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Evaluation of Models for Analysis of Radioligand Binding Data

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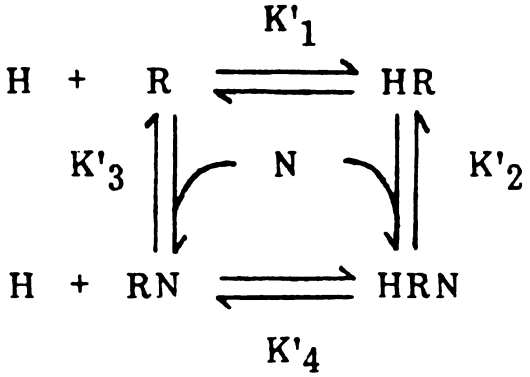
SUMMARY

In the presence of agonists, many neurotransmitter receptors interact with regulatory components, resulting in the formation of a ternary complex composed of agonist, receptor, and a regulatory component, and in biphasic or shallow dose response curves for inhibition of the binding of a radiolabeled antagonist by agonists. Complex dose response curves are often analyzed using equations that describe a model that assumes the presence of two independent populations of receptors (two-independent-receptor model) or equations that describe a model that assumes the reversible interaction of agonist-occupied receptors with a regulatory component (ternary complex model). In this study, the ability of these models to provide good estimates of the concentration of the regulatory component and of the affinities involved in formation of the ternary complex was evaluated. Dose response curves were generated by a computer using an equation that describes the ternary complex model. Analysis of the dose response curves with the two-independent-receptor model resulted in good estimates of the concentration of the regulatory component and of the affinity of the receptor for the agonist. Reliable estimates of the other affinity constants that relate to formation of the ternary complex could not be obtained. Analysis of the dose response curves with the ternary complex model resulted in good estimates of the concentration of the regulatory component and of the affinity constants that relate to formation of the ternary complex. Random error was added to the data points that made up the dose response curves to simulate error observed in experiments with biological systems. Analysis of the dose response curves that contained random error with the two-independent-receptor model yielded results similar to those obtained from analysis of dose response curves that did not contain random error. Analysis of the dose response curves that contained random error with the ternary complex model resulted in good estimates of the concentration of the regulatory component and of the affinity of the receptor for the agonist. However, reliable estimates of the other affinity constants involved in formation of the ternary complex could not be obtained. Thus, caution should be exercised when interpreting results of the analysis of dose response curves with either the two-independent-receptor model or the ternary complex model.

The adenylate cyclase system is composed of a catalytic unit, stimulatory and inhibitory guanine nucleotide-binding proteins (N_S and N_i, respectively), and receptors that either stimulate or inhibit the activity of the catalytic unit after occupation by an agonist. Investigation of the interactions of agonists and antagonists with receptors can yield important insights into the mechanisms through which receptors regulate the activity of adenylate cyclase. The affinity of a receptor for an unlabeled ligand can often be determined in a relatively direct manner by measuring the ability of the unlabeled ligand to inhibit the binding of a radiolabeled antagonist. When the unlabeled ligand is an antagonist, the result usually conforms to that expected for competitive binding of two ligands to a single homogeneous population of receptors. However, when the unlabeled ligand is an agonist, the result is often inconsistent with the existence of a single homogeneous population of receptors. Binding of agonists is often described by Hill coefficients that are <1, and the affinity of receptors for agonists is often decreased when assays are carried out in the presence of guanine nucleotides. Low Hill coefficients and guanine nucleotide-dependent decreases of the affinity of β-adrenergic receptors for agonists led to the proposal that agonist-occupied receptors interact with a regulatory component to form a ternary complex composed of agonist, receptor, and regulatory component (1-3).

The "ternary complex" model postulates that a receptor can exist in one of two distinct states with different affinities for agonists depending on whether the regulatory component is associated with the receptor. The model can be written as follows:



Model 1. The ternary-complex model.

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where R is the receptor, H is the agonist, N is the regulatory component, K_1' represents the affinity of the receptor for the agonist, K_2' represents the affinity of the agonist-occupied receptor for the regulatory component, K_3' represents the affinity of the receptor for the regulatory component, and K'_4 represents the affinity of the receptor-regulatory component complex for the agonist. Many of the theoretical properties of this model have been investigated, and the model is capable of explaining a number of experimental observations (4–6). For instance, curved Scatchard plots and low Hill coefficients for agonists, a lower apparent density of sites for radiolabeled agonists versus radiolabeled antagonists, biphasic dose response curves for inhibition of a radiolabeled antagonist by agonists, and effects of GTP on the ability of receptors to bind agonists can all be explained by the ternary complex model.

A ternary complex model can be proposed for most receptors that regulate the activity of adenylate cyclase, including receptors for epinephrine (1–3, 7), norepinephrine (8, 9), dopamine (6, 10, 11), acetylcholine (12, 13), serotonin (14, 15), opiates (16), angiotensin (17), γ-aminobutyric acid (18), and adenosine (19). Although it is not known whether a ternary complex model is the correct model, equations that describe the ternary complex model have been used for the analysis of radioligand binding data from a number of different receptor systems (2, 6, 20–22).

Many investigators simplify the analysis of radioligand binding data by assuming that agonists recognize a heterogeneous population of two independent and noninterconvertible classes of receptors (7–9, 11, 13, 15, 17, 19). This "two-independent-receptor" model can be written as follows:

$$H + R_1 \overset{K_1}{\rightleftharpoons} HR_1$$

$$H + R_2 \overset{K_2}{\rightleftharpoons} HR_2$$

Model 2. The two-independent-receptor model.

where R_1 and R_2 are independent populations of receptors with different affinities (K_1 and K_2, respectively) for the hormone H.

Analysis of radioligand binding data with a two-independent-receptor model has been used successfully for investigations of systems where independent classes of receptor subtypes actually exist (23–27). However, analysis of the behavior of a complex receptor system, such as a receptor coupled to a regulatory component, with a two-independent-receptor model may result in the loss of potentially important information. Furthermore, it is unclear how the parameters of the two-independent-receptor model relate to the actual reactions thought to be taking place in the membrane.

The present study was designed to evaluate the validity of results obtained from analysis of radioligand binding data with the ternary complex and the two-independent-receptor models. A computer was used to generate data from an equation that describes the ternary complex model, and systematic variations in the concentration of the regulatory component and of the values for the affinities involved in formation of the ternary complex resulted in computer-generated dose response curves that approximated those obtained in experiments with biological systems. The computer-generated dose response curves were then analyzed by fitting the data to an equation that describes the two-independent-receptor model and to an equation that describes the ternary complex model. The ability of each of these models to provide accurate estimates of the affinities that were used to generate the data was determined. In addition to the analysis of ideal data, random error was added to the computer-generated data to approximate error observed in experiments with biological systems.

Methods

Simulation of kinetic models. Equations that described kinetic models were simulated using MLAB (28) and programs written in PL-PROPHET (29) on a DEC10 computer. All of the equations were written to describe dose response curves of inhibition of the binding of a radiolabeled antagonist by an unlabeled agonist. Reactions were assumed to be competitive, reversible, and at equilibrium. The radioligand was assumed to be nonselective for different classes of receptors or for different forms of the same receptor. The concentration of radioligand was assumed to be lower than the affinity of the receptor for the radioligand, such that the concentration of receptors occupied by the radioligand was negligible relative to that of other forms of the receptor. A result of this assumption is that the observed EC_{50} value for a competing ligand is independent of the concentration of the radioligand and is equal to the K_d value of the receptor for the ligand. The concentration of receptor was assumed to be lower than its affinity for either the radioligand or the agonist, such that an insignificant fraction of the total concentration of either ligand was bound to the receptor at equilibrium. A result of this assumption is that the concentrations of free radioligand and free agonist are not affected by the amount of ligand bound to the receptor.

The equation used to describe the interaction of an agonist with a single homogeneous population of receptors (one-receptor model) was derived from the appropriate mass action and conservation of mass equations (30). These equations can be manipulated algebraically to yield

$$(HR) = (R_t)(H)/(K_d + H) \quad \text{(A1)}$$

where H is the concentration of free agonist, R_t is the total concentration of receptor, K_d is the equilibrium dissociation constant representing the affinity of the receptor for the agonist, and HR is the concentration of agonist-occupied receptors. The conservation of mass equation can be written as

$$(R) = (R_t) - (HR) \quad \text{(A2)}$$

where R is the concentration of free receptor. The final form of the equation used to simulate the one-receptor model was obtained by substitution of Eq. A1 for HR in Eq. A2.

The equation used to describe the interaction of an agonist with two independent populations of receptors (two-independent-receptor model; Model 2) was derived from the appropriate mass action and conservation of mass equations (30). These equations can be manipulated algebraically to yield

$$(HR_1) = (R_{1t})(H)/(K_1 + H) \quad \text{(B1)}$$

and

$$(HR_2) = (R_{2t})(H)/(K_2 + H) \quad \text{(B2)}$$

where H is the concentration of free agonist, R_{1t} and R_{2t} are the total concentrations of two independent populations of receptors, K_1 and K_2

are equilibrium dissociation constants representing the affinity of the receptors for the agonist, and HR_1 and HR_2 are the concentrations of agonist-occupied receptors. The conservation of mass equation can be expressed as

$$(R_1 + R_2) = (R_{1t} + R_{2t}) - (HR_1) - (HR_2) \quad \text{(B3)}$$

where R_1 and R_2 are the concentrations of free receptors. The final form of the equation used to simulate the two-independent-receptor model was obtained by substitution of Eqs. B1 and B2 for HR_1 and HR_2, respectively, in Eq. B3.

The equation used to describe the reversible interaction of agonist-occupied receptors with a regulatory component (ternary complex model; Model 1) was derived from the appropriate mass action and conservation of mass equations (4, 30). The mass action equations can be expressed as

$$K_1' = (H)(R)/(HR) \quad \text{(C1)}$$

$$K_2' = (HR)(N)/(HRN) \quad \text{(C2)}$$

$$K_3' = (R)(N)/(RN) \quad \text{(C3)}$$

$$K_4' = (H)(RN)/(HRN) \quad \text{(C4)}$$

where H is the concentration of agonist, R and N are the concentrations of free receptor and free regulatory component, respectively, HR is the concentration of agonist-occupied receptor, RN is the concentration of receptor-regulatory component complex, and HRN is the concentration of agonist-receptor-regulatory component complex (i.e., the ternary complex). The equilibrium dissociation constant K_1' represents the affinity of the receptor for the agonist, K_2' represents the affinity of the agonist-occupied receptor for the regulatory component, K_3' represents the affinity of the receptor for the regulatory component, and K_4' represents the affinity of the receptor-regulatory component complex for the agonist. The conservation of mass equations can be written as

$$(R_t) = (R) + (HR) + (RN) + (HRN) \quad \text{(C5)}$$

$$(N_t) = (N) + (RN) + (HRN) \quad \text{(C6)}$$

where R_t and N_t are the total concentrations of the receptor and the regulatory component, respectively.

The concentration of HRN can be expressed in terms of H, R_t, N_t, K_1', K_2', and K_4'. Dividing both sides of Eq. C5 by HRN and rearranging yields

$$(HRN)/(R_t) = (HRN)/(R + HR + RN + HRN) \quad \text{(C7)}$$

If the numerator and denominator of the right side of Eq. C7 are each multiplied by $(H)(N)/(HRN)$, and Eqs. C1, C2, C4, and C6 are rearranged and substituted for HR, HRN, RN, and N, respectively, then rearranging the resulting equation yields

$$[(H) + (2)(K_4') + (K_4')^2/(H)](HRN)^2 - [(K_1')(K_2') + (H)(K_2') + (K_4')(N_t) + (H)(N_t) + (R_t)(K_4') + (R_t)(H)](HRN) + [(H)(R_t)(N_t)] = 0 \quad \text{(C8)}$$

Eq. C8 is a quadratic equation in standard form and can be solved to yield

$$HRN = [-b - \sqrt{b^2 - 4ac}]/(2a) \quad \text{(C9)}$$

where $a = [(H) + (2)(K_4') + (K_4')^2/(H)]$, $b = -[(K_1')(K_2') + (H)(K_2') + (K_4')(N_t) + (H)(N_t) + (R_t)(K_4') + (R_t)(H)]$, and $c = [(H)(R_t)(N_t)]$.

The concentration of HR can be expressed in terms of H, N_t, HRN, K_2', and K_4' by rearranging Eqs. C6 and C4 and substituting them for N and RN, respectively, in Eq. C2 and then rearranging to yield

$$HR = (K_2')(HRN)/[(N_t) - (K_4')(HRN)/(H) - (HRN)] \quad \text{(C10)}$$

Since the radioligand was assumed to be nonselective and the concentration of receptors occupied by the radioligand was assumed to be negligible relative to that of other forms of the receptor, the final form of the equation used to simulate the ternary complex model was obtained by substituting Eqs. C9 and C10 for HRN and HR, respectively, in the conservation of mass equation (C5), and then solving for $(R + RN)$.

The equilibrium dissociation constant K_3', the affinity of the receptor for the regulatory component, does not appear in the final equation used to describe the ternary complex model. However, since the overall free energy necessary to go from R to HRN must be the same regardless of the path taken, the value of K_3' can be determined from the values of the other equilibrium dissociation constants using the equation

$$(K_1')(K_2') = (K_3')(K_4')$$

Analysis of computer-generated data. The equation that describes the ternary complex model (Model 1) was used to generate data for 28 or 56 concentrations of an agonist spaced equally on a log scale between 10^{-12} M and 10^{-3} M. The values of N_t, K_2', and K_4' were systematically changed, and the resulting dose response curves were analyzed using equations that described the two-independent-receptor and ternary complex models. Analysis involved nonlinear least squares regression of the data using MLAB (28) and programs written in PL-PROPHET (29) on a DEC10 computer. This computer program converges on the same final parameter estimates even if the initial estimates vary over a large range. A common set of initial estimates was, therefore, used for all of the iterative, nonlinear regression procedures. The initial estimates used were: $R_t = 1.0$, $N_t = 0.5$, R_{1t} and $R_{2t} = 0.5$, K_1 and $K_1' = 10^{-6}$, K_2 and $K_4' = 10^{-9}$, and $K_2' = 10^{-3}$.

Random error was added to each computer-generated data point to approximate experimental error observed for data derived from biological experiments. Random error was obtained by adding the results of the following equation to the computer-generated data:

$$\text{random error} = \frac{(0.02)(B_t)\left\{\sum_{i=1}^{n} RAN_i - (n/2)\right\}}{(n/12)}$$

where RAN is a uniformly distributed random number between 0 and 1 generated by the computer, B_t is the total amount of radioligand bound to receptors, and $n = 20$. The above equation uses the Central Limit Theorem to convert the sum of a series of uniformly distributed random numbers into a single random number from a normal distribution with a mean of 0 and SD of 2.0% of the total radioligand bound (31). This error function was chosen because it led to values that approximated the actual distribution of data points from replicate radioligand binding assays observed in our laboratory for a number of different receptors in a variety of tissues.

Dose response curves that included random error were analyzed by nonlinear least squares regression of the data using an equation that describes the ternary complex model. The values of N_t, K_2', and K_4' were systematically changed, and for each set of values, 10 dose response curves were analyzed. Means and standard errors were determined for the computer-calculated values of N_t, K_1', K_2', and K_4'. Because replicate K_d values of a drug have been found to conform to a normal distribution only after conversion of their negative logarithms (27, 32, 33), the values for all of the affinity constants were converted to their negative logarithms before determining the mean and standard error.

The nonlinear least squares regression analysis provided an approximation of the standard deviation associated with the computer-calculated value of each parameter (28, 29). The result of dividing the computer-calculated value of a parameter by its associated standard deviation follows Student's t distribution and can be used to obtain a p value associated with the parameter (28, 29). It was our empirical evidence that when the p value associated with a computer-calculated estimate was greater than 0.98, the estimate varied many orders of magnitude from the true value for that parameter. Thus, we chose an

arbitrary p value of 0.98 in order to have an objective criterion for excluding computer-calculated estimates that were unrealistically high or low. If any of the computer-calculated parameters obtained from the nonlinear least squares regression analysis of a given dose response curve had an associated $p > 0.98$, none of the parameters from analysis of that dose response curve were included in the determination of the mean and standard error.

Results

Simulation of kinetic models. The equation that describes the two-independent-receptor model (Model 2) was simulated for various concentrations of agonist between 10^{-12} M and 10^{-3} M (Fig. 1). The ratio of K_2 to K_1 is a measure of the selectivity of the agonist for the two independent populations of receptors. When the magnitude of this ratio decreased from 10^{-1} to 10^{-5} (K_2 decreased from 10^{-7} M to 10^{-11} M), the resulting dose response curves became increasingly biphasic and the apparent affinity of some of the receptors for the agonist increased (Fig. 1). The computer-generated dose response curves resembled biologically derived dose response curves observed for agonists in a variety of receptor systems in the absence of GTP (7–9, 11, 13, 15, 17, 19).

The equation that describes the ternary complex model (Model 1) was simulated for various concentrations of agonist between 10^{-12} M and 10^{-3} M (Fig. 2). The ratio of K_4' (the affinity of RN for H) to K_1' (the affinity of R for H) is a measure of the selectivity of the agonist for the two states of the receptor. When the magnitude of this ratio decreased from 10^{-1} to 10^{-5} (K_4' decreased from 10^{-7} M to 10^{-11} M), the resulting dose response curves became increasingly biphasic and the apparent affinity of some of the receptors for the agonist increased (Fig. 2). The ratio of K_2' (the affinity of HR for N) to K_1' is a measure of the ability of the components of the ternary complex to associate in the form of HRN. When the magnitude of this ratio decreased from 10^5 to 1 (K_2' decreased from 10^{-1} M to 10^{-6} M), the resulting dose response curves became increasingly biphasic and the apparent affinity of some of the receptors for the agonist increased (Fig. 2). The computer-generated dose response curves resembled biologically derived dose response curves observed for agonists in a variety of receptor systems in the absence of GTP (7–9, 11, 13, 15, 17, 19). The maximum apparent affinity of the receptors for the agonist was limited by the value of K_4' (affinity of RN for H) no matter how much the value of K_2' (affinity of HR for N) favored formation of the ternary complex.

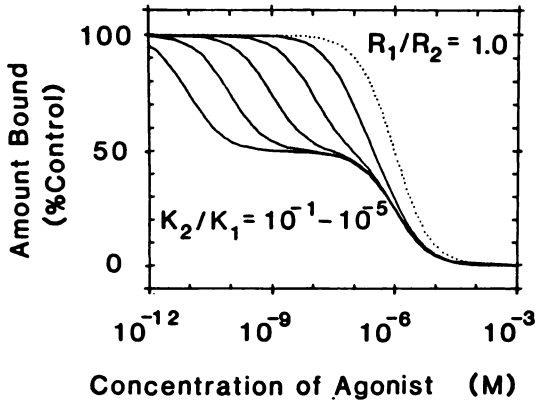

Fig. 1. Simulation of the two-independent-receptor model: effect of varying the ratio of K_1 to K_2. The equation that describes the two-independent-receptor model was simulated for various concentrations of agonist. The results are depicted as the amount (% control) of radioligand bound versus the concentration (M) of agonist. For all simulations, K_1 was 10^{-6} M and R_1/R_2 was 1.0. The *solid curves* become increasingly biphasic as K_2 decreases from 10^{-7} M to 10^{-8} M, 10^{-9} M, 10^{-10} M, and 10^{-11} M. The *dotted curve* is presented as a reference and represents the results of simulation of the equation that describes the interaction of an agonist with a single homogeneous population of receptors with a K_d of 10^{-6}.

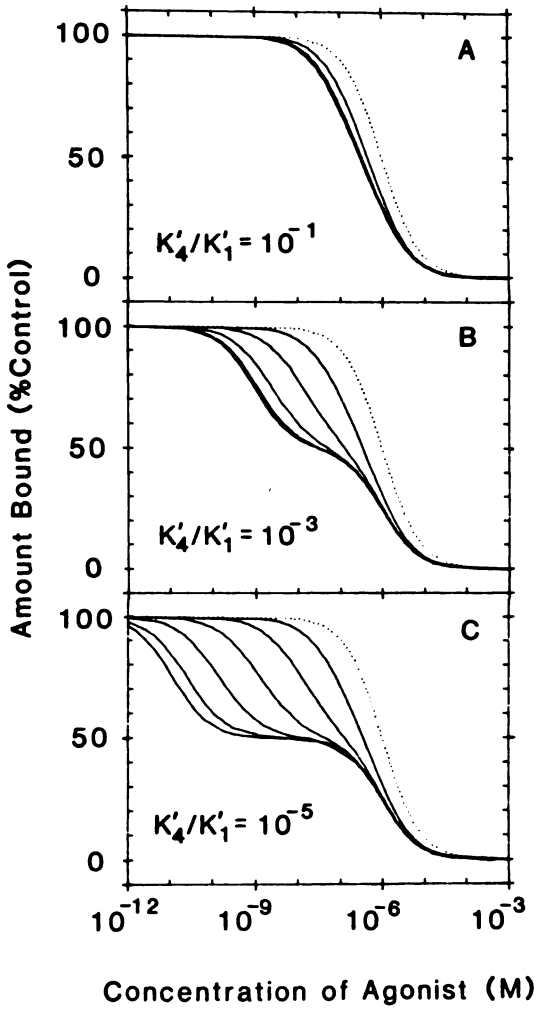

Fig. 2. Simulation of the ternary complex model: effect of varying the ratios of K_2' to K_1' and K_4' to K_1'. The equation that describes the ternary complex model was simulated for various concentrations of agonist. The results are depicted as the amount (% control) of radioligand bound versus the concentration (M) of agonist. For all simulations, K_1' was 10^{-6} M and N_t/R_t was 1.0. Within each panel the solid curves become increasingly biphasic as K_2' decreases from 10^{-1} M to 10^{-2} M, 10^{-3} M, 10^{-4} M, 10^{-5} M, and 10^{-6} M. The values of K_4' were: A, 10^{-7} M; B, 10^{-9} M; and C, 10^{-11} M. The *dotted curves* are presented as references and represent the results of simulation of the equation that describes the interaction of an agonist with a single homogeneous population of receptors with a K_d of 10^{-6}.

When the ratio of N_t to R_t was greater than 0.25 and less than 0.75, the resulting dose response curves were biphasic (Fig. 3, A–C). However, when the ratio of N_t to R_t was greater than 2.0, the resulting dose response curves were no longer biphasic (Fig. 3D). As the ratio of N_t to R_t increased, more of the receptors appeared to bind the agonist with high affinity and the apparent affinity of the receptor for the agonist increased. However, the maximum apparent affinity of the receptors for the agonist was limited by the value of K_4' (affinity of

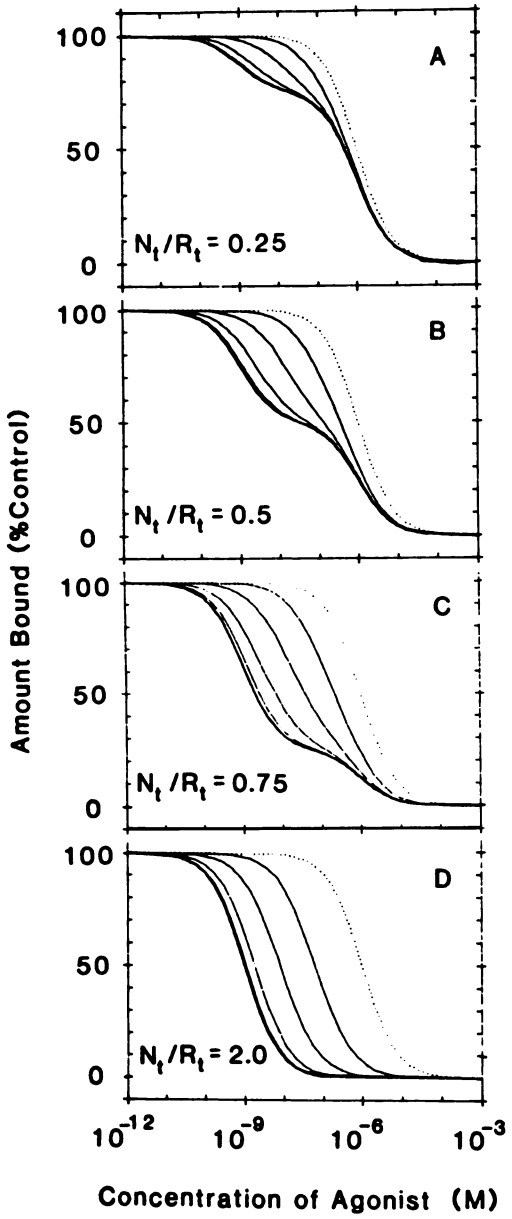

Fig. 3. Simulation of the ternary complex model: effect of varying the ratio of N_t to R_t and the ratio of K_2' to K_1'. The equation that describes the ternary complex model was simulated for various concentrations of agonist. The results are depicted as the amount (% control) of radioligand bound versus the concentration (M) of agonist. For all simulations, K_1' was 10^{-6} M and K_4' was 10^{-9} M. Within each panel the *solid curves* become increasingly biphasic as K_2' decreases from 10^{-1} M to 10^{-2} M, 10^{-3} M, 10^{-4} M, 10^{-5} M, and 10^{-6} M. The values of N_t/R_t were: A, 0.25; B, 0.5; C, 0.75; and D, 2.0. The *dotted curves* are presented as references and represent the results of simulation of the equation that describes the interaction of an agonist with a single homogeneous population of receptors with a K_d of 10^{-6}.

RN for *H*) regardless of the ratio of N_t to R_t or how much the value of K_2' (affinity of *HR* for *N*) favored formation of the ternary complex.

Analysis of computer-generated dose response curves. The equation that describes the ternary complex model (Model 1) was used to generate data for 28 concentrations of agonist spaced equally on a log scale between 10^{-12} M and 10^{-3} M. A series of dose response curves was simulated, keeping the values of R_t, N_t, and K_1' constant and systematically changing the values of K_2' and K_4'. These computer-generated dose response curves were analyzed by nonlinear least squares regression of the data using an equation that describes the two-independent-receptor model (Table 1). The computer-calculated value of K_1 appeared to be a very good approximation of the value of K_1', since it was always within 2.0% of the value of K_1'. Similarly, the computer-calculated value of R_{2t} appeared to be a relatively good approximation of the value of N_t, since it was always within 20% of the value of N_t. The computer-calculated value of R_{2t} was a better approximation of the value of N_t when the affinity of *HR* for *N* (K_2') favored formation of the ternary complex. At each value of K_4', the ability of the computer-calculated value of K_2 to approximate the value of K_4' increased as the affinity of *HR* for *N* (K_2') increased (Table 1, Fig. 4). However, the computer-calculated value of K_2 often substantially overestimated the value of K_4', especially when the affinity of *RN* for *H* (K_4') was high and the affinity of *HR* for *N* (K_2') was low. Similar results were obtained from analysis of dose response curves composed of data that included the addition of random error (results not shown).

The same computer-generated dose response curves that were analyzed using the equation that describes the two-independent-receptor model were also analyzed using the equation that describes the ternary complex model. Since no experimental error had been added to the computer-generated data, analysis with the same equation that was used to generate the data resulted in exact fits of the data to the equation (results not shown). However, data derived from biological experiments always contain experimental error. To approximate this error,

TABLE 1

Estimated parameters from the analysis of computer-generated data using the two-independent-receptor model

The equation that describes the ternary complex model was simulated for 28 concentrations of agonist spaced equally on a log scale between 10^{-12} M and 10^{-3} M. The resulting dose response curves were analyzed by nonlinear regression with the equation that describes the two-independent-receptor model. For all of the simulations, R_t was 1.0, N_t was 0.5, and K_1' was 10^{-6} M. The value of K_4' was decreased from 10^{-7} to 10^{-11} M, and at each value of K_4' the value of K_2' was increased from 10^{-6} to 10^{-1} M. The estimates of the parameters K_1 and K_2 are presented as the negative logarithm of the values of K_1 and K_2 calculated by the computer.

K_4'		K_2' (M)					
		10^{-6}	10^{-5}	10^{-4}	10^{-3}	10^{-2}	10^{-1}
M							
10^{-7}	R_{1t}	0.492	0.499	0.500	0.500	0.504	0.567
	R_{2t}	0.508	0.501	0.500	0.500	0.496	0.433
	K_1	5.99	6.00	6.00	6.00	6.00	6.02
	K_2	6.99	7.00	7.00	6.99	6.94	6.73
10^{-8}	R_{1t}	0.500	0.500	0.500	0.501	0.521	0.600
	R_{2t}	0.500	0.500	0.500	0.499	0.479	0.400
	K_1	6.00	6.00	6.00	6.00	6.02	6.05
	K_2	8.00	8.00	8.00	7.93	7.64	7.05
10^{-9}	R_{1t}	0.500	0.500	0.500	0.507	0.535	0.606
	R_{2t}	0.500	0.500	0.500	0.493	0.465	0.394
	K_1	6.00	6.00	6.00	6.01	6.04	6.06
	K_2	9.00	8.91	8.93	8.61	7.17	7.10
10^{-10}	R_{1t}	0.500	0.500	0.504	0.514	0.538	0.607
	R_{2t}	0.500	0.500	0.496	0.486	0.462	0.393
	K_1	6.00	6.00	6.01	6.03	6.05	6.06
	K_2	9.99	9.93	9.60	8.87	7.96	7.10
10^{-11}	R_{1t}	0.502	0.502	0.507	0.515	0.539	0.607
	R_{2t}	0.498	0.498	0.493	0.485	0.461	0.393
	K_1	6.01	6.00	6.02	6.03	6.05	6.06
	K_2	11.1	10.6	9.85	8.91	7.97	7.11

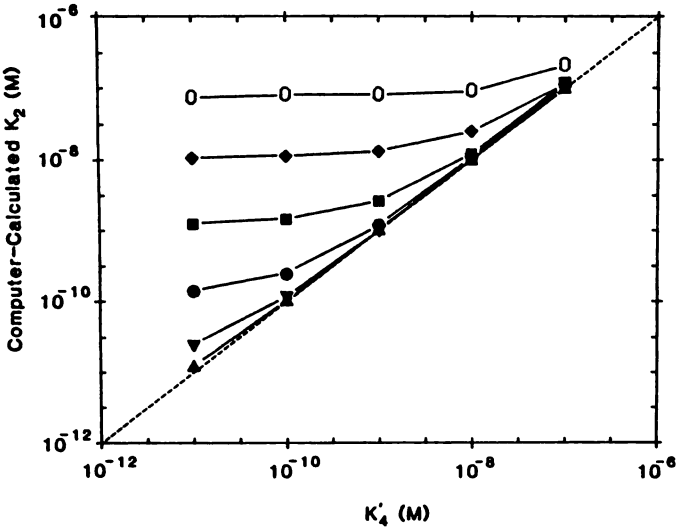

Fig. 4. Analysis of data using the two-independent-receptor model: comparison of the computer-calculated value of K_2 with the true value of K_4'. Computer-calculated values of K_2 (M) taken from Table 1 are plotted versus the true value of K_4' (*M*) used to generate the data. For all simulations, R_t was 1.0, N_t was 0.5, and K_1' was 10^{-6} M. The values of K_2' were: 10^{-6} M (▲), 10^{-5} M (▼), 10^{-4} M (●), 10^{-3} M (■), 10^{-2} M (♦), and 10^{-1} M (○). The line of identity (– – –) is shown for reference.

random error generated from a normal distribution with a mean of 0 and SD of 2.0% of the total radioligand bound was added to the computer-generated data. The distribution of data that included random error was similar to the distribution of data from replicate experiments observed in binding assays with a variety of radioligands (Fig. 5).

The equation that describes the ternary complex model (Model 1) was used to generate data for 28 concentrations of agonist spaced equally on a log scale between 10^{-12} M and 10^{-3} M. A series of dose response curves was simulated, keeping the values of R_t, N_t, and K_1' constant and systematically changing the values of K_2' and K_4'. For each set of parameters, 10 dose response curves to which random error had been added were analyzed by nonlinear least squares regression using an equation that describes the ternary complex model. For each set of parameter values, analysis of at least 3 of the 10 dose response curves resulted in useful estimates of all of the parameters (i.e., the associated significance determined for each of the parameters was $p < 0.98$), and the mean and standard error of the estimates were determined (Table 2). For each set of parameter values examined, the means of the computer-calculated values of K_1' and N_t were within 5% and 30% of the values of K_1' and N_t, respectively (Table 2). In general, at each value of K_4', the computer-calculated value of K_4' approached the value of K_4' as the affinity of *HR* for *N* (K_2') increased (Table 2, Fig. 6). However, the mean of the computer-calculated values of K_4' often substantially overestimated the value of K_4', especially when the affinity of *RN* for *H* (K_4') was high and the affinity of *HR* for *N* (K_2') was low. At each value of K_2', the computer-calculated value of K_2' approached the value of K_2' as the affinity of *RN* for *H* (K_4') increased (Table 2, Fig. 7). However, the mean of the computer-calculated values of K_2' often substantially overestimated the value of K_2', especially when the affinity of *HR* for *N* (K_2') was high and the affinity of *RN* for *H* (K_4') was low.

As long as the ratio of N_t to R_t was greater than 0.25 and less than 1.0, the computer-calculated value of N_t/R_t was a relatively good approximation of the value of N_t/R_t (Table 3). However, when the ratio of N_t to R_t was increased, the computer-calculated parameters had associated significance levels of $p > 0.98$ and were therefore not useful estimates of the parameters.

Discussion

Although some receptors are themselves enzymes or ion channels, others are without inherent activity. In such instances, neurotransmitter-occupied receptors must interact with another protein that possesses such activity. There is substantial evidence to support the hypothesis that many neurotransmitters participate in the formation of reversible complexes composed of neurotransmitter, receptor, and a regulatory component. A model that assumes a reversible interaction between agonist-occupied receptors and a regulatory component (ternary complex model) may apply to many of the receptors that either stimulate or inhibit the activity of adenylate cyclase. This model is similar to a model that assumes the reversible interaction of an enzyme with two substrates (30). However, the ternary complex model, as used here and elsewhere (2, 4, 5, 17, 20, 21), explicitly allows for a significant fraction of the regulatory component (i.e., the second substrate) to be bound to the receptor.

Simulations of the ternary complex model revealed that the apparent affinity of the receptor for an agonist depends on the concentration of the regulatory component relative to its affinity for the receptor. As long as the regulatory component is present in excess, the interaction of the receptor with an agonist will always appear as if it were a simple bimolecular reaction. However, if the regulatory component is present in stoichiometrically limiting concentrations, then binding of an agonist by the receptor can be complex and the receptor can demon-

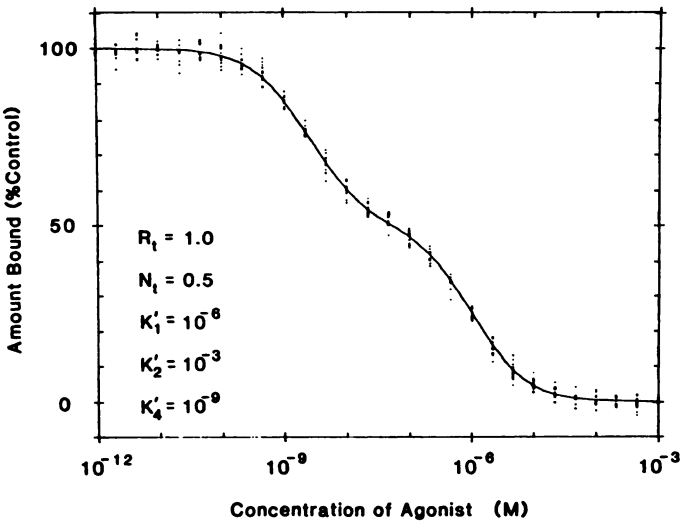

Fig. 5. Simulation of the ternary complex model: addition of random error. The *solid line* represents the results of simulation of the equation that describes the ternary complex model for concentrations of agonist between 10^{-12} M and 10^{-3} M. The results are depicted as the amount (% control) of radioligand bound versus the concentration (M) of agonist. This equation was also used to generate data points for 28 concentrations of agonist spaced equally on the log scale between 10^{-12} M and 10^{-3} M. Random error was then added to each of the data points, and the results of 10 simulations are depicted (·). For the simulations shown here, R_t was 1.0, N_t was 0.5, K_1' was 10^{-6} M, K_2' was 10^{-3} M, and K_4' was 10^{-9} M.

TABLE 2

Estimated parameters from the analysis of computer-generated data using the ternary complex model

The equation that describes the ternary complex model was simulated 10 separate times for 28 concentrations of agonist spaced equally on a log scale between 10^{-12} and 10^{-3} M. Random error was added to each data point and the resulting dose response curves were analyzed by nonlinear regression with the equation that describes the ternary complex model. For all of the simulations, R_t was 1.0, N_t was 0.5, and K_1' was 10^{-6} M. The value of K_4' was decreased from 10^{-7} to 10^{-11} M, and at each value of K_4' the value of K_2' was increased from 10^{-6} to 10^{-1} M. The estimates of the parameters K_1', K_2', and K_4' are presented as the negative logarithm of the values of K_1', K_2', and K_4' calculated by the computer. The mean ± standard error for the computer-calculated parameters from (n) successful analyses are presented.

K_4'		K_2' (M)					
		10^{-6}	10^{-5}	10^{-4}	10^{-3}	10^{-2}	10^{-1}
M							
10^{-7}	N_t	0.703 ± 0.088	0.560 ± 0.041	0.651 ± 0.091	0.636 ± 0.076	0.561 ± 0.058	0.648 ± 0.094
	K_1'	5.79 ± 0.099	5.97 ± 0.039	5.86 ± 0.081	5.86 ± 0.069	5.90 ± 0.091	5.91 ± 0.041
	K_2'	1.30 ± 0.099	1.39 ± 0.114	1.38 ± 0.081	1.45 ± 0.056	1.32 ± 0.171	0.974 ± 0.087
	K_4'	7.61 ± 0.112	7.22 ± 0.054	7.34 ± 0.113	7.28 ± 0.094	7.37 ± 0.152	7.01 ± 0.041
	(n)	(6)	(5)	(4)	(6)	(3)	(3)
10^{-8}	N_t	0.511 ± 0.008	0.502 ± 0.004	0.515 ± 0.008	0.505 ± 0.008	0.526 ± 0.012	0.541 ± 0.066
	K_1'	5.96 ± 0.019	5.97 ± 0.009	5.96 ± 0.015	5.97 ± 0.017	5.94 ± 0.024	5.94 ± 0.058
	K_2'	2.87 ± 0.052	2.66 ± 0.121	2.64 ± 0.085	2.69 ± 0.067	2.17 ± 0.041	1.31 ± 0.158
	K_4'	8.11 ± 0.028	8.21 ± 0.106	8.22 ± 0.065	8.07 ± 0.015	7.81 ± 0.059	7.49 ± 0.226
	(n)	(8)	(6)	(9)	(8)	(4)	(4)
10^{-9}	N_t	0.508 ± 0.003	0.495 ± 0.003	0.504 ± 0.004	0.499 ± 0.004	0.492 ± 0.011	0.523 ± 0.053
	K_1'	5.97 ± 0.009	5.99 ± 0.007	5.98 ± 0.012	5.98 ± 0.011	5.98 ± 0.021	5.95 ± 0.042
	K_2'	3.73 ± 0.163	4.02 ± 0.167	3.62 ± 0.138	3.30 ± 0.127	2.69 ± 0.204	1.37 ± 0.124
	K_4'	9.34 ± 0.102	9.14 ± 0.053	9.25 ± 0.135	8.93 ± 0.136	8.10 ± 0.153	7.46 ± 0.133
	(n)	(10)	(9)	(8)	(9)	(4)	(5)
10^{-10}	N_t	0.503 ± 0.004	0.492 ± 0.004	0.494 ± 0.003	0.495 ± 0.003	0.482 ± 0.003	0.470 ± 0.041
	K_1'	5.99 ± 0.009	6.01 ± 0.008	6.00 ± 0.011	5.99 ± 0.009	5.98 ± 0.015	6.00 ± 0.034
	K_2'	6.04 ± 0.087	5.96 ± 0.125	4.92 ± 0.195	3.36 ± 0.162	2.47 ± 0.212	1.34 ± 0.067
	K_4'	10.0 ± 0.078	10.1 ± 0.066	9.79 ± 0.125	9.34 ± 0.162	8.87 ± 0.404	7.44 ± 0.087
	(n)	(10)	(10)	(10)	(7)	(3)	(7)
10^{-11}	N_t	0.501 ± 0.002	0.510 ± 0.007	0.491 ± 0.004	0.492 ± 0.006	0.456 ± 0.016	0.348 ± 0.075
	K_1'	5.98 ± 0.005	5.97 ± 0.011	6.01 ± 0.017	6.00 ± 0.014	6.05 ± 0.025	6.10 ± 0.063
	K_2'	5.69 ± 0.099	5.66 ± 0.248	4.24 ± 0.265	3.34 ± 0.162	2.41 ± 0.168	1.66 ± 0.449
	K_4'	11.1 ± 0.063	10.7 ± 0.197	10.1 ± 0.099	9.56 ± 0.141	8.48 ± 0.306	7.96 ± 0.571
	(n)	(10)	(9)	(8)	(9)	(4)	(3)

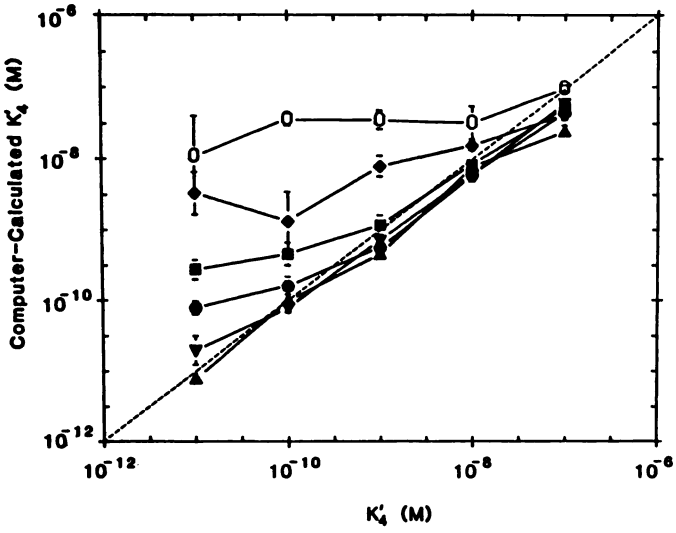

Fig. 6. Analysis of the data using the ternary complex model: comparison of the computer-calculated value of K_4' with the true value of K_4'. Computer-calculated values of K_4' (M) taken from Table 2 are plotted versus the true value of K_4' (M) used to generate the data. Random error was included as defined in Fig. 5. For all simulations, R_t was 1.0, N_t was 0.5, and K_1' was 10^{-6} M. The values of K_2' were: 10^{-6} M (▲), 10^{-5} M (▼), 10^{-4} M (●), 10^{-3} M (■), 10^{-2} M (♦), and 10^{-1} M (○). The line identity (– – –) is shown for reference.

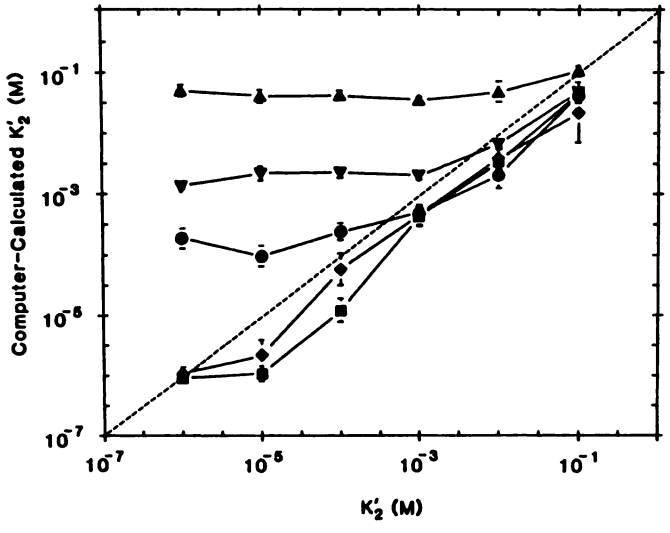

Fig. 7. Analysis of data using the ternary complex model: comparison of the computer-calculated value of K_2' with the true value of K_2'. Computer-calculated values of K_2' (M) taken from Table 2 are plotted versus the true value of K_2' (M) used to generate the data. Random error was included as defined in Fig. 5. For all simulations, R_t was 1.0, N_t was 0.5, and K_1' was 10^{-6} M. The values of K_4' were: 10^{-11} M (♦), 10^{-10} M (■), 10^{-9} M (●), 10^{-8} M (▼), and 10^{-7} M (▲). The line of identity (– – –) is shown for reference.

strate two apparent affinities for the agonist (i.e., K_1' and K_4'). The apparent affinity of the receptor for the agonist increases as the affinity of HR for N (K_2') increasingly favors formation of the ternary complex, but the maximum apparent affinity of the receptor for the agonist is limited by the affinity of RN for H (K_4'), no matter how much the value of K_2' favors formation of the ternary complex.

Complex dose response curves for agonists are often analyzed

TABLE 3

Estimated parameters from the analysis of computer-generated data using the ternary complex model: varying the ratio of N_t to R_t

The equation that describes the ternary complex model was simulated 10 separate times for 28 concentrations of agonist spaced equally on a log scale between 10^{-12} and 10^{-3} M. Random error was added to each data point and the resulting dose response curves were analyzed by nonlinear regression with the equation that describes the ternary complex model. For all of the simulations, K_1' was 10^{-6} M, K_4' was 10^{-9} M, and K_2' was 10^{-3} M. The mean ± standard error for the computer-calculated parameters from (n) successful analyses are presented.

	N_t/R_t					
	0.25	0.5	0.75	1.0	2.0	10
N_t/R_t	0.248 ± 0.006	0.498 ± 0.006	0.726 ± 0.008	0.974 ± 0.016		
(n)	(6)	(9)	(5)	(8)		

to obtain information about the affinity of receptors for agonists. The model chosen for such analysis may or may not accurately reflect the biological reactions that result in the observed data. In most cases, analysis of dose response curves is performed using a model that assumes the presence of a single population of receptors capable of cooperative interactions among binding sites (1, 10, 17, 34), or using a model that assumes the presence of two independent classes of receptor subtypes (7–9, 11, 13, 15, 17, 19). These models have been used to analyze data from experiments with receptors that regulate the activity of adenylate cyclase, and have provided useful information about the underlying reactions, even though they are not thought to be mechanistically realistic models. In a few cases, dose response curves have been analyzed by nonlinear least squares regression methods using models that assume the reversible interaction of agonist-occupied receptors with a regulatory component (2, 6, 21, 22). The use of these models allows determination of values for the affinities of the receptor for an agonist. However, it is not clear how these values relate to the true underlying affinities involved in formation of the ternary complex. To address this question, a computer was used to simulate dose response data from an equation that describes the ternary complex model. The computer-generated dose response curves were then analyzed by nonlinear least squares regression methods using equations that describe the two-independent-receptor and ternary complex models.

The results of this study, as well as others (2, 6, 7), demonstrate an analogy between K_1 and K_2 from the two-independent-receptor model and K_1' and K_4' from the ternary complex model. All four of these equilibrium dissociation constants describe hormone-binding reactions that take place in the total volume of the reaction. However, the equilibrium dissociation constants K_2' and K_3' do not relate to any of the parameters of the two-independent-receptor model. These two constants describe binding reactions between the receptor and the regulatory component that take place in the plane of the membrane rather than in the total volume of the reaction. Since it is difficult to estimate the area of the membrane, K_2' and K_4' can be expressed as functions of the concentration of receptor (2, 4) or, as in this study, they can be expressed simply as apparent equilibrium dissociation constants that have not been corrected for the difference between total volume and membrane volume.

When computer-generated dose response curves were analyzed with the two-independent-receptor model, the computer-calculated values of R_{2t} and K_1 were found to be reasonably good estimates of the values of N_t and K_1', respectively. However, the computer-calculated value of K_2 was not a reliable estimate of the value of K_4'.

When computer-generated dose response curves that included the addition of random error were analyzed with the ternary complex model, the computer-calculated values of N_t and K_1' were found to be reasonably accurate estimates of the values of N_t and K_1', respectively. The computer-calculated value of K_2' was a good estimate of the value of K_2' when the affinity of RN for H (K_4') was high. However, as the affinity of RN for H (K_4') decreased, less HRN formed and the computer-calculated value of K_2' substantially overestimated the value of K_2'. Similarly, the computer-calculated value of K_4' was a good estimate of the value of K_4' when the affinity of HR for N (K_2') was high. However, as the affinity of HR for N (K_2') decreased, less HRN formed and the computer-calculated value of K_4' substantially overestimated the value of K_4'. Since the ability of the computer to determine the value of K_2' was dependent upon the value of K_4' and vice versa, the computer-calculated values of K_2' and K_4' were not reliable estimates for the values of K_2' and K_4'.

There are three approaches that could conceivably increase the ability of the ternary complex model to provide accurate estimates of the true values of K_2' and K_4'. We have demonstrated in this study that the analysis of data with no associated error resulted in accurate estimates of all of the affinities involved in formation of the ternary complex. Therefore, if the error involved in determining the occupancy of the receptor by an agonist could be reduced, it might be possible to determine accurately all of the affinities involved in formation of the ternary complex. However, it may not be possible to reduce the error associated with experimental measurements to a sufficient extent. A second approach involves increasing the number of data points to provide better definition of the shape of the dose response relationship. However, when the number of data points per dose response curve was increased from 28 to 56, there was only a marginal increase in the ability of the ternary complex model to provide accurate estimates of the true values of K_2' and K_4' (results not shown).

A third approach, not tested in this study, would involve the simultaneous analysis (35) of dose response curves constructed using different known concentrations of the regulatory component. The ability to reconstitute purified receptors with purified guanine nucleotide-binding proteins (36–41) could enable one to construct dose response curves for an agonist in the presence of various concentrations of a guanine nucleotide-binding protein. Thus, rather than analyzing a single dose response curve with an unknown concentration of regulatory component, a series of dose response curves constructed using different known concentrations of regulatory component could be analyzed simultaneously.

In conclusion the results of this study suggest that caution should be exercised in interpretation of results based on analysis of dose response curves for agonist by either the two-independent-receptor model or the ternary complex model. Both models can be used to assess the relative amount of

regulatory component capable of functionally interacting with a receptor. This substantiates the conclusions of those who have performed such analyses to assess the amount of N_S functionally available to interact with β-adrenergic receptors (20, 21). Although the affinity of the initial hormone-binding reaction (K_1') could be reliably assessed with either the two-independent-receptor model or the ternary complex model, the other affinities involved in formation of the ternary complex (K_2', K_3', and K_4') cannot be reliably assessed with either model. Estimates of these affinities should be considered descriptive values, rather than accurate estimates of the true affinities.

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