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A Quantitative Analysis of *Beta*-Adrenergic Receptor Interactions: Resolution of High and Low Affinity States of the Receptor by Computer Modeling of Ligand Binding Data

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SUMMARY

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The properties of ligand binding to the *beta*-adrenergic receptor have been studied using a computer modeling technique to analyze data obtained by indirect binding methods. Antagonists are shown to bind to the receptor with one homogeneous state of uniform affinity, while agonists manifest heterogeneous binding. For agonists, two distinct binding states are apparent; one of high and one of low affinity. Affinity states of the receptor are characterized by specific macroscopic dissociation constants, and the proportion of total receptors in each state can be determined. The ability of an agonist to activate adenylate cyclase (intrinsic activity) correlates closely with the amount of high affinity state formed in the presence of the agonist ($p < 0.001$). A similar correlation exists between adenylate cyclase activation by an agonist and the ratio of dissociation constants of the agonist for the high and low affinity states of the receptor ($p < 0.001$). There is an apparent impairment of high affinity state formation in membranes derived from desensitized cells. Along with a decrease in the total number of receptors, this impairment of high affinity state formation may be responsible for the decreased activation of adenylate cyclase observed after desensitization. Guanyl nucleotides mediate the transition of high affinity receptors to the low affinity state. The extent of this transition is dose dependent and is evident even at low nucleotide concentrations. Our data indicate that high and low affinity states of the *beta*-adrenergic receptor are interconvertible, a finding incompatible with models of ligand–receptor interaction involving independent binding sites. Computer modeling of receptor binding data significantly enhances the ability to describe the interactions for adenylate cyclase-coupled receptor systems and validates the concept and biological importance of receptor affinity states for the *beta*-adrenergic receptor.

INTRODUCTION

The detailed mechanisms of interaction between *beta*-adrenergic agonists, the *beta*-adrenergic receptor, and the enzyme adenylate cyclase remain unknown. In recent studies (1–4) several unique properties of agonists, not shared by antagonists, have emerged that provide insights into the process of agonist-induced adenylate cyclase activation. For example, it has been found that [^{3}H]antagonist/agonist competition curves tend to be shallow, with slope factors ("pseudo" Hill slopes) less than one (5), suggesting an apparent heterogeneity of the receptor population not found with [^{3}H]antagonist/antagonist competition curves. In the presence of guanyl nucleotides, [^{3}H]antagonist/agonist competition curves "shift to the right" (to lower affinities) and become steep, with slope factors approaching one (3–6), as if apparently distinct receptor affinity states were merging into a uniform state of lower affinity. Concomitant with this "shift" to lower affinities is a nucleotide-mediated increase in adenylate cyclase catalytic activity (5, 7). The competition curves of antagonists, which do not activate adenylate cyclase, are not affected by guanyl nucleotides. Similarly, a high affinity, slowly dissociable, magnesium-dependent state of the *beta*-adrenergic receptor, recently

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demonstrated by direct binding studies with the *beta*-agonist [^{3}H]hydroxybenzylisoproterenol ([^{3}H]HBI)[1] (1, 2, 8) as well as by indirect binding studies with (−)isoproterenol in competition with [^{3}H]dihydroalprenolol ([^{3}H]DHA) (8) and [^{125}I]iodohydroxybenzylpindolol ([^{125}I]HYP) (9), is not evident with antagonist binding.

The observed differences between agonist and antagonist binding properties have led to the development of a model of ligand–receptor interaction and enzyme activation for the *beta*-adrenergic receptor system (10). The model proposes that the *beta*-adrenergic receptor can exist in either a high or a low affinity state and appears to account for many features of agonist binding and adenylate cyclase activation. However, no rigorous attempt to quantitatively relate the biological properties of agonists to these putative "states" of the *beta*-adrenergic receptor has been reported.

In this communication we describe a systematic examination of agonist interaction with the *beta*-adrenergic receptor using computer modeling methods. We identify and quantify high and low affinity states of the receptor and demonstrate their biological significance. Computer modeling methods are also used to examine other properties of the *beta*-adrenergic receptor including the regulatory effects of guanyl nucleotides, desensitization-mediated changes in receptor affinity states, and the interconvertibility of high and low affinity receptors. The implications of our findings are discussed with reference to proposed models of hormone–receptor interactions.

MATERIALS AND METHODS

Materials. The sources of all drugs and chemicals used have been previously reported (11). Jumbo southern grass frogs were obtained from Nasco–Steinhilber.

Frog erythrocyte membranes. Washed frog erythrocytes were prepared as previously described (11). The washed cells were resuspended in cold water (2.5 ml of packed cells/30 ml of water) for 7 min followed by homogenization in a Potter–Elvehjem homogenizer (20 strokes). The lysate was mixed with an equal volume of cold buffer (75 mM Tris–HCl, 12.5 mM $MgCl_2$, 1.5 mM EDTA, pH 7.5) and centrifuged at 30,000*g* for 10 min. The resulting pellet from 2.5 ml packed cells was resuspended in 10 ml of cold buffer, layered over 50% sucrose containing 50 mM Tris–HCl and 12.5 mM $MgCl_2$ (pH 8.1) and centrifuged at 1,200*g* for 8 min at 4°. The material at the sucrose–buffer interface was harvested and was centrifuged at 30,000*g* for 10 min. The resulting pellet was resuspended in buffer to give a final protein concentration of 0.5–1.0 mg/ml which was used in all assays.

Whole cell desensitization. Desensitization of whole frog erythrocytes was performed as previously described (12) by incubation of the cells with 0.1 mM (−)isoproterenol for 1–3 hr.

[^{3}H]Dihydroalprenolol binding assay. Erythrocyte membranes were incubated with (−)[^{3}H]DHA, 1–3 nM, in a total volume of 1 ml containing 75 mM Tris–HCl, 12.5 mM $MgCl_2$, 1.5 mM EDTA, pH 7.5 for 20 min at 25° in the presence of the indicated concentrations of unlabeled ligands. Nonspecific binding was determined by computer analysis of the resulting competition curve and was generally less than 10% of total binding. In the figures and text "binding" refers to total binding (specific and nonspecific). Maximum binding refers to total binding of [^{3}H]DHA in the absence of competing ligand. Maximum binding of [^{3}H]DHA was generally approximately 400 fmol/mg protein at a ligand concentration of 1–3 nM with little day-to-day variation. This concentration of ligand was used in all competition experiments.

Adenylate cyclase. Adenylate cyclase activity was assayed as previously described (2) in the presence of 0.1 mM ATP and 1 mM GTP. The intrinsic activity of a *beta*-adrenergic compound was defined as the maximal activation of adenylate cyclase achieved by the compound proportioned to the maximal activation achieved by (−)isoproterenol. The amount of protein was determined by the method of Lowry *et al.* (13).

Data analysis. The indirect competitive binding assay data for the ligand involved were analyzed by a nonlinear least-squares curve fitting procedure using a generalized model for complex ligand–receptor systems as previously described (14–16).[2] The method, based on mass action law, provides estimates for the affinity of the ligands for the different states of the receptor and for the proportion of these states. The term "state" or "binding state" rather than "site" or "binding site" is used to signify that the *beta*-adrenergic receptor is capable of binding *beta*-adrenergic ligands with a variety of apparent affinities for the ligands. For each displacement curve of [^{3}H]DHA by competing agonist or antagonist, fitted estimates were provided for the data, assuming either one or two affinity states of the receptor, with deviation of the observed points from the predicted values being weighted according to the reciprocal of the predicted variance (17). Statistical analysis comparing "goodness of fit" between one and two affinity state models was also provided and was used to determine the most appropriate model for the ligand being examined. The goodness of fit was ultimately evaluated using the residual variance (the ratio of the sum of squares of residuals divided by degrees of freedom). For those compounds whose interaction with the *beta*-adrenergic receptor was best described by two states of binding we defined R_H as those receptors that bound the compound with high affinity (high affinity state receptors) when compared with the remaining lower affinity receptors (R_L). The macroscopic dissociation constants for these two affinity states were defined as K_H and K_L respectively.

In order to test the significance of differences between parameter estimates from different curves, each curve was first analyzed independently of the others allowing the parameter estimates for each curve to reach their optimal values. The curves were then analyzed simultaneously and the corresponding parameters from the individual curves were constrained to be estimated as a common value. The effect of sharing common parameters among the curves on the goodness of fit was tested and the shared parameters were considered statistically indistinguishable when the sharing process did not signifi-

[1] The abbreviations used are: [^{3}H]HBI, [^{3}H]hydroxybenzylisoproterenol; [^{3}H]DHA, [^{3}H]dihydroalprenolol, [^{125}I]HYP, [^{125}I]iodohydroxybenzylpindolol.

[2] A complete list of programs is available from the authors on request.

cantly worsen the fit of all the curves. Such a procedure was used: (1) to compare the dissociation constants (K_L) for the low affinity state in the absence and in the presence of guanyl nucleotides; (2) to compare the proportion of high affinity states (% R_H) observed for agonists of different intrinsic activities; (3) to compare the dissociation constants (K_H, K_L) observed before and after desensitization of the receptor.

Slope factors ("pseudo" Hill coefficients) were determined by fitting the data to a four parameter logistic equation as previously described (18). Because indirect rather than direct binding was evaluated in this study, these slope factors are not true Hill coefficients. Fitted estimates and statistical analysis were provided as described. All computations were performed using iterative programs in PL/1 for a PDP 11/45. Results are reported as the mean ± SEM for each variable, determined from several experiments. Statistical significance was established by Student's *t* test for paired data. Standard errors for the various parameters from individual experiments were generally smaller than the standard error of their average from several experiments (reported in Table 1).

Radiolabeled antagonist binding. The dissociation constant (K_D) for the radiolabeled antagonist [^{3}H]DHA was determined by direct ligand binding studies to be 2 nM in this membrane preparation. Detailed computer analysis of the binding isotherm revealed only one affinity state of the receptor for the radioligand (data not shown). For the purposes of computer analysis, therefore, [^{3}H]DHA as a competing radioligand was considered to bind with equal affinity to all states of the receptor present. This assumption is consistent both with the computer analysis of direct [^{3}H]DHA binding and with the results obtained in the study of [^{3}H]DHA/(−)alprenolol competition curves as shown below.

Experimental procedure. Ten compounds including eight agonists and two antagonists were studied. "Weak" and "strong" agonists were defined by their relative intrinsic activities, not by their relative potencies. Two agonists, (−)isoproterenol and (±)soterenol, were studied both in the presence and absence of saturating concentrations of guanyl nucleotides (10^{-4} M GTP or 10^{-4} M Gpp(NH)p). Detailed [^{3}H]DHA competition curves were performed in a number of experiments with each compound. For each experiment, computer modeling determined (1) whether the data were more consistent with one or two binding affinity states of the receptor, (2) the percentage of the total receptor population in each (high and low) affinity state, (3) the dissociation constant of the ligand for each affinity state, and (4) the slope factor of the competition curve.

RESULTS

Comparison of agonist and antagonist binding curves and the effects of nucleotides. Figure 1 is an example of experimental data and a computer modeled curve for the antagonist (−)alprenolol. Analysis revealed only one binding state of the receptor with K_D = 12 nM. The slope factor of 0.98 supports one affinity state for the antagonist. The curve is of "normal" steepness consistent with these findings. ("Normal" steepness is defined by ED_{90}/ED_{10} = 81 (19) and a slope factor of one. These findings

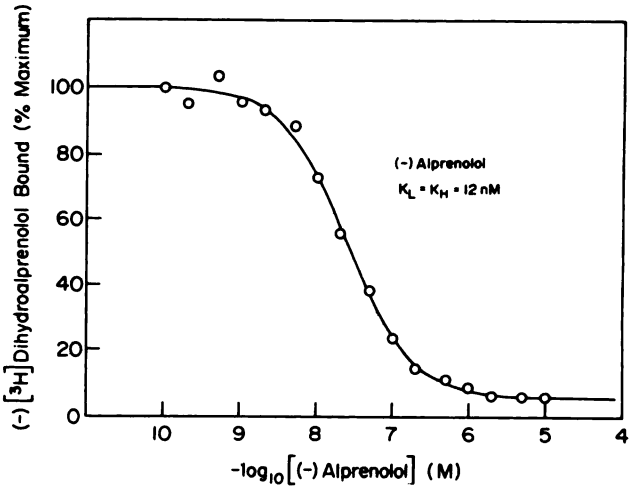

FIG. 1. *Computerized curve fitting of binding data from displacement of [^{3}H]DHA by (−)alprenolol (antagonist binding)*

Frog erythrocyte membranes were incubated with 1.5 nM [^{3}H]DHA in competition with increasing concentrations of the *beta*-adrenergic antagonist (−)alprenolol and then assayed for [^{3}H]DHA binding. Maximum binding represents the binding of [^{3}H]DHA in the absence of competing (−)alprenolol. The data points obtained are shown by open circles and represent the mean of duplicate determinations in a single representative experiment. The solid line is a computer generated curve fitting the observed data points. The data in this experiment model to one state of homogeneous receptor affinity with K_D = 12 ± 1 nM. As heterogeneity of antagonist binding is not apparent, K_L = K_H = K_D. The slope factor is 0.98 ± 0.05.

are expected in a ligand–receptor binding system of homogeneous affinity.) Four additional antagonists, butoxamine, practolol, dichlorisoproterenol, and metoprolol, have been similarly studied (data not shown) and have been shown to model to one homogeneous state of binding with slope factors equal to one.[3]

In contrast, Figs. 2 and 3 show the results of the modeling of competition curves from experiments employing the strong *beta*-agonist (−)isoproterenol (intrinsic activity = 1.0) and the weak *beta*-agonist (±)soterenol (intrinsic activity = 0.08). It can be seen that the competition curves in the absence of nucleotides (solid lines) are "shallow" (i.e., show a trailing "foot" in the high dose range with $ED_{90}/ED_{10} > 81$), and deviate from the "normal" steepness demonstrated by the [^{3}H]DHA/(−)alprenolol curve (Fig. 1). This difference is more marked for (−)isoproterenol (Fig. 2) than for (±)soterenol (Fig. 3). Computer analysis of these data indicated that in the absence of nucleotides the curves for both agonists were best explained by two affinity states of the receptor. For (−)isoproterenol (Fig. 2), 77% of the total receptor population was of high affinity (R_H) with a dissociation constant (K_H) of 24 nM; 23% of the receptors were of low affinity (R_L) with a dissociation constant (K_L) of 1400 nM. These values can be contrasted to those of (±)soterenol (Fig. 3) where % R_H = 65% (K_H = 120 nM), and % R_L = 35% (K_L = 710 nM). The percentage of total receptors in the high affinity state was larger for the stronger agonist. Similarly the ratio of dissociation constants for the high and low affinity states (K_L/K_H) was larger for

[3] A. Hancock, A. De Lean and R. Lefkowitz, unpublished observations.

(−)isoproterenol (58.3) than for (±)soterenql (5.9). The slope factor for each curve was less than one (0.73 for (−)isoproterenol and 0.87 for (±)soterenol). Apparent heterogeneity of receptor binding states is supported by these slope factors, i.e., the curves are "shallow." The ability of the two-state model to fit the data points can be appreciated by inspection of the curves. In both cases a two-state binding model markedly improved the fit when compared with attempts to fit the data to one

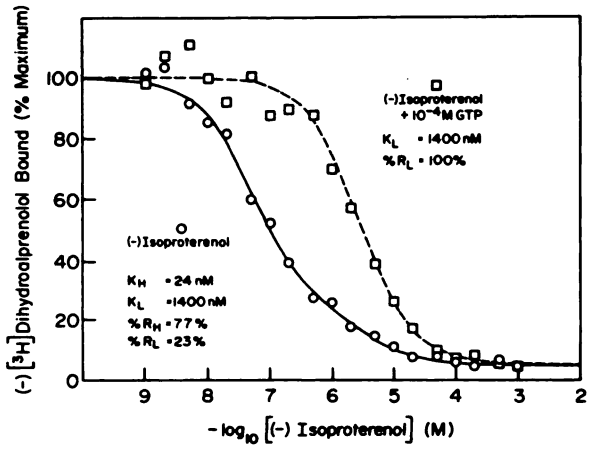

FIG. 2. *Computerized curve fitting of binding data from displacement of [^{3}H]DHA by (−)isoproterenol in the presence and absence of guanyl nucleotides (strong agonist binding)*

Frog erythrocyte membranes were incubated with 2.1 nM [^{3}H]DHA in competition with increasing concentrations of the strong *beta*-adrenergic agonist (−)isoproterenol, in both the absence and presence of 10^{-4} M GTP. In the absence of GTP receptor concentrations are: $[R_H] = 0.258 \pm 0.008$ nM ($K_H = 24 \pm 4$ nM) and $[R_L] = 0.078 \pm 0.007$ nM ($K_L = 1400 \pm 107$ nM). Thus, % R_H = 77% and % R_L = 23%. The slope factor is 0.73 ± 0.09. In the presence of 10^{-4} M GTP, $[R_L] = 0.336 \pm 0.015$ nM ($K_L = 1400 \pm 107$ nM). No R_H receptors are apparent; % R_L = 100%. The slope factor is 1.0 ± 0.1.

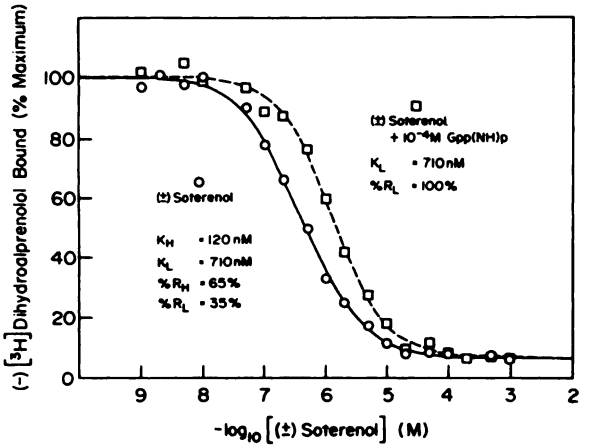

FIG. 3. *Computerized curve fitting of binding data from displacement of [^{3}H]DHA by (±)soterenol in the presence and absence of guanyl nucleotides (weak agonist binding)*

Analysis was as for Figs. 1 and 2. In the absence of Gpp(NH)p receptor concentrations are: $[R_H] = 0.199 \pm 0.028$ nM ($K_H = 120 \pm 43$ nM) and $[R_L] = 0.108 \pm 0.030$ nM ($K_L = 710 \pm 57$ nM). Thus, % R_H = 65% and % R_L = 35%. The slope factor is 0.87 ± 0.05. In the presence of 10^{-4} M Gpp(NH)p, $[R_L] = 0.307 \pm 0.058$ nM ($K_L = 710 \pm 57$ nM). No R_H receptors are apparent; % R_L = 100%. The slope factor is 0.95 ± 0.08.

homogeneous affinity state ($p < 0.01$ for (−)isoproterenol; $p < 0.025$ for (±)soterenol).

In the presence of saturating concentrations of guanyl nucleotides, the competition curves for both (−)isoproterenol and (±)soterenol shifted to the right and steepened (Figs. 2, 3, dashed lines), suggesting that under these conditions the receptor population manifested only one homogeneous state of lower affinity. Computer modeling confirmed that both (−)isoproterenol and (±)soterenol were in fact binding to a single low affinity form of the *beta*-adrenergic receptor; high affinity state receptor binding was no longer apparent. The increased slope factors from 0.73 to 1.0 for (−)isoproterenol and from 0.87 to 0.95 for (±)soterenol were also consistent with these results. Simultaneous curve fitting indicated that for both agonists the dissociation constants of the competition curves in the presence of guanyl nucleotides were not significantly different from the low affinity dissociation constants (K_L) in the absence of nucleotides ($p > 0.9$). Their common values are therefore reported in Figs. 2 and 3 (see MATERIALS AND METHODS). The actions of guanyl nucleotides therefore "shift" high affinity receptors to a low affinity form indistinguishable from that found in the absence of nucleotides.

Relationship between agonist binding properties and intrinsic activity. These initial observations suggested that the characteristics of ligand binding to the *beta*-adrenergic receptor might depend on ligand intrinsic activity. To further study the question we undertook a systematic examination of a number of *beta*-adrenergic agonists and antagonists. Table 1 is a summary of the compounds studied and their binding properties as determined by computer curve modeling. The intrinsic activities of the compounds ranged from zero for the two antagonists (±)propranolol and (−)alprenolol to 1.1 for the strong *beta*-agonist (±)hydroxybenzylisoproterenol (HBI).

The ratio K_L/K_H for an agonist was found to correlate strongly with the intrinsic activity of the agonist. From Table 1 it can be seen that K_L/K_H ranged from 14 for the weak partial agonist (±)AH4941 to 130 for the strong agonist HBI. Figure 4A graphically illustrates the close correlation and demonstrates that as intrinsic activity approaches zero (antagonist), K_L/K_H approaches 1. (Under conditions in which $K_L = K_H$, the ligand binds with homogeneous affinity to both states of the receptor. It therefore demonstrates apparent one-state binding as for an antagonist).

The mean percentage of total receptors in the high affinity state for an agonist in the absence of nucleotides also correlated strongly with the intrinsic activity of the agonist; % R_H ranging from 52% for the weak partial agonists (±)AH4941 and (±)soterenol to a maximum of 92% for the strong agonist HBI. A graph of % R_H vs intrinsic activity is presented in Fig. 4B. The increasing percentage of receptors in the high affinity state as intrinsic activity increases is evident from the data in the figure. As intrinsic activity approaches zero (antagonist), % R_H approaches 50% (see footnote *c*, Table 1). This is a somewhat unexpected result for which no clear explanation is available.

To assess the significance of the differences in %R_H

TABLE 1

Computer derived parameters from curves of [^{3}H]DHA in competition with beta-adrenergic agents of varying intrinsic activity

Intrinsic activity is defined under MATERIALS AND METHODS. For each experiment with the various compounds the computer programs provided estimates of the percentage of total receptors in the high affinity state (% R_H), the high (K_H) and low (K_L) affinity state dissociation constants, and the slope factor. All agonists were modeled best by two receptor affinity states, while both antagonists were best described by a single state of homogeneous receptor affinity. In the presence of either 10^{-4} M GTP or 10^{-4} M Gpp(NH)p both (−)isoproterenol and (±)soterenol modeled to a single homogeneous state of binding with a dissociation constant indistinguishable from the low affinity dissociation constant (K_L) of the control curve in the absence of nucleotides. *N* indicates the number of experiments. Results are expressed as mean ± SEM for the number of experiments indicated. For K_H, K_L, and K_L/K_H the data were transformed to log form and geometric means are shown.

Compound	Intrinsic activity	%R_H	K_H	K_L	K_L/K_H	Slope factor	*N*
			(nM)	(nM)			
(±)Hydroxybenzylisoproterenol	1.10 ± 0.03	92 ± 1	6 ± 2	800 ± 200	130 ± 16	0.72 ± 0.02	3
(−)Isoproterenol	1.0	79 ± 2	32 ± 9	2500 ± 400	78 ± 14	0.56 ± 0.04	5
(−)Isoproterenol + 10^{-4} M GTP[a]		0[b]		1800 ± 200		0.96 ± 0.04	3
(−)Epinephrine	0.92 ± 0.02	74 ± 2	80 ± 40	10,000 ± 4000	128 ± 14	0.48 ± 0.03	3
(−)Cobefrin	0.54 ± 0.02	70 ± 7	350 ± 130	26,000 ± 11,000	74 ± 13	0.53 ± 0.02	3
(±)Isoetharine	0.45 ± 0.02	65 ± 5	2000 ± 1000	99,000 ± 36,000	51 ± 18	0.56 ± 0.04	6
(±)Salbutamol	0.21 ± 0.02	66 ± 6	280 ± 60	5800 ± 100	20 ± 4	0.70 ± 0.02	2
(±)Soterenol	0.08 ± 0.02	52 ± 8	40 ± 16	600 ± 200	16 ± 4	0.79 ± 0.02	7
(±)Soterenol + 10^{-4} M Gpp(NH)p		0[b]		600 ± 100		0.91 ± 0.04	2
(±)AH4941	0.08 ± 0.01	52 ± 6	1800 ± 900	25,000 ± 5000	14 ± 4	0.76 ± 0.04	5
(−)Alprenolol	0	*[c]	6 ± 3[d]	6 ± 3[d]	1	1.00 ± 0.02	3
(±)Propranolol	0	*[c]	6[d]	6[d]	1	1.1	1

[a] Includes one experiment done in the presence of 10^{-4} M Gpp(NH)p.

[b] Models to one state, all in low affinity form (% R_L = 100%).

[c] Antagonist; models to one state of homogeneous affinity. When $K_H = K_L$, % R_H and % R_L become indeterminate variables; values cannot be ascribed to them through modeling of the competition curve. Although % R_H approaches 50% as intrinsic activity approaches zero (Fig. 4B), % R_H in the presence of an antagonist or in the absence of ligand is unknown.

[d] Antagonist; models to one state of homogeneous affinity ($K_H = K_L$).

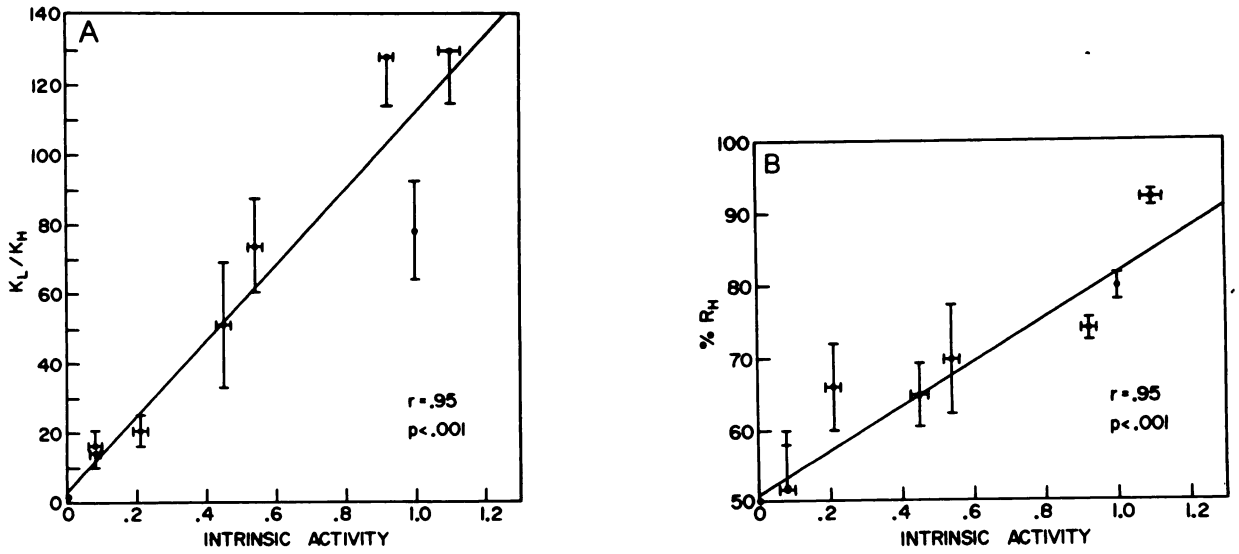

FIG. 4. *Relationship between computer derived receptor parameters and the intrinsic activity of beta-adrenergic agonists and antagonists*

(A) Correlation between the ratio of dissociation constants for high and low affinity states and intrinsic activity. Data from Table 1 and a least-squares linear regression analysis were used to determine the correlation between intrinsic activity and the ratio K_L/K_H. The two antagonist points (intrinsic activity = 0) were placed at K_L/K_H = 1. This did not alter the statistical significance of the regression line. Data are shown as mean ± SEM. The intrinsic activity of (−)isoproterenol is defined as 1.0. (B) Correlation between the percentage of receptors in the high affinity state and intrinsic activity. Analysis was as for Fig. 4A. The two antagonist points (intrinsic activity = 0) were placed at % R_H = 50%. This did not alter the statistical significance of the regression line.

among the various agonists, simultaneous curve fitting was performed with the meaned data from seven agonists (see MATERIALS AND METHODS). The results of this analysis are shown in Table 2. All possible pairs of agonists were tested. The pattern of statistical significance observed is that expected based on the intrinsic activity and % R_H data in Table 1, i.e., %R_H values for agonists of similar intrinsic activity do not differ significantly, while the % R_H values for agonists of more widely differing intrinsic activities do show highly significant differences.

TABLE 2

Analysis of the significance of differing proportions of high and low affinity state receptors among the various agonists

The experimental results from seven agonists (three to seven experiments per agonist; Table 1) were meaned. Simultaneous curve fitting was performed with the meaned data from all possible pairs of agonists to test the hypothesis that the % R_H values between each pair differ significantly. The F ratio was obtained by comparing the individual fits for the two agonists under no constraints vs a simultaneous fit with the constraint that their % R_H parameters be estimated as a common value. The p values shown were derived from the F ratio and represent the probability that the % R_H values for any pair of agonists are actually statistically equal, and that the experimental differences seen were due only to chance (see MATERIALS AND METHODS).

	(±)HBI (1.1)[a]	(−)Isoproterenol (1.0)	(−)Epinephrine (0.92)	(−)Cobefrin (0.54)	(±)Isoetharine (0.45)	(±)Soterenol (0.08)	(±)AH4941 (0.08)
(±)HBI	—						
(−)Isoproterenol	<0.01* 8.94**	—					
(−)Epinephrine	<0.01 5.80	>0.05 1.49	—				
(−)Cobefrin	<0.01 7.83	<0.05 4.08	>0.05 0.62	—			
(±)Isoetharine	<0.01 8.30	<0.01 9.19	>0.05 1.09	>0.05 1.15	—		
(±)Soterenol	<0.05 3.92	<0.01 9.14	<0.05 4.52	>0.05 3.29	<0.01 5.57	—	
(±)AH4941	<0.01 6.69	<0.01 7.58	<0.05 3.73	>0.05 3.29	<0.05 4.51	>0.05 0.25	—

[a] ( )Intrinsic activity.
* p value.
** F test ratio.

The highly significant results and appropriate pattern of significance refute the null hypothesis that differences in $\%R_H$ are due only to chance and indicate that the *beta*-adrenergic receptor population is not a fixed entity but is distributed into high and low affinity states according to the intrinsic activity of the agonist examined.

As expected, a close correlation was also found between K_L/K_H and % R_H ($r = 0.90$, $p < 0.001$; data not shown). For the purpose of data analysis, the antagonist points in Figs. 4A and 4B were placed at (0, 1) and (0, 50%), respectively. The inclusion of these points did not alter the statistical significance or general conclusions of the correlation plots.

Table 1 also summarizes the mean slope factors for all agonists and antagonists studied. Slope factors are less than one in ligand–receptor systems with heterogeneous affinity states, and approach one as heterogeneity of receptor binding affinities is lost. Heterogeneity of the receptor population, as evidenced by slope factors less than one, is lost as intrinsic activity approaches zero because K_H approaches K_L, and the ligand binds as if one homogeneous affinity state of the receptor population were present. Likewise, heterogeneity of the receptor population is lost at very high intrinsic activities because % R_H approaches 100%. Deviation from homogeneous one-state binding is greatest and the slope factor least for agonists of intermediate and high intrinsic activity because appreciable amounts of both high and low affinity state receptors are present and K_L/K_H is large.

Graded effects of guanyl nucleotides. Figure 5 is an example of an experiment designed to study the graded effects of guanyl nucleotides on high and low affinity receptor populations. [^{3}H]DHA/(−)isoproterenol competition curves were performed both in the absence of nucleotides and with the indicated concentrations of Gpp(NH)p. Analysis of these data revealed a concentration-dependent effect of nucleotides. In the absence of nucleotides 69% of the total receptor population was in the high affinity state. This number fell to 26 and 16% in the presence of 10^{-7} M Gpp(NH)p and 10^{-6} M Gpp(NH)p, respectively. In the presence of 10^{-4} M Gpp(NH)p no high affinity receptors were apparent. In a second experiment (curves not shown) examining a slightly lower concentration range, % R_H was 78% in the absence of nucleotides and fell to 68%, 43% and 0% in the presence of 10^{-8} M, 10^{-7} M, and 10^{-4} M Gpp(NH)p, respectively. To assess the significance of nucleotide-induced changes in

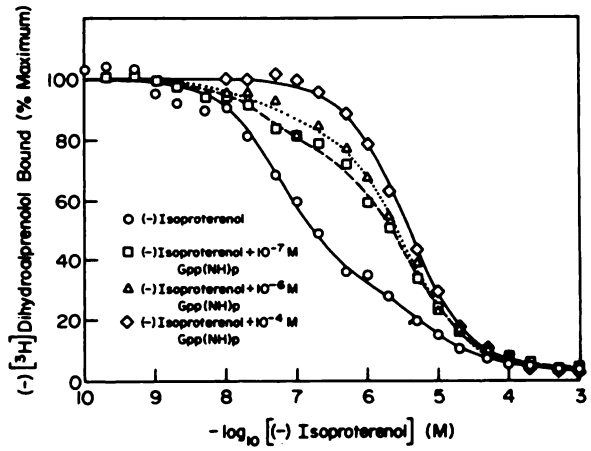

FIG. 5. *Computerized curve fitting of binding data from displacement of [^{3}H]DHA by (−)isoproterenol in the presence of graded concentrations of guanyl nucleotides*

Receptor concentrations and percentages are: Control: [R_H] = 0.322 ± 0.012 nM, [R_L] = 0.143 ± 0.010 nM, % R_H = 69%; 10^{-7} M Gpp(NH)p: [R_H] = 0.123 ± 0.019 nM, [R_L] = 0.342 ± 0.018 nM, % R_H = 26%; 10^{-6} M Gpp(NH)p: [R_H] = 0.076 ± 0.037 nM, [R_L] = 0.392 ± 0.023 nM, % R_H = 16%; 10^{-4}M Gpp(NH)p: [R_H] = 0, [R_L] = 0.463 ± 0.012 nM, % R_H = 0%, % R_L = 100%.

% R_H, simultaneous curve fitting of the data in Fig. 5 was performed (see MATERIALS AND METHODS). The curves were fitted to test the null hypothesis that the differences in % R_H among the curves were due only to chance and that these data could be equally well described by a single % R_H value. By statistically comparing an unconstrained fit (allowing all % R_H values to vary) with a fit in which all % R_H were constrained to be estimated as a common value, the null hypothesis was rejected ($F = 7.17$; $p < 0.01$), again indicating that true differences in % R_H do exist in the presence of graded nucleotide concentrations and that the concept of fixed, non-varying R_H and R_L states is untenable for the *beta*-adrenergic receptor. Thus the distribution of the *beta*-adrenergic receptor into high and low affinity forms is quantitatively modulated by guanyl nucleotides.

Effects of desensitization on high and low affinity state receptors. Desensitization, the property of decreased responsiveness to hormonal stimulation after chronic agonist exposure, is characteristic of the *beta*-adrenergic receptor of frog erythrocytes (10, 12). We have used the technique of computer modeling to study receptor desensitization as a tool to better define high and low affinity receptor states. Desensitization of frog erythrocytes was carried out by incubation of whole cells with 10^{-4} M (−)isoproterenol for 60–180 min. Computer curve fitting was done on [^{3}H]DHA/(−)isoproterenol curves performed in both control membranes and membranes derived from desensitized cells. Data from all experiments were pooled for analysis. An example of data from control and postdesensitization membranes with computer modeled curves is presented in Fig. 6. In this

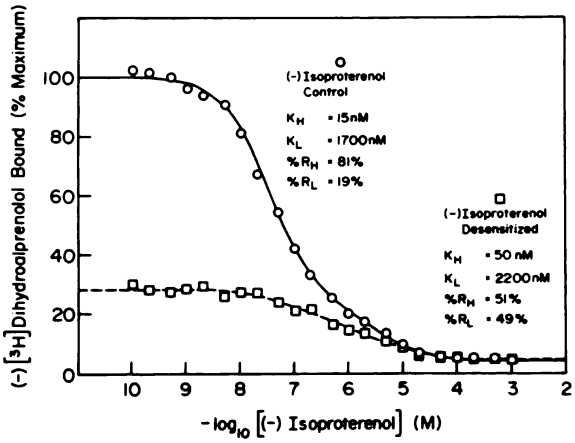

FIG. 6. *Computerized curve fitting of binding data from displacement of [^{3}H]DHA by (−)isoproterenol in control membranes and membranes derived from desensitized cells*

Whole frog erythrocytes were desensitized by incubation of the cells with 0.1 mM (−)isoproterenol for 3 hr. Erythrocyte membranes were prepared as described in MATERIALS AND METHODS using both desensitized and control whole cells. The solid and dashed lines are computer generated curves fitting the points obtained in these control and desensitized preparations, respectively. The total decrease in maximal [^{3}H]-DHA binding induced by desensitization was 72%. In control membranes: [R_H] = 0.191 ± 0.004 nM (K_H = 15 ± 2 nM), [R_L] = 0.046 ± 0.003 nM (K_L = 1700 ± 280 nM), % R_H = 81%, K_L/K_H = 113. In membranes from desensitized cells: [R_H] = 0.030 ± 0.004 nM (K_H = 50 ± 21 nM), [R_L] = 0.028 ± 0.004 nM (K_L = 2200 ± 320 nM), % R_H = 51%, K_L/K_H = 44.

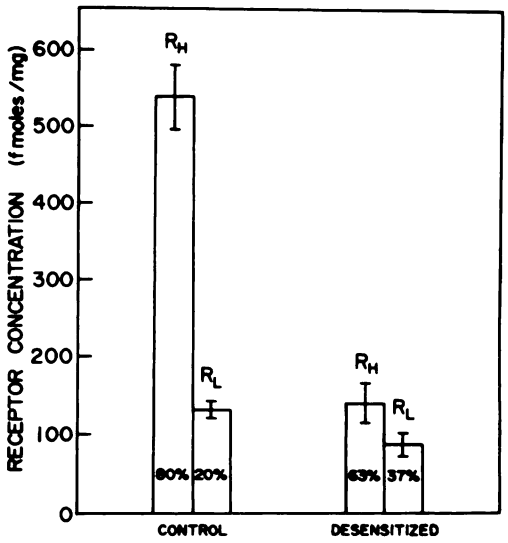

FIG. 7. *Effect of desensitization on high and low affinity state receptor concentrations*

Whole erythrocyte desensitization was carried out as described in MATERIALS AND METHODS. [^{3}H]DHA/(−)isoproterenol competition curves were performed in control membranes and membranes derived from desensitized cells, and the concentrations of high (R_H) and low (R_L) affinity state receptors were determined by computer modeling. The results are expressed as mean ± SEM for five experiments. Total mean decrease in maximal [^{3}H]DHA binding after desensitization was 65%. After desensitization the proportion of high affinity receptors decreased significantly in membranes derived from desensitized cells when compared with control (80% of all receptors were in the high affinity state in control membranes vs 63% in desensitized; $p < 0.05$).

experiment 72% of the [^{3}H]DHA binding sites present in the control membranes were lost during desensitization. The data demonstrate a decrease in % R_H in membranes derived from desensitized cells compared to control (81% control vs 51% desensitized) as well as a threefold increase in K_H (15 nM control vs 50 nM desensitized) and a much smaller increase in K_L (1700 nM, control vs 2200 nM, desensitized). K_L/K_H decreased from 113 in the control membranes to 44 after desensitization.

Figure 7 is a graphical summary of the changes in high and low affinity state receptor concentrations in five experiments. The mean loss of total receptor population due to desensitization was 65%, i.e., there were only 35% as many [^{3}H]DHA binding sites in desensitized as opposed to control membranes. High affinity state receptors comprised a smaller proportion of the total receptor population in the membranes derived from desensitized cells (80% control vs 63% desensitized, $p < 0.05$). In all five experiments, changes in the dissociation constants for the two affinity states similar to those shown in Fig. 6 were observed. After desensitization, K_H increased (25 ± 4 nM control; 53 ± 21 nM desensitized; $p < 0.05$), K_L did not change significantly (2300 ± 600 nM control; 3050 ± 1100 nM desensitized; p = NS), and K_L/K_H decreased (90 ± 14 nM control; 56 ± 7 nM desensitized; $p < 0.05$). To further substantiate these findings and to test the null hypothesis that the changes in K_H and K_L/K_H were due to chance alone, the mean curves from five experiments were fit simultaneously (see MATERIALS AND METHODS). When compared with an unconstrained fit in which K_H

and K_L varied freely, significant deterioration in the simultaneous fit occurred when mean control and desensitized curves were constrained to share the same estimates for K_L and K_H ($F = 23.33$; $p < 0.001$). Thus, the null hypothesis is rejected and we conclude that the noted changes in K_H and K_L/K_H after desensitization, although not of large magnitude, are highly significant. Desensitization, therefore, induces changes in agonist–receptor interaction in addition to the actual loss of receptors. The ability of the residual receptors in membranes derived from desensitized cells to form high affinity state seems to be altered, and significant changes occur in the dissociation constants for the two affinity states.

DISCUSSION

The results of this investigation validate the concept of high and low affinity receptor populations in the *beta*-adrenergic receptor system of the frog erythrocyte and demonstrate their biological significance. It is evident from our data that a fundamental property of *beta*-adrenergic agonists, not shared by antagonists, is the ability to allow formation of a high affinity, nucleotide sensitive, state of the *beta*-adrenergic receptor. Using computerized methods, not previously applied to an adenylate cyclase-coupled receptor, we have quantitated high affinity state formation for a number of *beta*-adrenergic agonists and have shown a strong correlation between the percentage of high affinity receptors found in the presence of an agonist and the ability of the agonist to activate adenylate cyclase (Fig. 4B). It is thereby suggested that high affinity state formation is associated with the activation of adenylate cyclase. That these differences in % R_H are significant is substantiated by the analysis shown in Table 2; the ability to demonstrate high affinity binding varies significantly among agonists of differing intrinsic activity.

A strong correlation also exists between the ratio of dissociation constants of an agonist for the two affinity states (K_L/K_H) and the ability of the agonist to activate adenylate cyclase (Fig. 4A). The ability of an agonist to favor high affinity state formation and thus cyclase activation may be related to its relatively greater affinity for R_H compared with R_L, as indicated by a large value of K_L/K_H. It is not unexpected, therefore, that K_L/K_H, although determined independently from intrinsic activity and % R_H, correlates strongly with both of these variables. Our results also provide an explanation for "shallow" agonist competition curves and slope factors less than one; two major binding affinity states of the *beta*-adrenergic receptor account for this apparent heterogeneity of agonist binding.

Computer analysis has allowed the quantification of the graded effects of guanyl nucleotides on the *beta*-adrenergic receptor. Even very low concentrations of nucleotides can markedly alter agonist binding properties, decreasing the number of high affinity receptors apparent (Fig. 5), and shifting the competition curve incrementally to the right. At high GTP or Gpp(NH)p concentrations agonists bind to an apparently homogenous receptor population of low affinity (Figs. 2, 3), indistinguishable from the low affinity state of the receptor in the absence of nucleotides. The changes in % R_H in response to guanyl nucleotides are highly significant indicating quantitative conversion of high affinity receptors to a low affinity form.

The ability to form high affinity state is markedly decreased in membranes derived from desensitized cells (Figs. 6, 7) and indeed the interactions between agonists and the *beta*-adrenergic receptor may be altered by desensitization. Not only is (−)isoproterenol associated with a smaller proportion of high affinity receptors after desensitization (63%) compared with control (80%) (Fig. 7), but the ratio of dissociation constants for the two affinity states (K_L/K_H) is also significantly decreased by the process of desensitization. Isoproterenol, a full agonist in control membranes, therefore models as a partial agonist in membranes derived from desensitized cells (decreased % R_H and decreased K_L/K_H). From these considerations it would be predicted that adenylate cyclase activation would fall after desensitization for two reasons: (1) There is a fall in the total number of *beta*-adrenergic receptors as measured by [^{3}H]DHA binding ([^{3}H]DHA binding is reduced by 72% after desensitization in the example shown in Fig. 6); and (2) of those receptors which do remain after desensitization, a smaller percentage are capable of forming high affinity state when compared to control membranes. (High affinity state receptors represent 81% of the total receptor population in the control curve shown in Fig. 6 but only 51% after desensitization.) One would expect, therefore, that in membranes derived from desensitized cells, the percent loss of adenylate cyclase activity would exceed the percent loss of total receptor population as measured by [^{3}H]DHA binding. This phenomenon has in fact been demonstrated in the *beta*-adrenergic receptor of the frog erythrocyte (21).

The significant differences found in % R_H in association with agonists of varying intrinsic activities (Tables 1 and 2) as well as with varying concentrations of guanyl nucleotides (Fig. 5) are incompatible with fixed, noninterconvertible states of the *beta*-adrenergic receptor. Receptor state interconvertibility is strongly suggested, and proposed models attempting to explain *beta*-adrenergic receptor function must be consistent with these observations. Although theoretically possible, it is unlikely that guanyl nucleotides mediate the transition of both high and low affinity receptors to an independent low affinity state; low affinity binding in the presence of high concentrations of guanyl nucleotides is indistinguishable from low affinity binding in the control state (Figs. 2 and 3).

The present data readily explain the previously reported relationship between agonist intrinsic activity and the "fold shift" of agonist competition binding curves induced by guanyl nucleotides (Figs. 2, 3) (4). The fold shift is determined for each agonist by comparing 50% displacement points (EC_{50}) in the absence or presence of 10^{-4} GTP or Gpp(NH)p, and defined by the ratio EC_{50} in the presence of nucleotides/EC_{50} in the absence of nucleotides. For the two agonists (−)isoproterenol and (±)soterenol in five experiments, done both in the absence and presence of guanyl nucleotides (Table 1), this ratio was found to correlate closely with K_L/K_H ($r = 0.94$,

$p < 0.02$; data not shown). As K_L/K_H strongly correlates with agonist intrinsic activity (Fig. 4A), it is expected that the nucleotide-induced fold shift will likewise exhibit a close correlation with intrinsic activity.

Another finding from earlier studies now readily explained is the discrepancy previously noted between radiolabeled agonist and antagonist binding (21). The *beta*-adrenergic agonist [^{3}H]HBI was found to label only 60%–65% of the receptor population labeled with [^{3}H]DHA (21). Although this [^{3}H]HBI binding was recognized as of high affinity, the significance of the discrepancy between the quantitative extent of agonist and antagonist binding was not appreciated. That only 60–65% of the receptors formed high affinity state in the presence of [^{3}H]HBI was likely a reflection of the membrane preparation being used at that time. Our current membrane preparation is more "purified" (1000 fmol/mg, receptor concentration), presumably less contaminated with guanyl nucleotides, and able to form as much as 92% high affinity state in response to HBI occupancy (Table 1). We have recently performed [^{3}H]DHA/HBI competition curves in our previous "crude" (150 fmol/mg, receptor concentration) membrane preparation. Computer curve fitting of the data revealed only 60–70% of the receptor population in the high affinity form (data not shown), not greatly different from the estimate obtained by direct [^{3}H]HBI binding in earlier studies. We have also extended these observations by demonstrating the ability of [^{3}H]HBI to directly label greater than 90% of the receptor population labelled with [^{3}H]DHA in our "purified" membranes.[4]

It is evident, therefore, that the ability to discern high affinity state is critically dependent on the membrane preparation used: R_H must accumulate to be measurable. This accumulation is facilitated by a pure membrane preparation with negligible nucleotide contamination. In whole cells, because of endogenous GTP, high affinity state should not be demonstrable with either direct or indirect binding methods, an expectation further suggested by recent experimental data (24). We have, in fact, performed [^{3}H]DHA/(−)isoproterenol competition curves in whole frog erythrocytes. As predicted, the data model to a single low affinity state of agonist binding. The K_D for (−)isoproterenol in whole cells (3500 ± 1200 nM, data not shown) is virtually equal to the low affinity state dissociation constant (K_L) found in purified membrane preparations (2500 ± 400 nM, Table 1).

One other major study has used detailed computer modeling in the analysis of ligand–receptor interactions (25). Birdsall and colleagues, using the muscarinic cholinergic receptor from rat cerebral cortex and methodological approaches similar to those described in this report, have convincingly demonstrated heterogeneous agonist binding properties in this receptor system. In an extensive investigation they have related these properties to two states of agonist binding and have determined the proportions of high and low affinity receptors as well as their respective affinity constants. While several of their results and conclusions are similar to those presented here for the *beta*-adrenergic receptor, certain critical differences do exist. Thus, in the muscarinic cholinergic model, the high affinity state represented only a minority of the total receptor population, regardless of the agonist tested (19–49%). Also, while the ratio of affinity constants for the receptor binding states ranged from 10 to 275, there was no correlation between this ratio and the percentage of receptors in the high affinity state ($r = 0.08$, $p > 0.7$; calculated from data in Ref. (25), Table 3). Similarly, in contrast to the results presented here for the *beta*-adrenergic receptor, the authors' data indicated that interconversion of muscarinic cholinergic receptor states does not take place.

The significance of these differences between the two receptor systems is not yet clear. It should be noted, however, that the muscarinic cholinergic receptor of the rat cerebral cortex presents certain inherent methodological difficulties not found in the frog erythrocyte *beta*-adrenergic receptor system. Thus, since homogenates of a complex organ such as brain rather than a homogeneous cell population were used, the heterogeneity of agonist binding observed could relate to receptors derived from different cell types. Also, no direct measure of intrinsic activity is available for the muscarinic cholinergic receptor system of the rat cerebral cortex. Finally, the possibility must be considered that there are regulatory agents in the muscarinic cholinergic system that are analogous to GTP in the *beta*-receptor system. The presence of such regulators (e.g., ions, nucleotides, etc.) in the brain homogenates used for the cholinergic receptor studies could markedly alter the binding patterns observed.

The results presented here do not permit the determination of a definitive model of agonist binding to the *beta*-adrenergic receptor. However, several models can be exluded as inconsistent with the data derived from computer modeling of [^{3}H]antagonist/agonist competition curves. For example, we can reject any model based on two fixed, noninterconverting receptor states, e.g., the concept of "agonist and antagonist states" (26) cannot apply to the *beta*-adrenergic receptor. Classical allosteric theory as applied to receptor theory by Karlin (27) and subsequently by Thron (28) and Colquhoun (29) contains features similar to our findings in the *beta*-adrenergic receptor. "Active" and "inactive" states of the receptor are assumed to preexist even in the absence of ligand, and agonist efficacy is defined in terms of the ratio of the microscopic dissociation constants of the agonist for the two states (27–29). As predicted by the allosteric model, we have found that K_L/K_H correlates well with intrinsic activity (efficacy) (Fig. 4A). From theoretical considerations, however, allosteric theory cannot explain the apparent heterogeneity of agonist binding demonstrated by the *beta*-adrenergic receptor. Mathematically, allosteric theory predicts steep [^{3}H]antagonist/agonist competition curves and slope factors of one or greater (30). High affinity state, although quantitatively present in the system, cannot be estimated from competition curves or by analysis of slope factors. Our results, therefore, are not consistent with an allosteric mechanism of *beta*-adrenergic receptor function. Similarly, "collision coupling," a model recently proposed by Tolkovsky and Levitzki to explain the interactions of the *beta*-adrenergic receptor

[4] A. De Lean, J. M. Stadel and R. J. Lefkowitz, manuscript submitted for publication.

in the turkey erythrocyte (31), can be rejected as an adequate model for *beta*-adrenergic receptor interactions in isolated frog erythrocyte membranes. In this model, the "coupled" form of the receptor does not accumulate, and analysis of the model reveals steep competition curves without heterogeneity of agonist binding affinities. It should be noted, however, that the collison coupling model may be valid in intact frog erythrocytes where high affinity state is presumably a transient phenomenon and accumulation of high affinity receptors may not occur.

Several proposed models of ligand–receptor interaction cannot be excluded by our findings. These models share the property of containing a ternary complex, generally formed by the interaction of ligand and receptor with an effector subunit. This high affinity complex may accumulate and therefore be evident as shallow agonist competition curves or slope factors less than one. A general model of this type has been developed by Boeynaems and Dumont (32). Jacobs and Cuatrecasas have applied the model to the insulin receptor with considerable success in explaining many of its interactions (33). We have proposed a model involving interaction of the ligand–receptor complex with an enzyme effector (10). We currently believe that the effector may in fact be a nucleotide regulatory site. The model postulates a high affinity ternary complex of ligand, receptor, and effector which itself does not activate adenylate cyclase but rather acts to "prime" the enzyme for activation by guanyl nucleotides. Concomitant with enzyme activation is a nucleotide-induced decrease in the affinity of the receptor for the ligand, resulting in the production of the low affinity state. This model has been successful in explaining the behavior of the *beta*-adrenergic receptor in several earlier studies and is consistent with the data presented in this report. These models are preliminary formulations based on available data and will undoubtedly undergo considerable modification as knowledge of *beta*-adrenergic receptor function increases.

Note Added in Proof: It has been recently demonstrated by computer modeling that the high affinity state of the receptor induced by agonists in frog erythrocyte membranes can be explained by a ternary complex of agonist, receptor and an additional membrane component. (A. De Lean, J. M. Stadel and R. J. Lefkowitz, manuscript submitted for publication.)

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