Because signal transduction depends on the interaction of R with G, the existence of a positive interaction (free energy of coupling < 0, $\alpha > 1$) constitutes a molecular stimulus (the ligand H facilitates the binding of the ligand G). In pharmacological terms, this indicates that H has agonistic activity, the extent of which corresponds to the value of free energy coupling between H and G. In contrast, in the presence of either null or negative interactions (free energy coupling ≥ 0, $\alpha = 1$ or $\alpha < 1$), the ligand H does not facilitate or even deters the binding of R to G. Because such ligands can compete with agonists for the binding center of the receptor, they both can act as antagonists, and distinction can be made between the neutral and the negative kinds. Thus, drug efficacy is a molecular property of the receptor and both its partners, hormonal ligand and G protein. The process of signal transduction at the molecular level can be viewed and quantified as the result of energy transduction within this complex of interacting protein molecules (6).

The ternary complex model is a minimal representation of the actual interactions taking place in the membrane. The heterotrimeric G protein itself is a functional dimer, which consists of α and $\beta\gamma$ subunits, establishing multiple equilibria involving receptor and guanine nucleotides (5). Thus, a complete thermodynamic description of the process should depict energetic interactions between two small ligands (hormone and guanine nucleotide) via three distinct protein domains (receptor, G_α, and $G_{\beta\gamma}$) and obviously involves a much greater number of parameters and computations. Extensions of the model that explicitly consider nucleotide binding have been proposed recently, using either equilibrium conditions (18) or a steady state approach (19). Even these versions, however, do not address the dissociation of $\beta\gamma$. It is still possible to utilize the simpler ternary complex model to demonstrate the relation between free energy coupling and ligand efficacy and to predict the differences between antagonists, as long as a number of reasonable assumptions on the relation between receptor-G protein binding and GTPase response are made, as follows.

In an experimental context, such as that of biochemical studies in isolated membranes, two forms of output responses can be measured after addition of the ligand. One is ligand binding, i.e., the sum of the receptor species bound to ligand, [HRG] + [HR]. A second is the extent of G protein activation, which is reflected in an increase of steady state GTP hydrolysis. Studies on the intrinsic fluorescence of purified G protein subunits (20) or on the GTP hydrolysis of receptor-G protein

[1] The nomenclature used here is a combination of those used by DeLean *et al.* (7) and by Ehlert (17).

complexes reconstituted into liposomes (reviewed in Ref. 21) are consistent with the idea that the rate of nucleotide exchange, but not the intrinsic catalytic rate for GTP hydrolysis, is the limiting step for steady state GTPase activity. Thus, receptors allow G protein α subunits to reach their maximal catalytic rate by increasing the k_{on} for GTP and accelerating the k_{off} for GDP, an event that is mechanistically coupled to receptor-induced dissociation of $\beta\gamma$ subunits (5, 20–21). This results in a net reduction of the equilibrium constant for the nucleotide and net increase in apparent turnover number for GTP hydrolysis. Accordingly, a simple way to simulate hormone-stimulated steady state GTPase activity in a membrane using the ternary complex model is to evaluate the amount of G proteins bound to the receptor. Data are shown and analyzed as the sum of the concentrations of RG and HRG complexes that are formed in response to increases of the total concentration of the ligand. We refer to this quantity as G protein response.

Computer Simulations

In its most general form, the ternary complex model can describe the interaction between n ligands, m receptors, and l G proteins. For the sake of simplicity, we have implemented a form of the model that examines n ligands and l G proteins interacting with a single type of receptor. This is the most common situation encountered in an experimental setting, where an investigator uses cell membranes containing a homogeneous population of receptors that can potentially interact with a number of G protein subtypes and that is studied using a variety of agonists and antagonists. Input parameters for the simulations are the total concentrations of the reactants, i.e., receptor (R), G proteins (G_j), and ligands (T_i), plus the affinity constants (K_i and M_j) and allosteric factors (α_{ij}) that govern their interaction. Unknown variables are the concentrations of all the free and bound species. When multiple ligands are present, only one is allowed to vary. To compute the concentrations of the bound and free species, the following set of equations must be solved:

$$E = \frac{R_t}{1 + \mathrm{SUM}(R)} \tag{4}$$

$$U_j = \frac{G_{t_j}}{1 + \mathrm{SUM}(G)_j} \tag{5}$$

$$F_i = \frac{T_i}{1 + \mathrm{SUM}(B)_i} \tag{6}$$

where E, U, and F indicate concentrations of empty receptor, [R], uncoupled G proteins, $[G]_j$, and free ligands, $[H]_i$, respectively; T is the total concentration of ligand i, and the terms SUM(R), SUM(G), and SUM(F) indicate the sum of the bound species normalized to their free concentrations, as follows:

$$\mathrm{SUM}(R) = \sum_{i=1}^{n} K_i F_i + \sum_{j=1}^{l} M_j U_j + \sum_{i=1}^{n}\sum_{j=1}^{l} (\alpha_{ij} K_i F_i M_j U_j)$$

$$\mathrm{SUM}(G)_j = M_j E + \sum_{i=1}^{n} (\alpha_{ij} K_i F_i M_j E)$$

$$\mathrm{SUM}(F)_i = K_i E + \sum_{j=1}^{l} (\alpha_{ij} K_i E M_j U_j)$$

To obtain a simultaneous solution of eqs. 4–6, we used a simplified numerical procedure. First, the equations were solved sequentially, using as values for the concentrations of free species the corresponding concentrations of total species present. Next, the computed new values for the free species were replaced in the equations, and a new set of solutions was obtained. Iterations were repeated until the concentration of each free ligand present reached a stable value (i.e., when the relative difference $| F_{i_{new}} - F_{i_{old}} | / F_{i_{old}} < 10^{-7}$). In the absence of any ligand, checks for convergence were done with respect to the concentrations of uncoupled G proteins.

Data Analysis and Nomenclature

The results of the simulations were, when necessary, further analyzed as experimental data would be. Binding isotherms expressed as fraction of bound ligand (B/T) versus concentration of total ligand and ligand-mediated G protein response (i.e., [HRG] + [RG] versus the concentration of total ligand) were analyzed using a four-parameter logistic equation (22) in order to compute slope factors, EC_{50} or IC_{50}, and upper and lower horizontal asymtotes of the curves. Binding isotherms were also analyzed with a mass-action law model for multiple receptor sites using the program LIGAND (23), in order to evaluate the relationship between the parameters of the ternary complex model and the apparent number of classes of binding sites and associated affinity constants that would best describe the data when analyzed using the "incorrect" model. In some cases the curves were reanalyzed after the inclusion of "experimental error." This was generated using a random error simulator and included in the curves assuming a constant percentage error of the independent variable across the whole range of variation of the dependent variable. The following nomenclature was used. Parameters of the ternary complex model were K, the affinity constant of H (eq. 1) usually expressed as a dissociation constant, $K_d = 1/K$; M, the affinity constant of G (eq. 2); and α, the coupling constant (eq. 3), also referred to as "molecular efficacy." Parameters derived from data analysis were K_{ap}, the apparent dissociation constant of the ligand computed from bimolecular mass-action law analysis of binding curves (23); and K_i, the apparent dissociation constant of the antagonist derived from Schild plots, $K_i = 10^{-pA_2}$.

Experimental Procedures

Materials. [γ-^{32}P]GTP (6000 Ci/mmol) was purchased from DuPont New England Nuclear (Boston, MA). Adenosine 3′,5′-cyclic phosphoric acid 2′-*O*-succinyl-3-[^{125}I]iodotyrosine methyl ester (2000 Ci/mmol) was from Hazlton Laboratories (Vienna, VA). Neuroblastoma NG108-15 cells were cultured and maintained according to previously published procedures (24).

Assay of GTPase. Membranes from NG108-15 cells were prepared as described previously and stored at −70° until used. Determination of GTP hydrolysis was performed as described in a previous report, using a sodium-free buffer system (15). Briefly, the reaction was conducted at 37° for 10 min using 5–10 μg of membrane proteins in a total volume of 100 μl, in the presence of either NaCl or KCl, and was arrested by the addition of 250 μl of H_3PO_4. The measurement of the extent of P_i released (using charcoal) and the calculation of high affinity GTPase activity were performed as described (15).

Assessment of receptor-mediated inhibition of cAMP accumulation. NG108-15 cells were harvested and resuspended in a HEPES-buffered isotonic solution of the following composition (in mM): Na-HEPES, 20; NaCl, 135; KCl, 4.5; $CaCl_2$, 2; $MgSO_4$, 1; EGTA, 1; supplemented with 0.1% (w/v) bovine serum albumin. Cells (5×10^4) were incubated at 37° in the same buffer supplemented with 100 μM Ro 20-1724, in the presence of opioid agonists and antagonists, using a total volume of 100 μl. The incubation lasted 30 min and was terminated by the addition of 1 ml of ice-cold 0.1 M HCl. The concentration of cAMP was determined by radioimmunoassay, as described previously (25).

Radioligand binding studies. Binding of opioid ligands was measured as competition for the binding sites labeled by [^{3}H]diprenorphine (0.25–0.5 nM). The reaction was conducted in a buffer identical to that used for GTPase, in the presence of either NaCl or KCl, at 22° for 90 min and was terminated by filtration. Experimental details have been described previously (15, 26).

Results

Coupling constant α as an index of molecular efficacy. Fig. 1 demonstrates the interaction between H, R, and G according to the ternary complex model. To simulate the situation of an experimental setting, the concentrations of R_t and

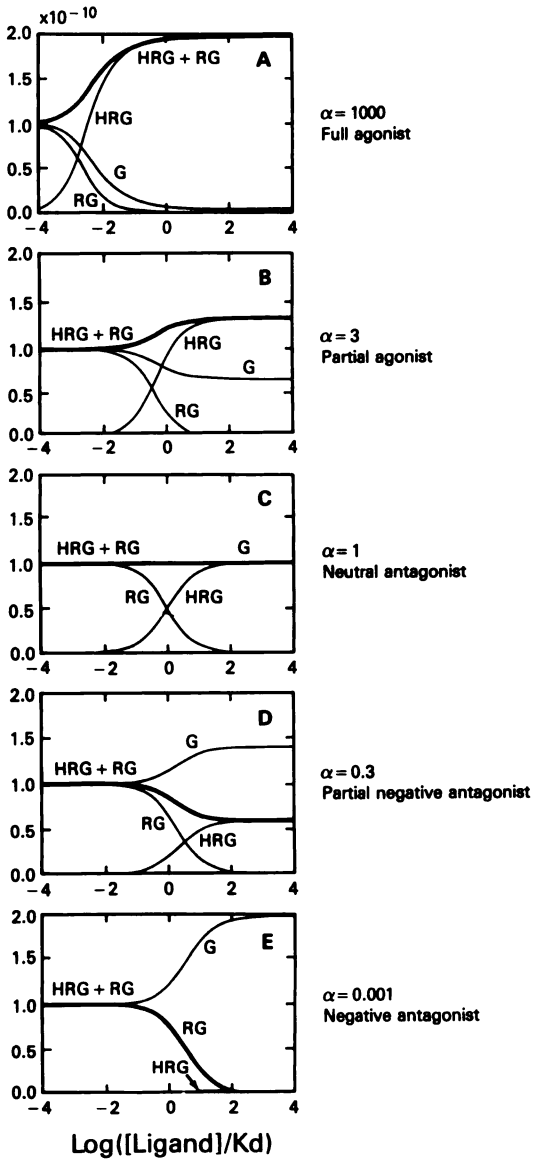

Fig. 1. Relation between α and ligand efficacy. Data were simulated according to the ternary complex model, using the following parameters: $R_t = G_t = 0.2$ nM, $M = 1 \times 10^{10}$ M^{-1}, and $K_d = 100$ nM. Each *panel* corresponds to a ligand of different α value, as indicated. Plotted are the concentrations (mol/liter) of the following species: G, uncoupled G protein; RG, ligand-independent complex; HRG, ternary complex; HRG + RG, G protein response (*thick lines*). *Abscissae*, logarithms of the molar concentration of the ligand normalized to the K_d.

G_t are fixed while that of the ligand (H_t) is the manipulated experimental variable. The simulations displayed in all panels of Fig. 1 were obtained using identical values for K, M, and $R_t = G_t$ (see legend to Fig. 1), but each panel corresponds to a ligand having a different value of the coupling constant α. In each panel the concentrations of G protein species (i.e., uncoupled, [G], binary complex, [RG], ternary complex, [HRG], and their sum, [RG + HRG]) are plotted as a function of the logarithm of the total concentration of ligand normalized to the ligand's dissociation constant for the receptor (H_t/K_d). All ligands induce a redistribution of G protein species, the extent and the direction of which depends on the value of α. The ligand with the highest coupling constant ($\alpha = 1000$) (Fig. 1A) is the most efficient in generating ternary complex at the expense of both free G and RG complex. As α decreases, the amount of HRG formed in excess of the initial level of RG diminishes and, accordingly, the reduction of free G also becomes less prominent (Fig. 1B). The concentration of ligand yielding half-saturation of the G protein response (EC_{50} of the solid lines in Fig. 1) is smaller than the ligand K_d when α is large (Fig. 1A) and tends to approach the K_d value as α approaches 1. Because the extent of receptor-G protein complex formation determines the size of the stimulus induced, the ligand with the highest value of α is a full agonist, in comparison with those having lower α values (partial agonists). For $\alpha = 1$, the amount of HRG formed as a function of the ligand concentration is exactly identical to that of RG that is disrupted. Thus, their sum and the concentration of free G are not altered by the ligand at any concentration (Fig. 1C). When α is less than 1, an opposite situation occurs, because the amount of HRG formed is smaller than the reduction of RG and the concentration of free G increases (Fig. 1, D and E). Thus, if we consider as output response of the system the sum of G protein species in receptor-bound form, it is clear that ligands fall into three classes, those producing a positive response, or agonists ($\alpha > 1$), those producing no response, or neutral antagonists ($\alpha = 1$), and those that inhibit basal activity, or negative antagonists ($\alpha < 1$). This is explained by the fact that, as free energy of coupling ranges from positive to negative values, the system reaches a point ($\alpha = 1$, i.e., free energy of coupling = 0) where it is just as probable to find H and G bound to distinct molecules of R as it is to observe their simultaneous binding to the same molecule of R (6). The receptor bound to an antagonist with $\alpha = 1$ is energetically indistinguishable from an "empty" receptor, and, therefore, such an antagonist can be defined as neutral. Neither ability to produce the ternary complex nor magnitude of ternary complex formation is a criterion of agonism or antagonism, because it is the relationship between the ligand-induced formation of HRG and dissolution of RG that determines the output from the system.

Competition between ligands having different α values. To further illustrate how α can be used to define the pharmacological concept of efficacy, it is instructive to examine the manner in which mixtures of ligands having different values of α will compete with each other in inducing receptor coupling to G. This situation simulates experiments in which the response of the receptor is investigated using mixtures of ligands of different efficacies. Fig. 2A shows the G protein response ([HRG] + [RG]) generated in the presence of increasing concentrations of a full agonist ($\alpha_1 = 1000$) and examined either in the absence or in the presence of competing ligands having identical affinities for the receptor ($K_d = 10$ nM, in this example) but different values of α_2, with all being present at concentrations (100 nM) 10-fold greater than their dissociation constant. As expected, the concentration-response curve of the full agonist is modified in a fashion that depends on the coupling constant of the competing ligand (α_2). The neutral antagonist produces only a rightward shift of the curve, whereas partial agonists ($1 < \alpha < 1000$) and negative antagonists ($\alpha < 1$) also shift the lower asymptote of the curve in the upward or downward direction, respectively. Similar effects are seen when the same ligands are allowed to compete with a negative antagonist ($\alpha_1 = 0.001$) (Fig. 2B).

The shift in EC_{50} induced by each competing ligand in the concentration-response curve of the competed ligand is larger

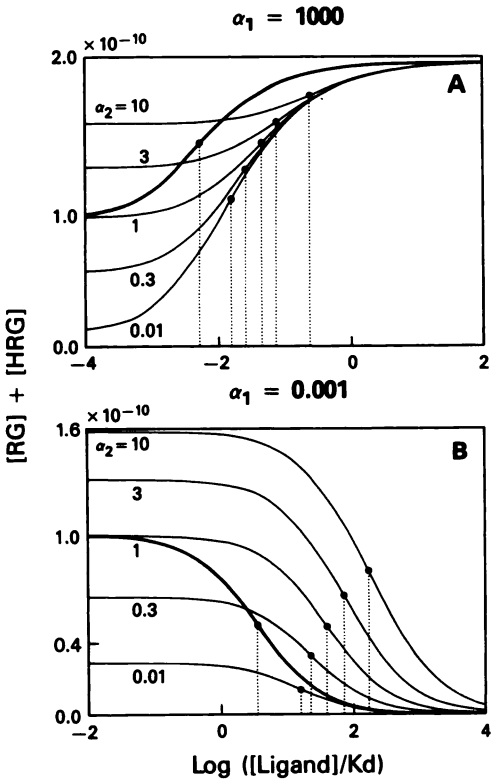

Fig. 2. Competition between ligands of different α values, in inducing a G protein response. The varying ligand H_1 is either a full agonist (α = 1000), (A) or a negative antagonist (α = 0.001) (B); both have the same receptor affinity (K_d = 100 nM). The *curves* in the absence of competing ligand are drawn as *thick lines*. All other *curves* were obtained in the presence of a fixed concentration (100 nM) of competing ligand H_2 (K_d = 10 nM), which has different values of α as indicated. *Ordinates*, total G protein response ($H_1RG + H_2RG + RG$); *abscissae*, normalized concentration of the varying ligand. Other parameters are $R_t = G_t$ = 0.2 nM, and $M = 1 \times 10^{10}$ liters/mol. EC_{50} values are shown as *black dots in each curve*. Because all competing ligands have the same K_d and are present at a concentration 10 times greater than the K_d, perfect competitive behavior would predict that each ligand would produce an identical shift in the concentration-response curve of the agonist. Instead, the shift induced is greater the larger α is.

the larger the value of α for the competing ligand. In fact, unlike classical receptor theory (1–3), the ternary complex model predicts that the apparent potency of a competing ligand depends on both receptor affinity (K) and efficacy (α). Thus, if two antagonists have identical affinities but different α values, the ligand with $\alpha < 1$ will be always less potent than that with $\alpha = 1$. This means that, other things being equal, a negative antagonist is a less efficient "agonist blocker" than a neutral antagonist. This departure from classical pure competitive behavior depends on the value of M. As M decreases, the discrepancy between competing potency and ligand affinity is progressively eliminated (data not shown). Because as M diminishes the concentration of RG complexes also tends to zero, it is clear that the ternary complex model predicts deviations from pure competitive behavior in antagonists only when the affinity between R and G is sufficient for spontaneous (ligand-independent) coupling to occur.

Yet, the potency of a neutral antagonist as determined by standard "null methods" remains, in most cases, a good approximation of the true affinity of this ligand for the corresponding binding center on the receptor. We simulated the ability of a neutral antagonist to antagonize the G protein response induced by either a full agonist or a negative antagonist. In both cases, the curves were shifted to the right as the concentration of neutral antagonist was increased, in a manner that closely approximates pure competitive behavior. In both cases, Schild analysis of these curves yielded slopes close to 1 and pA_2 values corresponding to the K_d of the antagonist for the combination neutral antagonist versus negative antagonist and only slightly divergent from the K_d for the pair neutral antagonist versus full agonist.[2] These results are in good agreement with the experimental data we reported previously in NG108-15 cells for opioid antagonists (14).

Effect of α on the binding properties of the ligand. Competition is often studied by experiments in which mixtures of ligands of different efficacies (one of which is radiolabeled) are examined for their ability to bind to the receptor. The effect of α under these conditions is best seen when ligands of identical receptor affinity but different α values are examined for their ability to compete for the receptor binding site labeled by a variety of radioligands with various values of α^*. Fig. 3 demonstrates how the IC_{50} of each ligand depends on both the α value of the ligand and the α^* value of the tracer. Although all competing ligands used in Fig. 3 share identical affinities, their competition isotherms exhibit a broad range of IC_{50} values, the relative ratios of which change for the two tracers used (Fig. 3).

When these data are analyzed using conventional models, assuming the existence of independent binding sites, the curves

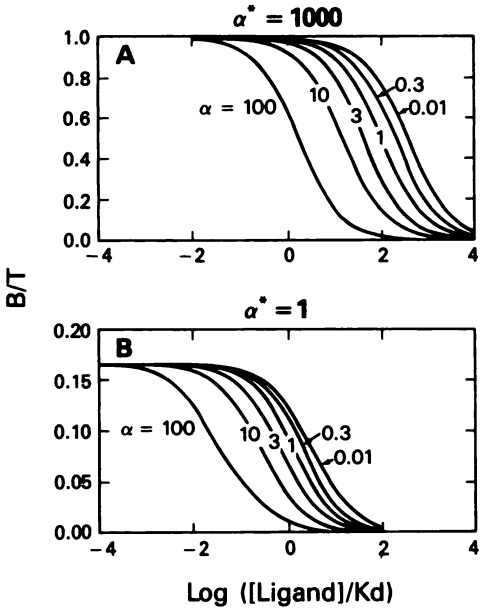

Fig. 3. Effect of α on the competition isotherms of ligands. R_t, G_t, and M are given in Fig. 1. Binding isotherms were generated for six ligands having identical affinities (K_d = 100 nM), competing with two radioligands (for both, K_d = 1 nM) present at tracer concentration (1 pM). Each *panel* corresponds to a different value of α^* for the radioligand, as indicated. The α values of the competing ligands are (from *left* to *right*) 100, 10, 3, 1, 0.3, and 0.01. *Ordinates*, fraction of bound tracer. *Abscissa*, normalized concentration of the competing ligands.

[2] We performed several simulations of the type described above, to assess under which conditions the discrepancy between apparent pA_2 (from Schild plots) and true dissociation constant of a neutral antagonist would become most noticeable. We found significant deviations only when $1/M \gg R_t$ or the antagonist was allowed to compete with an agonist of much higher affinity (data not shown).

for ligands having $\alpha \gtrless 1$ were consistent with multiple binding sites. The apparent affinities and the proportions of the sites depended on the particular combination of tracer/competitor used. One example of this analysis is shown in Table 1, from which it is clear that the apparent number of independent classes of sites found by the fitting routine depends in a complex manner on the ratio of α values for tracer and competing ligand (Table 1). Thus, in the case of G protein-linked receptors, analysis by models involving independent classes of sites may give inappropriate and misleading results (7, 8, 18).

Effect of the magnitude of M on receptor-mediated G protein response and ligand binding. How does the magnitude of the stability constant M affect the theoretical concentration-response curves for various ligands? Fig. 4 shows that, as M is varied over 6 orders of magnitude, the concentration-response curves for agonist- and antagonist-mediated G protein responses undergo complex changes. It is convenient to examine these changes as zones within which the dissociation constant $1/M$ varies with respect to the concentration of receptors, because the interaction between R and G (governed by M) occurs within the plane of the membrane. Zone 1 corresponds to an increase of the dissociation constant between receptor and G protein from a small value to one close to the concentration of receptors (i.e., $1/M \leq R_{total}$). Under these conditions, the decrease of M primarily changes ligand-independent activity without altering the EC_{50} and the maximal effect of the concentration-response curves of the agonist (Fig. 4A), whereas, for the negative antagonist (Fig. 4B), the IC_{50} values decrease and maximal effects are augmented as M diminishes. In Zone 2, when $1/M \geq R_{total}$, the EC_{50} values of the agonist are progressively increased (Fig. 4A), whereas those of the negative antagonist no longer change (Fig. 4B). In Zone 3, further increases of $1/M$ (i.e., $1/M \gg R_{total}$) produce only changes in the maximal effects of the agonist (Fig. 4B), because under these conditions [RG] is negligible and little or no spontaneous activity occurs. Thus, zone 3 describes a situation in which coupling between R and G is absolutely dependent on the presence of the ligand, and the distinction between neutral and negative antagonists is no longer possible.

TABLE 1

Analysis with the program LIGAND of binding isotherms of ligands of different α competing with a radiotracer having $\alpha^* = 1$

The analyzed curves correspond to those shown in Fig. 3B (tracer $\alpha^* = 1$). Parameters used for the simulations are $R_t = G_t = 0.2$ nM, K_d of competing ligands = 100 nM, K_d^* of tracer = 1 nM (present at 1 pM), and all other parameters as in Fig. 3. Data were analyzed both before and after the addition of experimental noise, as explained in Materials and Methods. The parameters tabulated are those computed from error-free data and correspond to the most complex model that could produce a significant improvement of the fit (23). Fitted parameters are apparent dissociation constants (K_{ap}) and density of binding sites (R). The level of significance was taken as $p = 0.05$.

Parameters	$\alpha = 100^a$	$\alpha = 10$	$\alpha = 3$	$\alpha = 1$	$\alpha = 0.3$	$\alpha = 0.01$
K_{ap_1} (nM)	1.5	14	42	100	220	740
K_{ap_2} (nM)	10	43	84		120	360
K_{ap_3} (nM)	71	93				124
R_1 (pM)	150	135	136	200	100	20
R_2 (pM)	40	47	64		100	88
R_3 (pM)	13	18				91
Sites detected with no error[b]	3	3	2	1	2	3
Sites detected with 3% error[b]	3	2	1	1	1	2

[a] α value of the competing ligand.
[b] This indicates the number of apparent binding sites that would have been inferred from the analysis of binding curves.

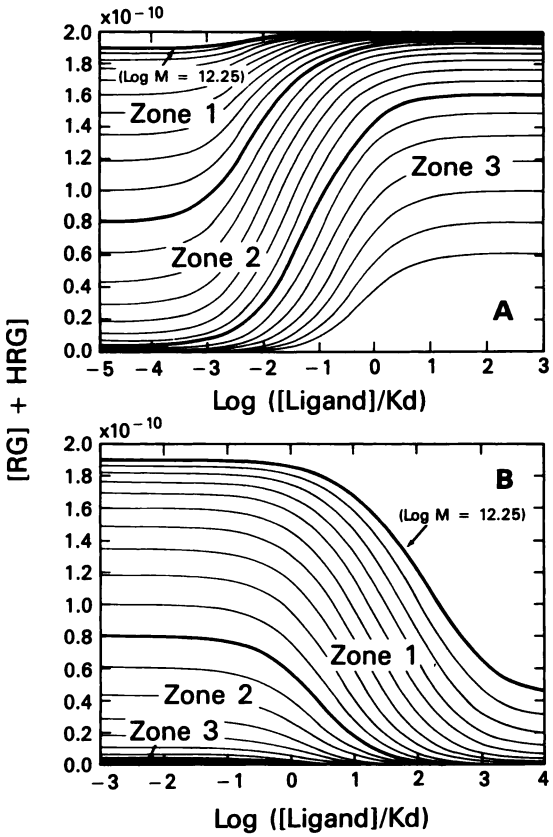

Fig. 4. Effect of reductions of M on the G protein response. The varying ligand is either a full agonist (α = 1000) (A) or a negative antagonist (α = 0.001) (B). Both ligands have the same affinity (K_d = 100 nM); $R_t = G_t$ = 0.2 nM. Each *curve* corresponds to a different value of the parameter M, which decreases in logarithmic steps of 0.25 starting from the first curve, as indicated.

The corresponding effects of varying M on the binding of the ligand are documented in Fig. 5, where binding isotherms for a full agonist (Fig. 5A) and a negative antagonist (Fig. 5B) in competition with a tracer concentration of neutral antagonist are displayed for several values of $1/M$. As observed for receptor-mediated activation of G, the decrease of M (i.e., increase of $1/M$) affects the binding of agonist and negative antagonist in opposite directions, and the effect of change in M is most prominent in zone 1 for the negative antagonist and in zones 2 and 3 for the agonist (compare Fig. 5, A and B).

It is clear from inspection of Fig. 5 that, the larger the value of M, the more the ratio between the EC_{50} and K_d of the ligand differs from unity (<1 for agonists and >1 for negative antagonists). Such departure of EC_{50}/K_d from unity is larger the more α differs from 1 (data not shown). Thus, the reduction of affinity between R and G affects the concentration-response curves of ligands in a direction determined by, and to an extent proportional to, the efficacy of the ligand. The relationship between a ligand's EC_{50} and $1/M$ is also influenced by the stoichiometry between R_t and G_t. We found a 10-fold rightward shift in the relationship between change in EC_{50} for both agonist and antagonist and increase of $1/M$ when there was a 10-fold molar excess of G proteins relative to the concentration of receptors (data not shown).

Interpretation of "sodium effect" in terms of equilib-

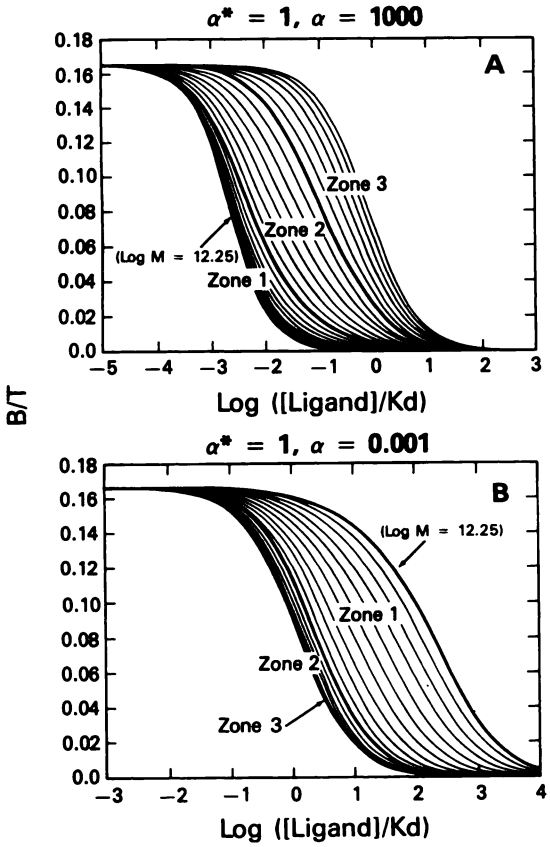

Fig. 5. Effects of reduction of the stability constant M on the binding isotherms. Ligand is either a full agonist (α = 1000) (A) or a negative antagonist (α = 0.001) (B). Parameters were as in Fig. 4. Both varying ligands (A and B) (K_d = 100 nM) compete for the binding sites labeled by a neutral antagonist radioligand (K_d = 100 pM, α = 1) present at tracer concentration (1 pM).

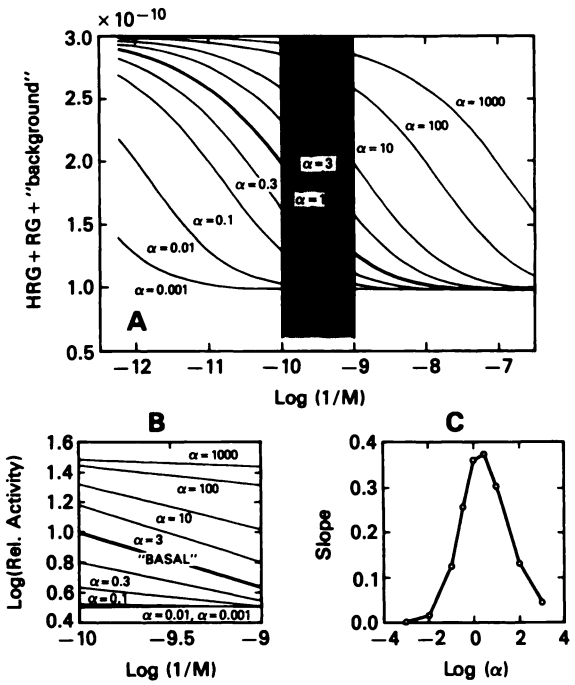

Fig. 6. Simulation of sodium effect on the G protein response A, Parameters were as in Figs. 4 and 5. The G protein response (HRG + RG) computed at maximal concentration of ligand (1000 × EC_{50}) is plotted for different values of log(1/M). Each *curve* corresponds to the response generated in the presence of a ligand of different α value, as indicated. The *thick solid line* corresponds to the basal response measured in the absence of ligand (which is identical to that in the presence of a ligand with α = 1). To simulate experimental data, an arbitrary background of 1 × 10^{-10} M has been added to the sum of receptor-bound G protein species and it represents the GTPase hydrolysis due to free G. B, The reduction of maximal G protein responses observed within the "window" of 1/M values of 0.1–1 nM (see A *shaded area*) has been normalized to the basal response observed at 1/M = 0.1 nM in the presence of a ligand with α = 1. C, The slopes computed from the curves of B were replotted as a function of the logarithm of α.

rium coupling of R and G, i.e., as a reduction of *M*. The effect of M on the curves for receptor-mediated activation of G protein closely resembles the effect of sodium ions on agonist-mediated stimulation of GTPase activity. Using opioid receptors in membranes prepared from NG108-15 cells, we have shown previously that there is an inverse relationship between intrinsic activity of the ligand and the ability of sodium ions to reduce GTPase in the presence of that ligand (15). Here we show that those findings can be simulated assuming that sodium ion results in a discrete reduction of M. The reduction of G protein response in the presence of saturating concentrations of ligand, as a function of M, depends on the α value for the ligand (Fig. 6A). If we examine the range of 1/M values between 0.1 and 1 nM (Fig. 6A), the decreases of G protein responses are linear with log(1/M) (the departure from a linear relation would not be expected to be detectable in the presence of experimental noise). The slopes of such lines are measures of sodium effect (Fig. 6B). The slopes are larger as the value of α approaches 1 and progressively decrease as α becomes either very large or very small. Thus, the relation between simulated sodium effect (slopes of the curves in Fig. 6B) and α is bell-shaped (Fig. 6C) and closely matches the previously reported relationship between experimental sodium effect and apparent intrinsic activity of the ligand (see Fig. 5 in Ref. 15). We did not observe a similar pattern of decrease of responsiveness when other parameters of the model (such as reduction of K_d or α of the ligand) were selectively varied (data not shown).

Effect of sodium on the binding isotherms of opioid ligands, showing apparent multiple affinity states. To further explore the relationship between sodium effect and reduction of M, we used a combination of experimental data and simulation according to the ternary complex model. Fig. 7 illustrates how the replacement of potassium by sodium in the reaction affects the binding isotherms of a full opioid agonist (DADLE) and two antagonists, a putative negative antagonist (ICI174) and a putative neutral antagonist (MR2266), as determined from their effects on GTPase in NG108-15 cell membranes (14, 15). Sodium induced a rightward shift in the isotherm of the agonist (Fig. 7A) and a leftward shift in that of the negative antagonist (Fig. 7B). In contrast, the binding isotherms of MR2266 remained unchanged (Fig. 7C). Analysis according to mass action law of these data suggests complex and divergent effects of sodium on the curves of the agonist and antagonist. The binding isotherms for the agonist are consistent with three apparent states of different affinities in the absence of sodium, very high (K_{ap_1}), high (K_{ap_2}), and low (K_{ap_3}), with dissociation constants ± standard errors (three experiments) of 0.55 ± 0.02 nM, 33 ± 12 nM, and 7.2 ± 2.5 μM, respectively. The corresponding proportions of sites existing in the three affinity states are R_1, 85 ± 10%; R_2, 10 ± 5%; and R_3, 5 ± 2%. When sodium was replaced by potassium, only two affinity states were detectable (K_{ap_1}, 1.5 ± 0.3 nM; K_{ap_2}, 170 ±

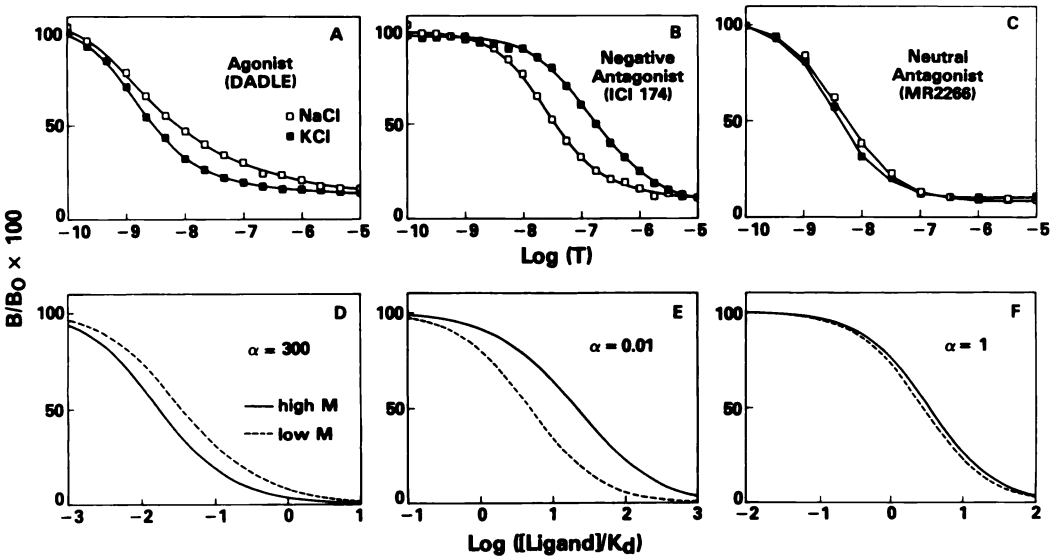

Fig. 7. Similarity between effect of sodium on experimental binding isotherms (A, B, and C) and reduction of *M* on simulated binding curves (D, E, and F). *Upper*, ligands are a full agonist (DADLE) (A), a putative negative antagonist (ICI 174) (B), and a putative neutral antagonist (MR2266) (C), in competition for the sites labeled by the partial agonist [^{3}H]diprenorphine (250 pM) in the presence of 150 mM levels of either KCl (■) or NaCl (□). Binding was measured using 230 μg/ml cell membranes in Tris/HEPES (50 mM), pH 7.5, containing 10 mM $MgCl_2$, 0.2 mM dithiothreitiol, 0.2 mM EGTA, 50 μg/ml bacitracin, and either KCl or NaCl as indicated. The incubation lasted 60 min at 20° and was terminated by filtration. Data are expressed are percentage of the binding of tracer observed in the absence of competing ligand. The *B/T* of the tracer was 0.144 and 0.120 in the presence of KCl and NaCl, respectively. The *lines* represent best fit of the data according to a mass action law model. *Lower*, simulated competition isotherms. The radioligand is a partial agonist ($\alpha^* = 0.3$, $K_d^* = 0.31$ nM, present at a fixed concentration of 0.25 nM); competitors are a full agonist ($\alpha = 300$, $K_d = 316$ nM) (D), a negative antagonist ($\alpha = 0.01$, $K_d = 25$ nM) (E), and a neutral antagonist ($\alpha = 1$, $K_d = 2$ nM) (F). Each *panel* shows the binding curve obtained at high (4×10^{10} M^{-1}) and low (2×10^9 M^{-1}) values of the stability constant *M*. Other parameters are $R_t = G_t = 0.2$ nM. Data are plotted as in *upper*, but the *abscissae* show the normalized concentration of total ligand.

50 nM; R_1, 75 ± 6%; R_2, 25 ± 5%). The curves for the negative antagonist indicated two affinity states in potassium (K_{a_1}, 540 ± 150 nM; K_{a_2}, 60 ± 12 nM; R_1, 70 ± 10%; R_2, 30 ± 4%). Replacement with sodium increased the K_a of the high affinity state and reduced the proportion of the low affinity state (K_{ap_1}, 800 ± 350 nM; K_{ap_2}, 20 ± 2 nM; R_1, 8 ± 2.5%; R_2, 92 ± 8%). The binding isotherms of MR2266 are consistent with a single affinity form of the receptor in both KCl and NaCl (K_{ap}, 3.8 ± 0.4 and 2.9 ± 0.5 nM, respectively).

Simulated binding isotherms for a full agonist ($\alpha = 300$), a negative antagonist ($\alpha = 0.01$), and a neutral antagonist ($\alpha = 1$) in competition for the binding of a partial agonist ($\alpha = 3$) were generated for two values of *M* (Fig. 7, D, E, and F). A 20-fold reduction of *M* alters the binding isotherms of the agonist and negative antagonist in a way that closely resembles the effect of sodium on experimental binding data. When the same data (to which random gaussian error with 3% coefficient of variation was added) are analyzed by the program LIGAND, the binding curves for the agonist and negative antagonist (but not those for the neutral antagonist) are consistent with multiple binding sites, the proportion and affinities of which are altered by a reduction of *M* in a pattern very similar to that observed for sodium in experimental data.

Negative antagonists as probes for the existence of ligand-independent receptor coupling. Because the competing potency of a negative antagonist is inversely related to the magnitude of *M*, it is to be expected that the apparent pA_2 of such antagonists would be increased by a diminution of *M* and, therefore, by an increase of sodium concentration. Fig. 8A shows the theoretical Schild plots for a negative antagonist ($\alpha = 0.01$) in competition with a full agonist, obtained from simulated curves generated using two different values of *M*. The concentrations of negative antagonist that produce equivalent shifts in the concentration-response curves of the agonist are reduced as *M* diminishes (Fig. 8A). Consequently, the apparent K_i (10^{-pA_2}) of the negative antagonist approaches the true K_d value when *M* becomes smaller (see legend to Fig. 8A). In Fig. 8B we show corresponding experimental data obtained using NG108-15 cell membranes. Concentration-response curves for DADLE-stimulated GTPase activity in the presence of increasing concentrations of the negative antagonist ICI174 were obtained in either NaCl or KCl. The apparent K_i value for ICI174 was decreased in the presence of NaCl, which is consistent with the idea that sodium acts by reducing *M*. In contrast, Schild plots for MR 2266 were virtually superimposable in NaCl and KCl (data not shown). The theoretical Schild plots of the negative antagonist at high values of *M* exhibit a slight but clear deviation from linearity (Fig. 8A). However, this deviation is too small to be detectable in the presence of experimental error and should not be used as a diagnostic tool for negative antagonism. The sodium-induced shift of the Schild plot of the antagonist is more readily accessible to experimental detection and less prone to be overlooked, even in the presence of realistic experimental error.

Lack of spontaneous receptor activity in intact cells. To investigate whether spontaneous receptor activity, which clearly exists in isolated membrane preparations, also occurs in intact cells, we studied the behavior of the negative antagonist ICI174 as a blocker of agonist-mediated inhibition of accumulation of cAMP levels in intact NG108-15 cells. Experiments designed to assess the effect of ICI174 on basal cAMP accumulation gave inconsistent results. Although in some experiments we observed an antagonist-induced enhancement of basal activity (22%), the effect did not exhibit clear concentra-

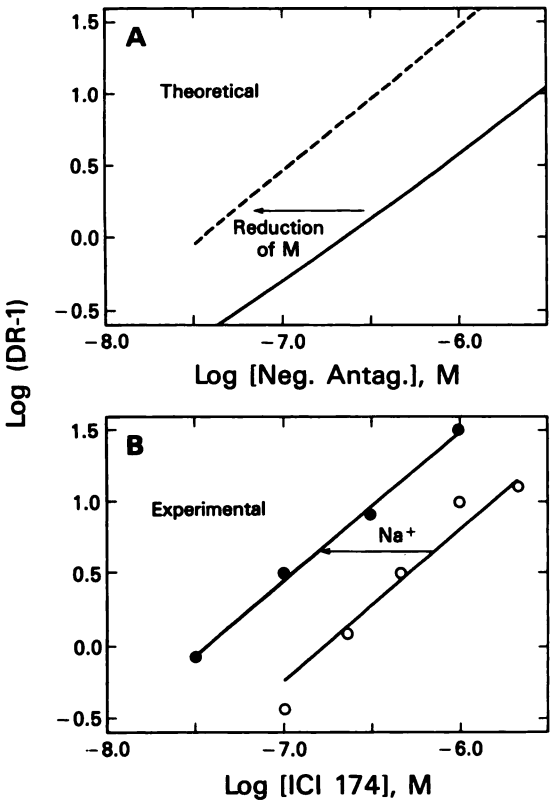

Fig. 8. Schild plots of a negative antagonist: effect predicted for a reduction of M (A) or experimentally determined for an increase of Na^+ (B). *Ordinates*, log(DR−1), where DR is does ratio, i.e., the ratio of EC_{50} values for the concentration-response curves of the agonist in the presence and absence of antagonist. *Abscissae*, logarithm of the concentration of antagonist. A, Concentration-response curves similar to those in Fig. 4 were generated in the absence and presence of increasing concentrations of a neutral antagonist. Parameters were $R_t = G_t = 0.2$ nM; varying ligand (agonist), $\alpha = 300$, $K_d = 316$ nM; and competing ligand (negative antagonist), $\alpha = 0.01$, $K_d = 25$ nM. The two plots were generated using different values of M (in liter/mol), $M = 4 \times 10^{10}$ (——) and $M = 1 \times 10^9$ (– – –). The corresponding parameter estimates by linear regression are $K_i = 213.4$ nM and slope = 0.87 for the high value of M and $K_i = 35$ nM and slope = 0.99 for the low value of M. Note that the loss of linearity at the highest value of M is minimal, and it would be difficult to detect in the presence of experimental error. B, Concentration-response curves for DADLE-mediated stimulation of GTPase activity in NG108-15 membranes in the presence of increasing concentrations of ICI174 were generated using a reaction mixture containing 150 mM levels of either KCl (○) or NaCl (●). A four-parameter logistic model (22) was used to compute EC_{50} values and their relative ratios; data were replotted in Schild coordinates, and *lines* are the corresponding linear regressions. The following values were computed: in KCl (○), $pA_2 = 6.78 \pm 0.15$ (K_i, 167 nM), and slope = 1.2 ± 0.25; in NaCl (●), $pA_2 = 7.43 \pm 0.05$ (K_i, 37 nM) and slope = 1.02 ± 0.04.

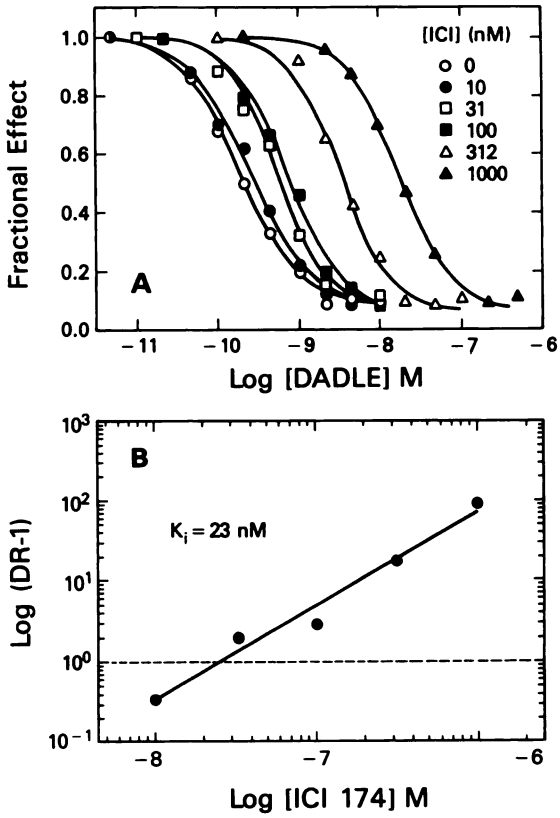

Fig. 9. Antagonism by ICI174 of agonist-mediated inhibition of cAMP accumulation in intact NG108-15 cells. Confluent NG108-15 cells were mechanically harvested and resuspended in assay buffer (24) containing 100 μM phosphodiesterase inhibitor Ro 20-1724 at 37° in the presence or absence of selected concentrations of DADLE and ICI174, as indicated. Incubation lasted 20 min. The total level of cAMP was determined by radioimmunoassay (24). A, Representative concentration-response curves fitted by a four-parameter logistic model (*solid lines*). Data were normalized as fractions of the levels of cyclic nucleotide measured in the absence of ligand. B, Schild plot of the data. In two additional experiments, K_i values were measured using only two curves, one in the presence and one in the absence of antagonist. The mean value averaged from the three experiments is given in the text.

tion dependency and was not reproducibly detected. To assess whether ICI174 has negative activity in intact cells, therefore, we relied on the measurement of its apparent K_i. Concentration-response curves for the agonist DADLE in the presence of increasing concentrations of the negative antagonist were examined (Fig. 9A). Schild analysis of these data (Fig. 9B) yields an apparent K_i value of 30 ± 7 nM, (three experiments), which is similar to the value measured in membranes at high concentrations of Na^+. This indicates that no ligand-independent activity is demonstrable and that there is no steady state accumulation of RG complexes in the membrane of intact cells.

Discussion

In this study we highlight three important features of G protein-linked receptors. 1) A ligand's efficacy, if defined in molecular terms, can span a continuous spectrum of amplitudes ranging from positive to negative values. 2) Modulators (GTP, sodium?) that reduce the stability constant M between receptor and G protein can affect the system in a manner that is inversely related to the efficacy of the ligand. 3) Receptor coupling in the absence of ligand will occur when the value of the stability constant M is not negligibly small. These points and their implications are discussed below.

"Molecular efficacy" and negative antagonism. Because signal transduction at G protein-linked receptors depends on the ability of H to induce binding of R to G, the coupling constant α, which measures amplitude and sign of such an allosteric effect, also defines the molecular efficacy of the ligand H. Because the logarithm of α can range between negative and positive values, ligands can display positive, neutral, or negative efficacy. Simulations according to this concept predict features that correspond very closely to experimental observations. 1) The model predicts that antagonists can inhibit G protein

activation according to the degree of negative efficacy and be partial or full with respect to the extent of inhibition, just as agonists do with respect to the extent of stimulation. As we have shown previously, opioid antagonists can exhibit a whole range of relative maximal effects for inhibition of GTPase, as opioid agonists do for stimulation (14, 15). 2) Under most conditions, a ligand with $\alpha = 1$ (neutral antagonist) will display similar potencies in competing for ligands having either $\alpha > 1$ or $\alpha < 1$. We have shown previously that the benzomorphan MR2266, which has minimal effects on GTPase (i.e., is a putative neutral antagonist), does indeed compete for the inhibitory effect of the peptidergic antagonist ICI174 (which maximally inhibits the enzyme) and for the stimulatory effect of the agonist (DADLE) with very similar pA_2 values (14). 3) The binding isotherm of a negative antagonist ($\alpha < 1$), unlike that of a neutral antagonist, can exhibit apparent heterogeneity of sites, depending on the value of α and the presence of G.

We have shown here (Fig. 7) that the binding isotherms of the negative antagonist (e.g., ICI174) are in fact consistent with multiple affinity states of the receptor, and this heterogeneity, as demonstrated previously (14), disappears upon inactivation of the G protein by pertussis toxin. It is important to note how this model addresses some fundamental questions of theoretical pharmacology. 1) What is the chemical nature of affinity and efficacy and what do these parameters tell about receptors? According to the model, affinity is the unconditional equilibrium binding constant (K) for the bimolecular interaction of H with R in the absence of G. Efficacy is, instead, a ratio (related to the difference of free energies) between the conditional affinity constant for the trimolecular interaction HR with G and the affinity K. Whereas K depends only on H and R and is a unique property of any ligand-receptor pair, α depends on H, R, and G, so that any ligand-receptor pair can have as many efficacies as there are types of G proteins it interacts with. Hence, affinity characterizes receptor binding sites, but efficacy also identifies receptor-G protein complexes, and the classification of receptors according to the true affinities of a ligand must inevitably be different from that based on the spectrum of efficacies of the ligand. 2) Can affinity and efficacy be distinguished and independently measured? Yes, if purified R and G are available. As elegantly shown by Weber (6), the half-saturation point for H binding to R in the absence of G defines $1/K$. The shift of this point in the presence of an excess of G yields α. For the special case of receptor coupled to pertussis toxin-sensitive G proteins, we propose a "noninvasive" version of this experiment; cells expressing a well identified receptor subtype could be transfected with a vector coding for a known α subunit of the G_i/G_o family of G proteins. If the level of expression can result in a large stoichiometric excess of G protein with respect to receptor, then ligand binding isotherms before and after inactivation by pertussis toxin can furnish a reasonable approximation of the true value of α. 3) Can affinity and efficacy be discriminated in pharmacological studies where only the concentrations of H and/or R are the known variables? No, as predicted by the reciprocal nature of the energetic linkage between the binding processes of H and G through R; the more the ligand H affects the binding of G protein to the receptor, the greater is the effect of G on the binding of H. Thus, any method of analysis based on the assumption that the observed occupancy of the ligand is independent of its efficacy will results in wrong estimates of the true affinity of the ligand. Mackay (19) and Kenakin and Morgan (27) have shown how the interaction of receptor with G proteins invalidates the determination of agonist affinity based on the analysis of agonist-mediated responses in isolated tissues. For radioligand binding studies, we show here, as others did before (8, 17), that the IC_{50} values of various ligands depend on their efficacies and on the efficacy of the tracer used to label R (Fig. 3). When these simulated data are subjected to analysis according to the mass-action law, it is possible to detect up to three apparent binding sites, even for a system simulated for a single type of receptor and G protein (Table 1). If we further postulate the presence of two different G proteins, both interacting with R (27), it is easy to imagine the case of a single receptor that can generate binding and pharmacological data that are perfectly consistent with the existence of several distinct and independent receptor types. The present study introduces two additional reasons for skepticism. First, the notion that antagonists may also exhibit vast differences in efficacy negates the common assumption that their use in the characterization of binding sites affords protection from the influence of receptor-associated G proteins. Second, the finding that the fraction of precoupled receptors may be much higher in isolated membranes than in whole cells suggests that, even for a single receptor-G protein pair, the spectrum of ligand efficacies seen *in vitro* varies dramatically with the degree of intactness of the experimental system. Thus the attempt to distinguish receptor heterogeneity from transducer heterogeneity using pharmacological studies and in the absence of additional molecular knowledge is bound to be an unfruitful undertaking.

Stability constant between receptor and G protein and effect of sodium. For any range of variation of the stability constant M, there are characteristic relationships between efficacy α and changes in binding properties and activity of the ligand. We noted previously (15) a paradoxical difference in the way sodium ions affect GTPase activity and ligand binding. There was an inverse relationship between the magnitude of the apparent intrinsic activity (either negative or positive) of the ligand for GTPase and the ability of sodium to inhibit GTPase in the presence of the ligand, suggesting that sodium effect is larger the closer α is to 1. However, different conclusions could be drawn from the effect of sodium on ligand binding. In this case, Na^+ increased the apparent affinity of antagonists that inhibit GTPase, reduced that of agonists, and only negligibly affected that of partial agonists and neutral antagonists. One might take this as strong phenomenological evidence that the effects of sodium on ligand binding and those on ligand-induced GTPase activity are two distinct and unrelated events. Instead, this dual effect of sodium can be adequately explained by a single mechanism, if we assume that the cation selectively reduces the magnitude of the affinity constant M in the membrane. Using a relatively high value of M (i.e., $1/M$ nearly equal to the receptor concentration) and the assumption that sodium causes a 10–20-fold reduction of this value, the model can mimic both the bell-shaped relation between the concentration of sodium and the intrinsic activity of the ligand for GTPase and the effect of sodium on the binding isotherms of ligands of different efficacies. We also provide experimental evidence, in isolated membranes, that the apparent pA_2 of a putative negative antagonist, unlike that of a neutral antagonist, is increased by sodium. This was predicted by simulations indicating that Schild plots of ligands with $\alpha < 1$, but not of

ligands with $\alpha = 1$, can be shifted to the left by reductions of M.

By suggesting that the effect of sodium acts like a reduction of M, we do not specify a precise molecular mechanism of interaction but simply manipulate an admittedly oversimplified model. Simulations only indicate that the phenomenology related to the introduction of sodium in the system is best mimicked by a selective reduction of the parameter M, rather than changes of K, α, or the ratio R_t/G_t. However, the effect of guanine nucleotides on the binding of H can also be modeled by a reduction of M (7). An "M effect" rules out trivial possibilities, such as sodium competing with H for the same site or changing the chemical properties of H. But a host of indistinguishable mechanisms remain possible, such as an action via G (like that of guanine nucleotides), a "syntopic" competition with G, or a direct effect on R at a distinct site. Biochemical evidence suggests that the latter is the most probable (28).

The net effect of a modulator that reduces the stability constant M depends on its initial magnitude and thus on the proportion of receptor "precoupling." This has two implications. 1) If a known receptor subtype is expressed in cells bearing different G proteins, qualitative and quantitative differences in the effect of Na^+ (and, similarly, in those of guanine nucleotides) may serve to indicate differences in the respective values of the stability constant M. 2) However, because sodium and similar modulators may affect the same receptor type differently in the presence of different G proteins, any effort to classify unknown receptor subtypes on the basis of sodium or GTP sensitivity is likely to be as futile as the endeavor to define receptors on the basis of the absolute potencies of agonists in different tissues.

Receptor activity in the absence of ligand and differences between membranes and isolated cells. The model predicts that a distinction between neutral and negative antagonism is only possible if the value of the affinity constant M is large enough for the spontaneous reaction between empty receptor and G protein to occur to a measurable extent. Therefore, the demonstration of negative antagonism in a biological response can allow one to determine whether unoccupied receptor-G protein complexes accumulate as stable intermediates. This is especially important for the study of intact systems, where biological responses are separated from the initial occupation of the binding site by a cascade of biochemical steps and, therefore, 'basal activity' is no longer a direct indication of the state of activation of the G protein. We show that ligand-independent receptor activity, which obviously exists in membranes, does not occur in the intact cell. The close similarity between the pA_2 of ICI174 obtained here in intact neuroblastoma cells and that measured previously in isolated mouse vas deferens (29) also suggests that the negative antagonism of this ligand and, consequently, spontaneous receptor activity are events only detectable in isolated membranes.

Why do unbound receptor-G protein complexes accumulate in the isolated membrane but not in the intact cell? A likely explanation is that, in the presence of cytosolic factors such as GTP, ions, and perhaps other as yet unknown regulators, the interaction between R and G is only transient and no longer describable by an equilibrium model (see also Ref. 19). This means that, upon separation of membranes from cytosol, spontaneous receptor activity is the inevitable consequence of an interrupted balance between the intrinsic high affinities of the components present in the membrane and the external constraints imposed by modulators existing in high concentrations in the intact cell. Negative efficacy for antagonists has been demonstrated at receptors that either stimulate (30, 31) or inhibit (32) adenylate cyclase. In all cases, isolated membranes or permeabilized cells have been used. Those data, therefore, further corroborate the idea that an intact intracellular environment is a key factor in preventing spontaneous receptor activity.

Are negative antagonists therapeutically useful and worth searching for? No, if competition with agonist is the main concern. In fact, affinities being equal, the neutral antagonist is a more efficient agonist blocker than is the negative kind. However, only a negative antagonist can suppress spontaneous receptor activity. Recent findings indicate that certain mutations in G protein-linked receptors result in constitutive receptor activity (33). If such mutations prove to occur in human pathology, the search for and development of negative antagonists is a novel important target of drug discovery.

Acknowledgments

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